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Comparisons of phenotype and immunomodulatory capacity among rhesus bone-marrow-derived mesenchymal stem/stromal cells, multipotent adult progenitor cells, and dermal fibroblasts

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Abstract

Background—Potent immunomodulatory effects have been reported for mesenchymal stem/stromal cells (MSCs), multipotent adult progenitor cells (MAPCs), and fibroblasts. However, side-by-side comparisons of these cells specifically regarding immunophenotype, gene expression, and suppression of proliferation of CD4⁺ and CD8⁺ lymphocyte populations have not been reported.

Methods—We developed MAPC and MSC lines from rhesus macaque bone marrow and fibroblast cell lines from rhesus dermis and assessed phenotypes based upon differentiation potential, flow cytometric analysis of immunophenotype, and quantitative RT-PCR analysis of gene expression. Using allogeneic lymphocyte proliferation assays, we compared the *in vitro* immunomodulatory potency of each cell type.

Results and Conclusions—Extensive phenotypic similarities exist among each cell type, although immunosuppressive potencies are distinct. MAPCs are most potent, and fibroblasts are

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the least potent cell type. All three cell types demonstrated immunomodulatory capacity such that each may have potential therapeutic applications such as in organ transplantation, where reduced local immune response is desirable.

Keywords

cellular therapy; immunophenotype; T-cell suppression

Introduction

Immunosuppressive therapies have wide clinical applications for the treatment of autoimmune diseases, graft-versus-host disease, and in the prevention of tissue rejection in organ transplantation. Despite an expanding repertoire of chemical immunosuppressive agents, their clinical use is currently limited by their lack of target specificity on the immune system. This may lead to untoward toxic side effects which may damage the very tissues that are intended to be protected from immune damage, as in the case of rapamycin damage to transplanted islet beta cells [22]. Thus, there has been an effort to establish therapies that more specifically target the harmful immune response and avoid the negative side effects associated with currently available drugs.

One promising approach is the use of naturally occurring immunosuppressive cells such as regulatory T lymphocytes (Tregs). FoxP3⁺ Treg cells, the classic immune cell known to serve this function, are currently being evaluated in this context [5]. However, the use of these cells is constrained by their systemic immunosuppression of non-specific antigens in addition to the difficulty of isolating an adequate supply. While current strategies are being developed to overcome these weaknesses, such as antigen-specific Tregs [9], the plasticity of Tregs will always be in question, while the use of different cell types may offer distinct advantages.

It has become evident that other cell types, such as MSCs, MAPCs, and fibroblasts, may also possess potent immunomodulatory functions that could be applied to therapeutic use. MSCs have particularly garnered increasing attention as a potential cellular therapeutic agent for treatment of a wide variety of disease conditions in which suppression of immune reactions is desired [1, 11, 17, 21, 29, 31]. The immunomodulatory capabilities of MSCs are well documented in both the human and mouse [1, 11, 17, 21, 31], and the immunomodulatory features of MAPCs have also been shown [14]. Likewise, fibroblasts have been noted to have significant dampening effects on immune reactions as have a variety of other mesenchymal-derived cell types [8, 13, 15, 18, 26, 30]. The potent immunomodulatory effects of these cell types have prompted investigations of their use, most notably MSCs and MAPCs, in the suppression of graft-versus-host disease, treatment of type 1 diabetes mellitus, and as an adjunct cellular therapy in pancreatic islet transplantation [1, 14, 25].

Prior to applications in humans, translational studies are necessary to understand the full therapeutic benefits, limitations, and side effects of these cell types and how to apply them appropriately for each disease state. Non-human primates, specifically macaque species, are excellent animal models in which potential therapeutic approaches may be studied.

Presently, relatively little is known about these cell types in non-human primates, and how they compare to each other. We therefore wished to assess whether MSCs, MAPCs, or fibroblasts of rhesus macaques would show immunosuppressive functions similar to human MSCs and therefore, whether any or all of these cell types might be used therapeutically where immunosuppressive effects are desired. Toward this goal, we documented and compared the phenotypes and *in vitro* immunosuppressive capacity of rhesus bone-marrow-derived MSCs and MAPCs and skin-derived fibroblasts.

Materials and methods

Humane care guidelines

All animal procedures are approved by the University of Minnesota Institutional Animal Care and Use Committee, are conducted in compliance with the Animal Welfare Act, and adhere to principles stated in the Guide for Care and Use of Laboratory Animals. See Table 1 for unique animal identifiers and location of animals used in this study.

Animals and tissue harvest

Rhesus 1—Bone marrow was obtained from a 1-year-old male rhesus macaque (*Macaca mulatta*) through the tissue procurement program at the California National Primate Research Center. After euthanasia, according to Primate Center standardized operating procedures, whole marrow was collected under aseptic conditions and placed into heparinized sterile glass tubes and shipped by overnight courier.

Rhesus 2 and 4—Bone marrow was obtained from one 4-year-old male and one 5-year-old female rhesus macaque at the NHLBI colony. Samples were harvested in heparin from iliac crest and shipped overnight on ice.

Rhesus 3 and 5—Two male rhesus macaques were enrolled as pancreas donors as part of an islet allotransplantation program. Both animals were age 6.6 years and weighed 14.9 kg and 13.1 kg. These animals were purpose bred and were acquired from a single institutionally approved vendor (Alpha Genesis, Inc., Yemassee, SC, USA). After total pancreatectomy, animals were euthanized with an overdose of pentobarbital sodium and phenytoin sodium (Beuthanasia-D; Schering-Plough Animal Health, Kenilworth, NJ, USA). Tissue was obtained via the tissue-sharing program at the University of Minnesota. Bone marrow was harvested immediately postmortem by aspiration from the femur and suspended in MSC or MAPC medium as appropriate. Skin was harvested postmortem from the thigh and abdomen and placed in RPMI (Life Technologies, Carlsbad, CA, USA) medium.

Derivation of cell lines

Fibroblasts—One square centimeter pieces of rhesus skin were incubated in either dispase (BD Bioscience, San Jose, CA, USA) or TrypLE (Life Technologies) at 37°C for 45 minutes. Using two sharp scalpels, the tissue was minced in either 0.25% trypsin-EDTA (Cellgro, Manassas, VA, USA) or TrypLE to form single cell suspensions. Cells were then washed with fibroblast growth medium and plated into one tissue culture-treated T25 flask coated with 1% gelatin (Cellgro).

MSCs—Bone marrow samples were processed through a ficoll separation gradient using Histopaque 1077 (Sigma-Aldrich, St. Louis, MO, USA) per manufacturer's instructions to obtain a population of cells enriched for nucleated cells or plated directly from the biopsy in MSC growth medium. Each 1 ml of biopsy was plated into one well of a six-well plate coated with 20 ng/ml fibronectin (Sigma-Aldrich) at density of approximately 100,000/cm² in MSC growth medium. After 24–48 hours, cells were washed gently with phosphate buffered saline (Cellgro) and growth medium was replaced. After about 10 days, when colonies emerged and appeared 100% confluent, cells were harvested and expanded as indicated below.

MAPCs—Bone marrow samples were processed through a ficoll separation gradient using Histopaque 1077 (Sigma-Aldrich) per manufacturer's instructions to obtain a population of cells enriched for nucleated cells or plated directly from the biopsy in MAPC growth medium. Each 1 ml of biopsy was plated into one well of a six-well plate coated with 20 ng/ml fibronectin (Sigma-Aldrich) at density of approximately 100,000/cm² in MAPC growth medium. After 24–48 hours, cells were washed gently with PBS (Cellgro) and growth medium was replaced. After about 10 days, when colonies emerged and appeared 100% confluent, cells were harvested and expanded as indicated below.

Culture conditions

Adult dermal fibroblast culture conditions—Cells were seeded at 3000–6000/cm² on flasks coated with 1% gelatin (Millipore, Billerica, MA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)-high glucose (Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), 1% non-essential amino acids (NEAA), 1% L-glutamine, 1% sodium pyruvate, and 1% pen per strep (Life Technologies). Cells were passaged after reaching 85–90% confluency or approximately every 3 days.

Rhesus MSC culture conditions—Cells were seeded at 1000/cm² in either untreated tissue culture flasks or flasks coated with fibronectin solution (20 ng/ml; Sigma-Aldrich) and cultured in DMEM low glucose (Life Technologies) supplemented with 20% FBS (Hyclone), 1% L-glutamine, 1% NEAA, and 1% pen per strep (Life Technologies). Cells were passaged after reaching 85–90% confluency (about every 4–6 days) with 0.25% trypsin–EDTA (Cellgro) or TrypLE (Life Technologies).

MAPC culture conditions—Cells were seeded at 500/cm² (Rhesus 4) or 1000/cm² (Rhesus 1, Rhesus 2) in flasks coated with fibronectin (20 ng/ml; Sigma-Aldrich). Cells were grown in MAPC medium containing: 60% DMEM low glucose (Life Technologies), 40% MCDB-201 buffered to pH 7.2, 1X insulin-transferrin-selenium, 1X linoleic acid bovine serum albumin, 5×10^{-8} M dexamethasone, 10^{-4} M ascorbic acid 3-phosphate (Sigma-Aldrich), 100 IU/ml penicillin and 100 mg/ml streptomycin (Life Technologies), 2% FBS (Hyclone), 10 ng/ml human platelet-derived growth factor (R&D Systems, Minneapolis, MN, USA), and 10 ng/ml human epidermal growth factor (R&D Systems). Cells were passaged every 3–4 days with 0.25% trypsin–EDTA (Cellgro) or TrypLE (Life Technologies).

KG1A and human MSC—Both cell lines (provided by Dr. Dan Kaufman, University of Minnesota) were grown according to published protocols [2, 10].

Differentiation assays

Adipocyte differentiation—Adipocyte differentiation was induced using StemPro Adipogenesis differentiation kit (Life Technologies) per manufacturer's recommendations. Differentiated cells were evaluated for lipid content with Oil Red O stain (Millipore) per manufacturer's instructions.

Cartilage—A total of 150,000–500,000 rhesus MSCs were pelleted into the bottom of a 15-ml conical tube in 0.5 ml of growth medium and placed in a 5% CO₂ incubator at 37°C. After 24 hours, medium was replaced with StemPro Chondrogenesis medium (Life Technologies). After 3 weeks, the pellet was fixed in 4% paraformaldehyde (ACROS) in phosphate buffered saline for 3 hours at 4°C and then stored in 70% ethanol until processed. Samples were routinely embedded in paraffin, sectioned at 5 µm thickness, and stained with Alcian blue.

Flow cytometry

Cells from each sample were harvested using TrypLE (Invitrogen, Carlsbad, CA, USA) and stained with primary antibody (Table 2) for one hour in staining buffer (PBS containing 2% fetal bovine serum) for CD34, CD44, CD45, CD73, CD90, MHCI, (BD Biosciences) CD105, CD133 (ebioscience, San Diego, CA, USA), CD146, (R&D Systems). If a secondary antibody was necessary, cells were washed twice in staining buffer and resuspended in goat anti-mouse IgG AlexaFluor-488 (Life Technologies) for one hour, washed twice in staining buffer, and evaluated with a FACS Caliber (Becton Dickinson, Franklin Lakes, NJ, USA). Appropriate isotype controls were assayed alongside the test samples. Data were analyzed using FLOJO Software (TreeStar Inc., Ashland, OR, USA).

Quantitative RT-PCR

Cells were collected and lysed in RLT buffer (Qiagen, Valencia, CA, USA) and stored at –80°C until processed. RNA was isolated from cell lysates using RNA easy microkit (Qiagen) according to manufacturer's instructions. Off column DNase treatment was performed using TURBO DNase (Life Technologies) according to manufacturer's instructions. cDNA was synthesized using Superscript III reverse transcriptase (Life Technologies). The PCR consisted of 20 ng cDNA samples, 6 µl 1X SYBR Green Mix PCR buffer (Life Technologies), and 100 nM primers (Table 2) plus RNase free water to equal 12 µl total. All reactions were run in duplicate or triplicate on a Realplex Master-cycler (Eppendorf, Hamburg, Germany) using the following program: 95°C for 10 minutes and 40 cycles at 95°C for 15 s and 57°C for 30 s, 68°C for 30 s followed by at 95°C for 15 s, 60°C for 15 s, and 95°C for 15 s.

Results of gene expression were calculated using expression relative to GAPDH, where $C_t = (C_t \text{ gene of interest} - C_t \text{ GAPDH})$. The $(-C_t)$ was then calculated relative to the positive control (total rhesus RNA; Biochain, Newark, CA, USA) where $(-C_t) = C_t \text{ gene} - C_t \text{ control}$. The fold change relative to the control for each gene was then calculated, where fold

change = $2^{- (C_t)}$. Samples with a C_t value greater than 35 were considered to represent no gene expression.

T-cell suppression assays

Rhesus macaque splenocytes were labeled with 5 μ M carboxyfluorescein succinimidyl ester (CFSE, ebioscience) and incubated at 37°C for 5 minutes. The labeled cells were then plated at 1×10^5 per well and stimulated with 1:1 ratio of α CD3 (SP34 clone; BD Biosciences) coated Dynabeads (Invitrogen) in the absence or presence of effectors serially diluted to 1:16 (effector: responder) equivalent in a 96-well u-bottom plate. Cells were cultured for 4 or 5 days, harvested, and immuno-stained with CD4 PerCP and CD8 APC antibodies (BD Biosciences). CD4 and CD8 T-cell proliferation, as measured by CFSE^{lo} expression was analyzed using FLOWJO software (Tree Star Inc., Ashland, OR, USA). Percent suppression was analyzed using the division index (determined using FLOWJO software), which is the average number of divisions of all cells from the parent generation including cells which never divide. Percent suppression was calculated using the software-calculated division index of responders with no effectors present as the zero percent suppression baseline for each ratio dilution, resulting in a reduction in percentage suppression as the suppressive cells are reduced in the ratio. The percentage suppression at 1:1 dilution was then used as a normalizer for each cell type to establish a diminishing effect curve where a fit line was used to calculate the effective concentration (EC50) ratio, where 50% of splenocyte proliferation was suppressed by each cell type for either CD4⁺ or CD8⁺ splenocyte populations.

Statistics

Lymphocyte proliferation assays—Means and standard deviations for %CD4 and %CD8 suppression were summarized by cell type. Analysis of variance (ANOVA) F-tests were performed to evaluate whether the means are significantly different from each other. To assess which means differ from which other means, multiple comparisons with Tukey–Kramer adjustment for the *P*-values were performed.

qRT-PCR—For those being scaled multiple times, the average delta-delta value was used in analysis. Means and standard deviations for delta-delta were summarized by cell type. Analysis of variance (ANOVA) F-tests were performed to evaluate whether the means are significantly different from each other. To assess which means differ from which other means, multiple comparisons with Tukey–Kramer adjustment for the *P*-values were performed.

Results

After harvest, dense colonies of cells began to emerge after about 10 days. Cell types were expanded, and characterizations were carried out between 21 and 27 population doublings, except for the MAPC line Rhesus 4, which was characterized at a population doubling of 70. This cell line was expanded for up to 180 population doublings without detectable chromosomal abnormalities (data not shown). All other cell lines underwent senescence before a population doubling of 30.

All MSC, MAPC, and fibroblast cell lines were found to be capable of differentiation *in vitro* into adipocytes and cartilage using identical differentiation protocols for each cell type (Fig. 1).

Flow cytometry analysis of surface immunophenotypes types led to remarkably similar results among all three cell types (Fig. 2). Comparisons of the canonical MSC surface markers, including CD44, CD73, CD90, CD105, and MHCI, showed essentially identical positive phenotypes for MSCs, MAPCs, and fibroblasts with the exception of one MAPC line (Rhesus 4), which showed a much lower population of CD90-positive cells than any other cell line. All cell lines were either negative for CD133 or were only dimly positive. CD146 expression, in comparison to the other markers, showed the greatest variability among cell lines, with MSCs tending to exhibit greater numbers of strongly positive cells than MAPCs, while the fibroblast lines showed high expression in Rhesus 3 and negligible expression in Rhesus 5. CD34 and CD45 were negative in all cell lines with the exception of Rhesus 4 which was CD34^{dim}.

Quantitative RT-PCR of selected markers revealed that all genes were expressed in all cell lines; however, no consistent or significant differences in quantity of expression among the three cell types for any marker were found (Table 3). Expression of the putative fibroblast markers S100A4 and type I collagen was nominally higher in the fibroblast cell lines in comparison to MSC or MAPC lines, but the differences did not achieve statistical significance ($P = 0.17$ and $P = 0.19$ respectively).

In T-cell suppression assays, all three cell types were shown to be capable of marked suppression of proliferation of both CD4⁺ and CD8⁺ allogeneic splenocytes (Fig. 3). CFSE-labeled CD4⁺ splenocyte cells showed a marked reduction in CFSE dilution with all three (MAPC, MSC, and fibroblast) cell types at a 1:1 ratio (Fig. 3A). This indicates that the splenocytes proliferated less in the presence of each cell type (bold black line of FACS plot) compared with splenocytes alone (gray dotted line of FACS plot), indicating that each cell type has a suppressive phenotype. When each cell line was diluted compared with the splenocyte responder cells, you can see an attenuation of the suppressive effects by each cell line compared with each 1:1 ratio, calculated by comparing the average number of cell divisions in treated vs. untreated splenocyte populations. We observed that the fibroblast suppression of splenocyte CD4⁺ cell proliferation quickly diluted starting at the 1:2 ratio compared with the other two lines, while the MAPC lines retained best suppression at lower dilutions such as 1:8 ratio (ANOVA F-test $P = 0.07$) and 1:16 ratio (ANOVA F-test $P = 0.05$; MAPC vs. MSC Tukey–Kramer $P = 0.05$) (Fig. 3B). ANOVA F-test comparing suppression by each cell type at the 1:8 and 1:16 showed. Using the data from Fig. 3B, each cell line was normalized to its suppression at 1:1 to calculate the effective concentration at 50% (EC50) value, which was 1:12.73 ratio for MAPC, 1:4.31 ratio for MSC, and 1:2.85 ratio for fibroblast in descending order, supporting our earlier observations (Fig. 3C). Similar results were observed for the suppression of CD8⁺ splenocyte cell suppression, with CFSE-stained responders having limited proliferation at 1:1 dilution for each of the three cell lines (bold black line of FACS plot) compared with CD8⁺ splenocytes alone (gray dotted line of FACS plot) (Fig. 3D). As with CD4⁺ splenocyte cells, fibroblast suppression of CD8⁺ splenocytes had the steepest decline of suppressive effects with dilutions beyond

1:1 ratio with significant differences at the 1:16 ratio (ANOVA F-test $P = 0.05$; MAPC vs. fibroblasts Tukey–Kramer $P = 0.05$). This was also reflected by a low EC50 (1.99 ratio) compared with the other two types (Fig. 3E,F). In contrast, unlike the CD4⁺ splenocytes where MAPC had the highest EC50, MSC and MAPC had very similar suppression of CD8⁺ splenocyte proliferation throughout the dilutions observed by percent suppression and reflected by their calculated EC50 values: 3.71 and 4.71, respectively (Fig. 3E,F).

Discussion

There is growing interest in the development of clinical applications of MSCs and similar cell types, particularly owing to their potent trophic and immunomodulatory functions [7, 16, 19, 20, 23, 25, 27, 29, 31]. The continued development of new clinical applications for these cell types will rely on preclinical investigation of new therapeutic approaches in animal models. Macaque species, owing to their close phylogenetic relationship to humans, are particularly valuable as animal models for preclinical studies. In view of this, we investigated the phenotypes and immunomodulatory properties of rhesus MSCs, MAPCs, and fibroblasts to justify their use in this animal model.

Each cell line included in the present study was derived from rhesus macaques according to published protocols for bone-marrow-derived rhesus MSCs, human MAPCs, and human dermal fibroblasts [3, 14, 18]. All cell lines examined displayed remarkable similarities, regardless of the derivation method. These included the capacity to differentiate toward mesenchymal cell types (adipose and cartilage), similar surface marker phenotype, similar gene expression levels for selected markers, and the ability to suppress CD4⁺ and CD8⁺ lymphocyte proliferation in *in vitro* assays at 1:1 dilution of suppressors to responders. These overall similarities conform to what has been previously described for human MSCs and fibroblasts obtained from a variety of tissues and suggest that these adult mesenchymal cell types in macaques and humans share common biologic features. These findings therefore, support the use of rhesus models for preclinical assessment of basic biology and therapeutic efficacy of mesenchymal cell therapies.

The ability of MSCs, MAPCs, and fibroblasts of human and mouse to differentiate into adipocytes and chondroblasts, as well as other mesenchymal cell lineages, is well documented [24, 27]. Thus, the ability of the rhesus MSCs, MAPCs, and fibroblasts in this study to differentiate into both adipocytes and chondroblasts confirms the multipotency of these cells. However, whether the rhesus cell lines show the same range of multipotency as their human or murine counterparts was not examined in the present study and remains to be proven.

Although extensively studied, no single, unique marker has been identified that can unequivocally identify MSCs in any species [4, 27]. At present, the generally accepted criteria for the identification of MSCs include adherence to plastic under standard cell culture conditions, expression of surface markers which include CD73, CD90, and CD105, lack of expression for CD11a, CD19, CD34, CD45, and HLA-DR, and ability to differentiate into two or more mesenchymal lineages [4, 27]. Cells with these MSC features have been cultured from a wide range of tissues [6, 12]. Comparisons of human MSCs and

fibroblasts obtained from a range of different tissues have shown similarities in surface phenotype and differentiation potential both within and between MSCs and fibroblasts. Our study in the rhesus macaque further illustrates these overall similarities and also demonstrates the similarities of the rhesus MSCs and fibroblasts to their human counterparts. Rhesus MAPCs likewise show overall high similarity to rhesus MSCs and fibroblasts consistent with a similar origin of these cells. In this regard, several studies have demonstrated that the likely cells of origin of MSCs are pericytes [4, 27], and given the close similarities observed among both rhesus and human MSCs, fibroblasts, and MAPCs, it is tempting to speculate that all of these cell types are indeed derived from pericytes.

Despite the similarity in surface marker phenotypes among MSCs, MAPCs, and fibroblasts, certain markers have been suggested as being potentially useful discriminators between MSCs, MAPCs, and fibroblasts [2, 24]. For example, MHCI was found to be a differentially expressed surface marker between human MSCs and MAPCs with lower expression among MAPCs [24]. However, this difference was not apparent among the rhesus cell lines. Another marker found to be useful in differentiating between human MAPCs and MSCs is CD140a (PDGFR1), which was found to be consistently expressed on MSCs while not expressed in MAPCs [24]. Unfortunately, no antibody reactive to rhesus CD140a was available for this study and so we were unable to assess this marker by flow cytometry. However, we did compare PDGFR1 gene expression among the three rhesus cell types by quantitative RT-PCR (qRT-PCR) and found no significant differences ($P = 0.72$). Additional markers that have been proposed as discriminators for MSCs, and fibroblasts include CD200 and the αV and $\beta 5$ integrins. Again, lacking suitable antibodies specific for rhesus versions of these markers we assessed gene expression in each of our rhesus cell types using qRT-PCR. No significant differences in gene expression were found among the three cell types. We also found no significant differences in gene expression among the three cell types for type 1 collagen or S100A4, both suggested as markers for human fibroblasts [28], or for toll-like receptors 3 and 4. Although not achieving statistical significance, both fibroblast cell lines did show higher gene expression of both type 1 collagen and S100A4 compared with MSC and MAPC lines, suggesting that this may be a real biologic difference which could not be confirmed statistically in this study. Moreover, as protein expression for these markers was not assessed in our study, it is possible that significant differences in protein levels do indeed exist among the three cell types. Thus, further analysis would be necessary to establish whether any of these markers might be useful discriminators of rhesus MSCs, MAPCs, and fibroblasts.

The overall effects of each of the rhesus cell lines on allogeneic lymphocyte proliferation were similar to those reported for human and murine MSCs and fibroblasts, and for murine MAPCs [7, 14, 30, 31]. Thus, it appears that the immunosuppressive activity of each of these mesenchymal cell types is conserved across all of these species, further supporting the use of rhesus models for translational studies using these cell types. While all three rhesus cell types showed immunomodulatory effects, MAPCs were more potent in suppression of CD4⁺ T lymphocytes compared with MSCs, which in turn were more potent than fibroblasts. MAPCs and MSCs were approximately equally potent in suppression of CD8⁺ lymphocytes, and both were more effective than fibroblasts. Whether these differences in

apparent potency of immunosuppression are sufficient to be clinically relevant for therapeutic applications remains to be determined. These differences, however, may serve to inform further experiments aimed at dissecting the mechanisms by which these cells suppress CD4⁺ and CD8⁺ T lymphocyte proliferation.

In summary, we have demonstrated that rhesus MSCs, MAPCs, and fibroblasts share a similar phenotype, are each multipotent, and all exhibit potent inhibition of *in vitro* lymphocyte proliferation. Each of the rhesus cell types shows similarity to their human and/or murine counterparts in each of these aspects, which supports the use of the rhesus macaque as an animal model for translational studies harnessing the therapeutic applications of these cells.

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Fig. 1. Differentiation of representative cell lines into adipocyte and chondroblast lineages. (**A–C**) Oil Red O stain of adipogenic differentiations: (**A**) Rhesus 3 MAPC, (**B**) Rhesus 3 MSC, and (**C**) Rhesus 5 fibroblast. (**D–F**) Alcian blue stain of chondrogenic differentiations: (**A**) Rhesus 4 MAPC, (**B**) Rhesus 3 MSC, and (**C**) Rhesus 3 fibroblast.

Fig. 2.
Flow cytometry evaluations of rhesus MSC, MAPC, and fibroblast cell lines with human MSC control, and KG1a cell line as negative control for CD73 and positive control for CD34 and CD45.

Fig. 3.

Effects of MSC, fibroblast, and MAPC lines on proliferation of allogeneic CD4⁺ and CD8⁺ lymphocytes stimulated by CD3 microbeads. **(A)** Representative plots showing suppression of CFSE dilution of CD4⁺ splenocyte proliferation assays for each cell type (bold black line) compared with splenocyte dilution alone (gray dotted line). **(B)** Percent suppression of CD4⁺ splenocyte proliferation for each cell type at dilutions from 1:1 (responder:suppressor) to 1:16 calculated using the average number of cell divisions in the population. **(C)** Data from part B graphed as relative suppression compared with 1:1 ratio with EC50 shown as dotted line for each cell line. **(D)** Representative plots showing suppression of CFSE dilution of CD8⁺ splenocyte proliferation assays for each cell type (bold black line) compared with splenocyte dilution alone (gray dotted line). **(E)** Percent suppression of CD8⁺ splenocyte proliferation for each cell type at dilutions from 1:1 (responder:suppressor) to 1:16 calculated using average number of cell divisions in the population. **(F)** Data from part E graphed as relative suppression to 1:1 ratio with EC50 shown as dotted line for each cell line.

Table 1

Animal samples in this study

Animal	Unique identifier	Location	Sample type(s) obtained	Cell type(s) derived
Rhesus 1	RY2	University of California Davis, Davis, CA	Bone marrow	MSC, MAPC
Rhesus 2	RG6848	National Institutes of Health, Bethesda, MD	Bone marrow	MSC, MAPC
Rhesus 3	Lear07CP16	University of Minnesota, St. Paul, MN	Bone marrow, dermis	MSC, fibroblast
Rhesus 4	RQ2633	National Institutes of Health, Bethesda, MD	Bone marrow	MAPC
Rhesus 5	Penn	University of Minnesota, St. Paul, MN	Dermis	Fibroblast

Table 2

Quantitative PCR primers

Primer	Sequence
GAPDH F	TGG TAT CGT GGA AGG ACT CAT GAC
GAPDH R	ATG CCA GTG AGC TTC CCG TTC AGC
Alpha-5 Integrin F	AGGAGGGCAAGTCCTCAAAT
Alpha-5 Integrin R	TGTTTCGACCTCACAGATGC
Beta-5 Integrin F	TCTGGGATCAGCCTGAAGAT
Beta-5 Integrin R	ACGTGCTCTGTGTGTCTGCT
CD200 F	TGGAATATCACCTGGAGGA
CD200 R	TGGCAGAGCAAGTGATGTTT
Type I Collagen F	GCCTCAAGGTATTGCTGGAC
Type I Collagen R	CACCACGATCACCACTCTTG
PDGF-R1 F	GAGAAGCAAGCCCTCATGTC
PDGF-R1 R	GCAGGTAGTCCACCAGGTCT
S100A4 F	GGTGTCCACCTTCCACAAGT
S100A4 R	GCTGTCCAAGTTGCTCATCA
TLR3 F	GCTGGAAAATCTCCAAGAGC
TLR3 R	CCGAATGCTTGTGTTTGCTA
TLR4 F	CATCCCCTTCTCAACCAAGA
TLR4 R	GGAGAGGTGGCTTAGGCTCT

Table 3

Quantitative RT-PCR analysis of expression of selected genes in bone-marrow-derived MAPC and MSC, and dermal fibroblasts

Gene	MAPC		MSC		Fibro		<i>P</i> -value from ANOVA F-test
	N	Mean (SD)	N	Mean (SD)	N	Mean (SD)	
Alpha-5 Integrin	4	0.10 (1.82)	4	-2.14 (1.31)	2	-3.08 (2.97)	0.15
Beta-5 Integrin	4	-0.86 (1.03)	4	-4.31 (3.19)	2	-2.70 (1.14)	0.16
CD200	4	2.18 (3.93)	4	-0.11 (1.85)	2	-0.66 (1.37)	0.45
Type I Collagen	4	-8.13 (0.97)	4	-7.73 (0.77)	2	-9.17 (0.01)	0.19
PDGF-R1	4	-3.15 (0.65)	4	-3.76 (2.01)	2	-4.12 (1.33)	0.72
S100A4	4	-3.86 (2.97)	4	-6.03 (1.27)	2	-7.56 (0.04)	0.17
TLR3	4	1.44 (0.54)	4	1.60 (1.43)	2	2.59 (0.30)	0.44
TLR4	5	6.68 (5.15)	3	2.11 (0.84)	2	0.15 (0.20)	0.15