

Phylogenetic distinction of iNOS and IDO function in mesenchymal stem cell-mediated immunosuppression in mammalian species

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Mammalian mesenchymal stem cells (MSCs) have been shown to be strongly immunosuppressive in both animal disease models and human clinical trials. We have reported that the key molecule mediating immunosuppression by MSCs is species dependent: indoleamine 2,3-dioxygenase (IDO) in human and inducible nitric oxide synthase (iNOS) in mouse. In the present study, we isolated MSCs from several mammalian species, each of a different genus, and investigated the involvement of IDO and iNOS during MSC-mediated immunosuppression. The characterization of MSCs from different species was by adherence to tissue culture plastic, morphology, specific marker expression, and differentiation potential. On the basis of the inducibility of IDO and iNOS by inflammatory cytokines in MSCs, the tested mammalian species fall into two distinct groups: IDO utilizers and iNOS utilizers. MSCs from monkey, pig, and human employ IDO to suppress immune responses, whereas MSCs from mouse, rat, rabbit, and hamster utilize iNOS. Interestingly, based on the limited number of species tested, the iNOS-utilizing species all belong to the phylogenetic clade, *Glires*. Although the evolutionary significance of this divergence is not known, we believe that this study provides critical guidance for choosing appropriate animal models for preclinical studies of MSCs.

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Mesenchymal stem cells (MSCs) exist in almost all tissues and can be easily obtained from the bone marrow (BM), adipose tissues, and other tissues.^{1,2} MSCs were first identified, by Friedenstein and Caplan, as spindle-shaped cells with the capacity for self-renewal and multipotent differentiation.³ Given the lack of universal criteria for defining MSCs, a standard panel of surface markers is employed to define MSCs for basic research and clinical studies. Recently, MSCs have been found to be highly immunosuppressive. As such, they have been used in both animal models and clinical trials to treat various immune disorders, including graft *versus* host disease (GvHD),^{4,5} systemic lupus erythematosus (SLE),⁶ and rheumatoid arthritis.⁷

Notably, MSCs are not innately immunosuppressive but acquire this important function in response to the pro-inflammatory cytokines interferon- γ or tumor necrosis factor- α in combination with interleukin (IL)-1 β . This process is orchestrated by chemokines, adhesion molecules and immunosuppressive molecules, and ultimately indoleamine 2,3-dioxygenase (IDO) in human, or inducible nitric oxide synthase (iNOS) in mouse.^{8,9} Immune cells are recruited by chemokines into the vicinity of MSCs where they are retained

by adhesion molecules, and then suppressed either by locally high concentrations of nitric oxide (NO) or by IDO-mediated tryptophan depletion.^{10,11}

Although MSCs have been proven to be very effective in inducing immunosuppression in both animal models and human trials, their reported effectiveness varies significantly.^{12–14} Given that the BM-derived MSCs from mouse differ in their immunomodulatory mechanism from that of humans, mouse models may be inappropriate for preclinical experiments to investigate the therapeutic effects of MSCs. Other animal models may better mimic human diseases, and also be more convenient than mouse models. In fact, immune disorder models established using rabbits, rats, pigs, and nonhuman primates can all be ameliorated or cured by MSC administration.^{15–19} The underlying mechanisms, however, remain to be elucidated.

To explore interspecies variation in the mechanisms of MSC-mediated immunosuppression, BM-MSCs were prepared from seven different species, including mouse, rat, hamster, rabbit, pig, monkey, and human. First, MSCs were defined by their morphology, marker expression, and differentiation ability. Then, their immunosuppressive capacity and

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Abbreviations: MSCs, mesenchymal stem cells; NO, nitric oxide; iNOS, inducible nitric oxide synthase; IDO, indoleamine 2,3-dioxygenase; IL-1 β , interleukin-1beta; CFSE, carboxy fluorescein diacetate succinimidyl ester; L-NMMA, NG-monomethyl-L-arginine acetate salt; 1-MT, 1-methyl-tryptophan; PBMCs, peripheral blood mononuclear cells; Spl, splenocyte

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the associated key molecules were investigated. We found that BM-MSCs isolated from the tested species conform to the current criteria for MSC identification; they appear fibroblast like, have the same surface markers, and can differentiate into adipocytes or osteoblasts. As expected, the immunosuppressive molecule that exerts the immunoregulatory function of BM-MSCs depended on the species. BM-MSCs derived from human, monkey, and pig utilized IDO for immunosuppression, whereas BM-MSCs from mouse, rat, hamster, and rabbit depended on iNOS expression. Interestingly, human, monkey, and pig are similarly positioned in the iNOS gene cladogram, whereas mouse, rat, hamster, and rabbit are all closely located in another branch. Therefore, our studies reveal not only the different molecules involved in MSC-mediated immunosuppression in distinct species but also the potential relationships in the evolution of this difference.

Results

Derivation of BM-MSCs from different species.

BM-MSCs from different species including human, monkey, pig, hamster, rabbit, mouse, and rat were obtained by adherent culture of BM cells, as described in Materials and Methods. All MSCs could be easily isolated and expanded to large-scale culture. They showed similar fibroblast-like cell morphology and formed homogenous colonies (Figure 1). Notably, MSCs derived from mouse and rat had to be passaged several times to achieve a high level of purity. For rabbit or pig BM-MSCs, non-adherent cells had to be removed at 24 h otherwise these cells would begin to adhere and thus contaminate MSCs. Although sharing the same morphology, BM-MSCs derived from human or monkey were bigger and had lower proliferation rates than other species. They required 7 days to achieve 90% confluence when started at 2×10^3 cells/cm², whereas mouse and rat BM-MSCs reached 90% confluence within only 2–3 days under similar plating densities. Therefore, although BM-MSCs from different species all had the similar spindle shape, they differed in cell size and growth rate.

Identification of BM-MSCs from different species.

Because of a lack of universal criteria for defining MSCs, a

panel looking at mRNA for surface markers was employed to exclude other cell types, and thereby identify them. At a minimum, MSCs should express stem cell markers like CD105, CD73, and CD29, and always be positive for CD44 and negative for CD45.^{20,21} As shown in Figure 2 and Table 1, BM-MSCs from the various species were remarkably consistent: human-, pig-, monkey-, rabbit-, and mouse-derived BM-MSCs all had the same phenotype: CD105⁺CD73⁺CD29⁺CD44⁺CD45⁻; rat- and hamster-derived BM-MSCs were CD105⁻CD73⁺CD29⁺CD44⁺CD45⁻, differing by their lack of CD105. Therefore, according to mRNA analyses of MSC surface markers, BM-MSCs derived

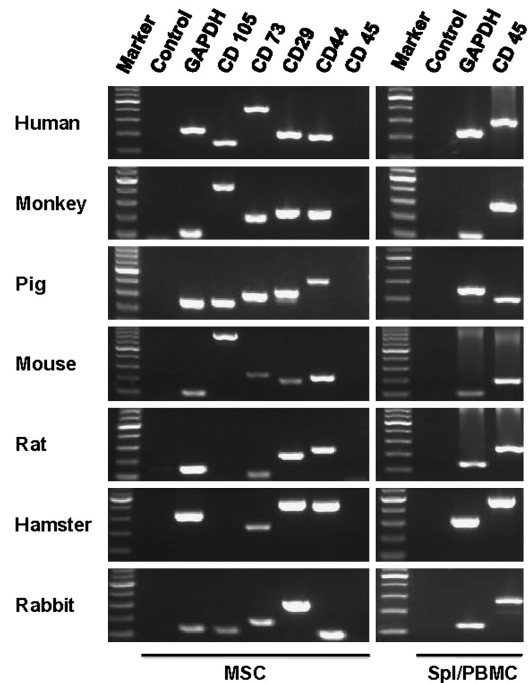


Figure 2 Surface markers of different species-derived BM-MSCs. All BM-MSCs were seeded in six-well plates and collected upon confluence for subsequent RNA extraction, RNA reverse transcription, and PCR assay. A panel of surface markers including CD105, CD73, CD29, CD44, and CD45 was detected for identification of BM-MSCs while GAPDH served as an input control

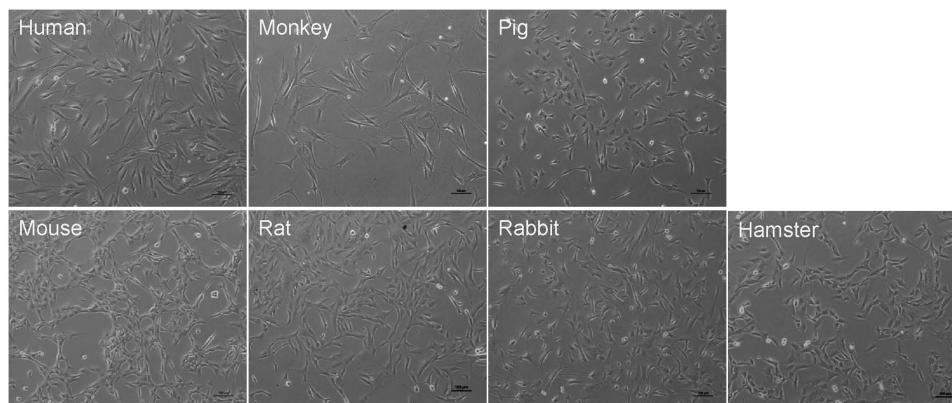


Figure 1 Morphology of BM-MSCs from different species. BM-MSCs before passage 10 from different species were isolated and maintained in DMEM complete medium as described. Their morphology was monitored under microscope and the scale bar indicates 100 μ m

Table 1 Summary of MSC marker expression by BM-MSCs derived from different species

	Human	Monkey	Pig	Mouse	Rat	Hamster	Rabbit
CD 105	+	+	+	+	-	-	+
CD 73	+	+	+	+	+	+	+
CD 29	+	+	+	+	+	+	+
CD 44	+	+	+	+	+	+	+
CD 45	-	-	-	-	-	-	-

from all seven species conform closely to the definition of MSCs.

Multipotent differentiation potential of MSCs from different species. Besides the mRNA analyses for surface antigen expression profile, another criteria used to define MSCs is their potential to differentiate into adipocytes and osteoblasts. Our laboratory has established a robust system to induce adipocyte and osteoblast differentiation by human and mouse MSCs. Accordingly, BM-MSCs from human, mouse, and other species were induced to undergo adipogenesis or osteogenesis using the respective culture conditions, with successful differentiation checked by staining with oil red O or Alizarin Red S, respectively. We found that BM-MSCs derived from all seven species could successfully differentiate into adipocytes (Figure 3a) or osteoblasts (Figure 3b), indicating that these BM-MSCs are indeed multipotent, further confirming their identity as MSCs.

Immunosuppression of BM-MSCs from different species. We have reported previously that the immunosuppressive function of MSCs is not intrinsic.^{10,22} Stimulation with pro-inflammatory cytokines is necessary to endow MSCs with the capacity to inhibit T-cell proliferation. The resultant immunosuppression is powerful; thus, MSCs are emerging as a promising tool in the treatment of immune disorders.^{23,24} Many laboratories have documented that the immunosuppressive effects of mouse MSCs on splenocyte (Spl) function is similar to that of human MSCs.^{23,25,26} This suggests that the same phenomenon may exist in many commonly used laboratory animals. To verify this, we tested the immunosuppressive function of MSCs derived from other five laboratory animals, including hamster, rabbit, rat, pig, and monkey. For each species, BM-MSCs were cocultured with stimulated Spls or peripheral blood mononuclear cells (PBMCs) at 1:10 ratio (MSC-to-Spl (or PBMC)) for 4 days. The proliferation of Spls or PBMCs was measured by the carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution assay. As shown in Figure 4, MSCs from all tested species were found to be highly immunosuppressive to Spls or PBMCs. This suggests that it may be prudent to create more suitable animal models besides mouse so as to verify MSC-mediated immunosuppression *in vivo*.

Differential mechanisms account for similar immunosuppression by MSCs in distinct species. In our previous work, we have demonstrated the mechanisms underlying MSC-mediated immunosuppression. They are not innately immunosuppressive, but become so after exposure to pro-inflammatory cytokines. With sufficient cytokine stimulation,

MSCs can produce large amounts of chemokines, which recruit immune cells into their proximity.¹¹ The recruited immune cells are then subject to the effects of locally high concentrations of immunosuppressive factors whose production is also induced by pro-inflammatory cytokines. Remarkably, the key molecule mediating this immunosuppression differs according to species; in mouse, it is NO produced by iNOS, whereas in human it is tryptophan depletion resulting from IDO upregulation.¹² Yet, most researches to date have employed mouse models to verify the therapeutic effect of MSCs. As the mechanisms of MSC-mediated immunosuppression differ between mouse and human, we propose that other *in vivo* animal models, more suitable than mouse, should be used to mimic the immunosuppressive function of MSCs in human. To this end, we used specific inhibitors of iNOS and IDO to test whether MSCs from other species also employ either of these two molecules for immunosuppression. Accordingly, NG-monomethyl-L-arginine acetate salt (L-NMMA) or 1-methyl-tryptophan (1-MT), specific inhibitors of iNOS and IDO, respectively, were added to the MSC-Spl (or PBMC) coculture system. Interestingly, we found that immunosuppression by MSCs from rat, hamster, and rabbit was reversible by L-NMMA, but not 1-MT, indicating that they share a similar mechanism with mouse MSCs. Conversely, monkey and pig MSCs were dependent on IDO, making them similar to human MSCs (Figure 5a). The similar results were obtained when we measured the NO production in the supernatant of the MSC-Spl (or PBMC) cocultures. High level of NO concentration was detected in mouse, rat, hamster, and rabbit coculture system, whereas little NO was detected in human, monkey, and pig cocultures (Figure 5b). Therefore, our findings strongly suggest that animal models using monkey or pig would be more appropriate for preclinical studies of the effect of MSC-based therapy in human patients.

Discussion

MSCs exist in almost all tissues. They can be easily isolated from multiple tissues and expanded in large scale. Although previous studies have revealed multiple potential effector molecules in MSC-mediated immunosuppression, including IDO,²⁷ prostaglandin E2,²⁸ transforming growth factor- β 1,²⁹ IL-10,³⁰ heme oxygenase-1,³¹ hepatocyte growth factor,³² iNOS,²³ IL-6,³³ LIF,³⁴ galectins, and soluble HLA-G5,^{25,35} these studies focused mostly on either human MSCs or mouse MSCs. The key effector in rat- and monkey-derived MSCs also has been investigated to some extent: rat shares the same factor with mouse, whereas monkey is similar to human.^{12,36} The mechanisms in pig, hamster, and rabbit are not known.^{37,38} Therefore, the potential immunomodulatory mechanisms of MSCs from other species deserve further investigation, as some studies have revealed potent therapeutic effects by MSCs *in vivo* using these laboratory animals. Our study reveals that distinct mechanisms are involved in the immunosuppressive abilities of MSCs derived from several different species of laboratory animal, some dependent on iNOS and others on IDO. Notably, we have demonstrated that the key effector employed by MSCs from

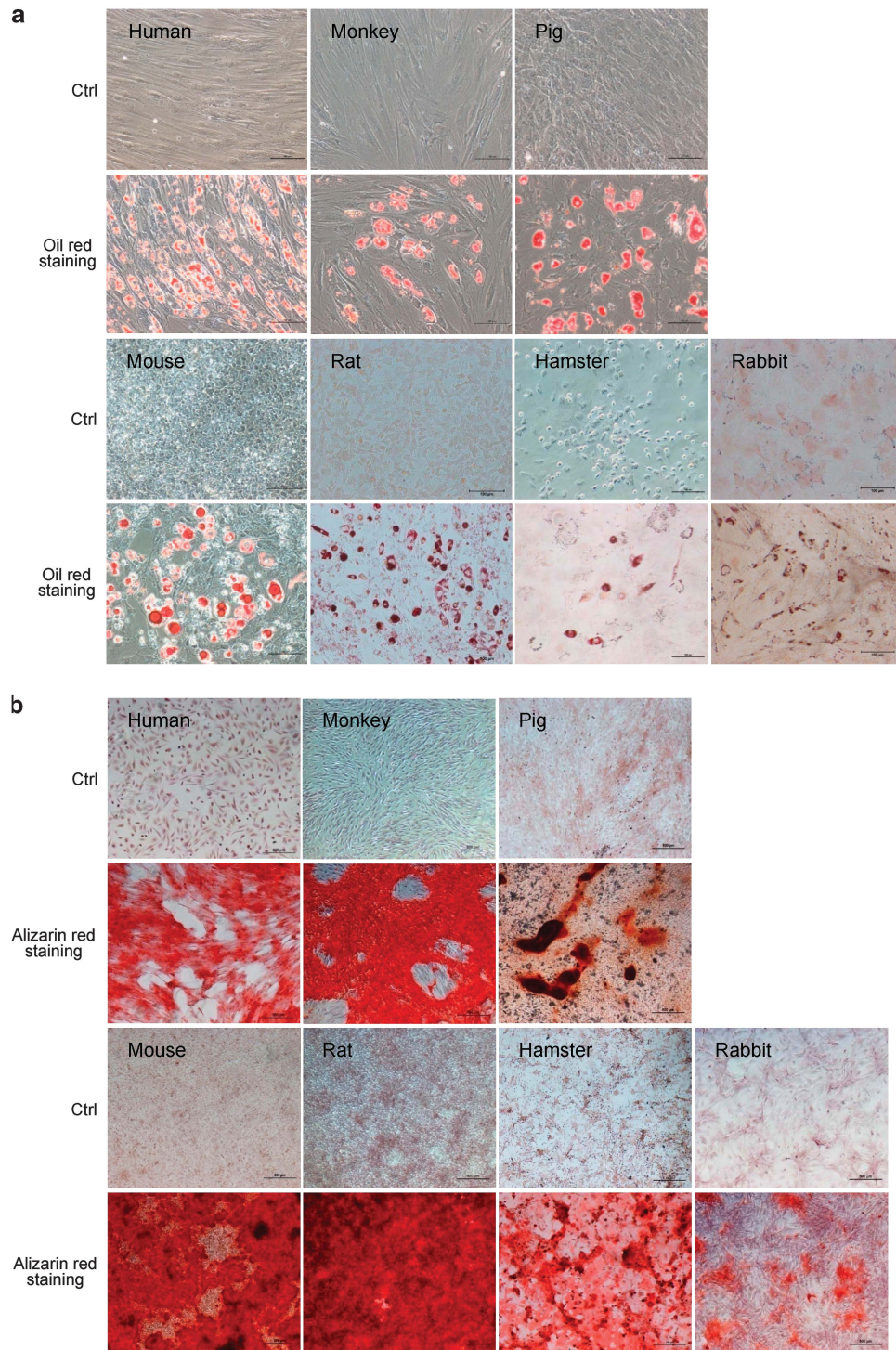


Figure 3 Adipocyte and osteoblast differentiation of BM-MSCs from different species. BM-MSCs before passage 12 were induced to differentiate into adipocytes and osteoblasts in differential medium and detected as described in Materials and Methods, untreated group used as control. (a) Adipocyte differentiation indicated by oil red O staining; the scale bar indicates 100 μm . (b) Osteoblast differentiation indicated by Alizarin red S staining; the scale bar indicates 500 μm

different species correlates with how closely they are related evolutionarily.

In addition to studies of the immunomodulatory effects of MSCs *in vitro*, many cases of successful MSC-based immunotherapy in mammalian species *in vivo* have been

reported for conditions such as GvHD,⁴ liver disease,^{39,40} chronic spinal cord injury,⁴¹ and other diseases. Moreover, many MSC-based treatments have been tested in various animal models: mouse MSCs have been used to improve liver fibrosis⁴² and SLE;⁶ rat MSCs were reported to cure

Parkinson's disease¹⁶ and liver cirrhosis;⁴³ and rabbit MSCs were of benefit in treating experimental retinal holes.¹⁵ Finally, MSCs derived from monkey and pig were effective in

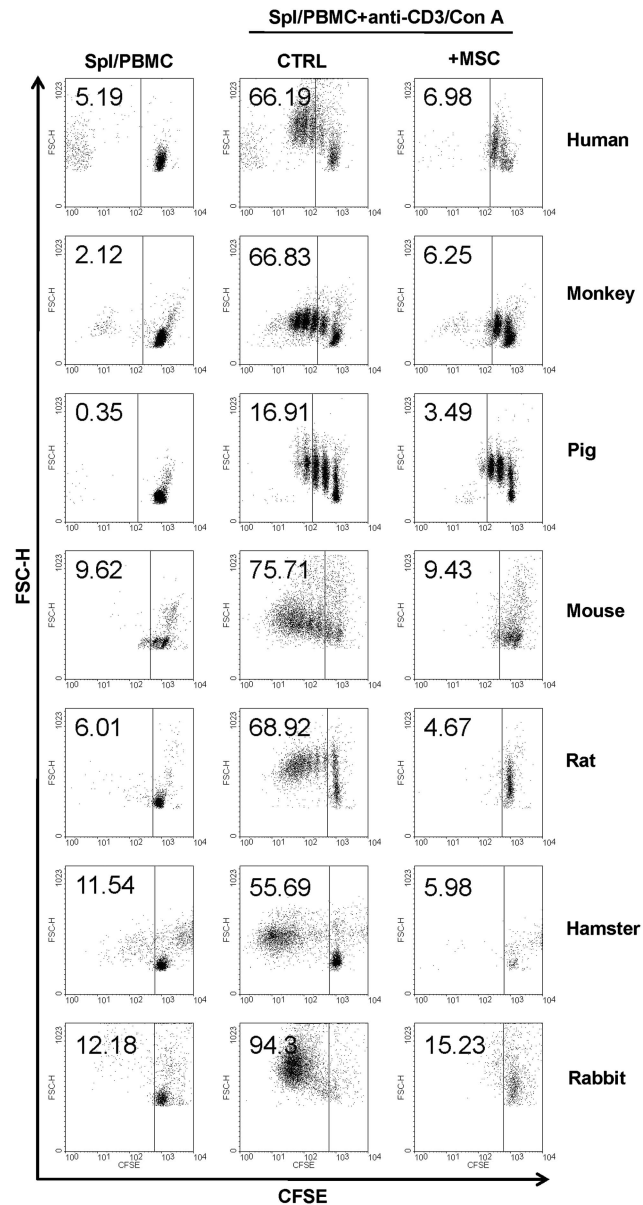


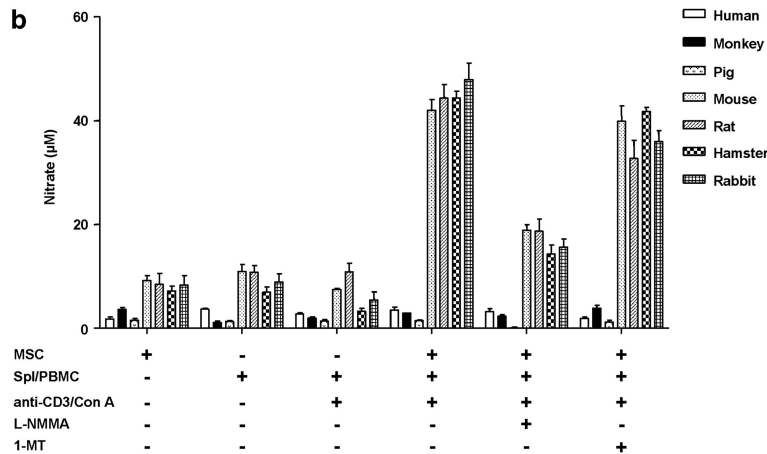
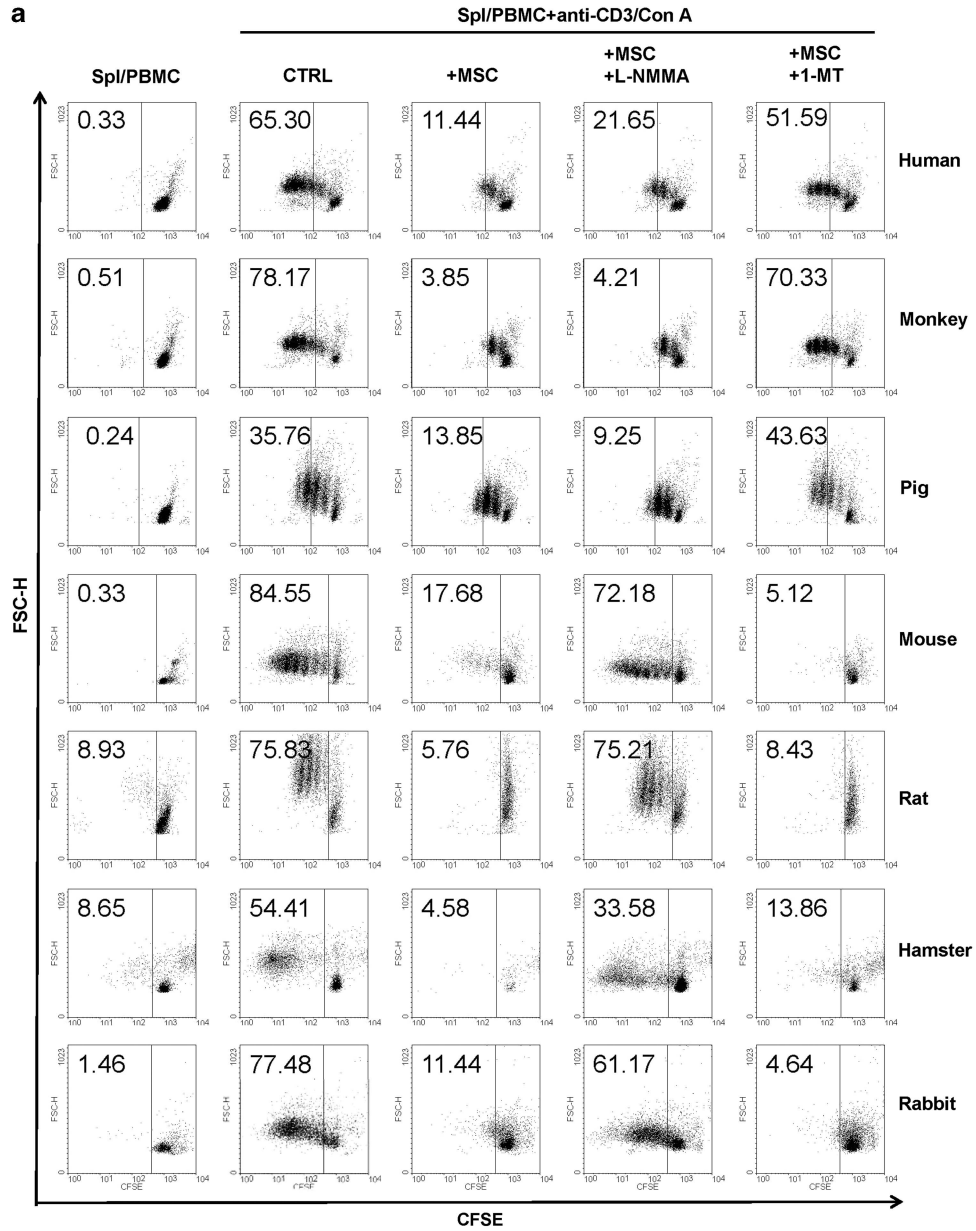
Figure 4 BM-MSCs isolated from different species have the same immunosuppression effects on Spls or PBMCs. PBMCs or Spls stained with CFSE were cocultured with BM-MSCs (2.0×10^4 cells per well in 96-well plate) at a ratio of 1 : 10 (MSC: T cell) for 96 h, in the presence of concanavalin A (Con A) ($1 \mu\text{g/ml}$; for rat, rabbit, hamster, and pig), or anti-CD3 ($1 \mu\text{g/ml}$; for human, monkey, and mouse). CFSE fluorescence intensity reduction of PBMCs or Spls was detected by flow cytometry. Data are representative of three independent experiments

peripheral nerve injury repair¹⁹ and myocardial infarction treatment,⁴⁴ respectively. Accordingly, MSCs have been proposed as a promising therapeutic strategy for disorders that benefit from their immunomodulation. Remarkably, MSCs from human and mouse exhibited distinct mechanisms of suppressing T-cell proliferation. In this study, we prepared BM-MSCs from human, monkey, pig, rabbit, hamster, rat, and mouse. These cells showed characteristics consistent with a series of universal standards for identifying MSCs: fibroblast-like morphology, the unique surface marker profile, $\text{CD105}^+/\text{CD73}^+ \text{CD29}^+ \text{CD44}^+ \text{CD45}^-$, and the ability to differentiate into adipocytes and osteoblasts. Experiments using specific inhibitors demonstrated that the effector molecules of MSC-mediated immunosuppression fall into two categories, mainly iNOS or mainly IDO, which is associated with the evolutionary relationships of the species.

Given that MSCs from different species employ distinct immunoregulatory molecules, we asked whether the divergent mechanisms could be ascribed to their different phylogenetic relationships. According to Carl von Linne's classification system, mouse, hamster, and rat are members of the taxonomic order rodentia. Notably, rabbit belongs to lagomorpha, which was formerly classified into rodentia. By contrast, human and monkey are primates, whereas pig belongs to artiodactyla. Recently, Song *et al.*⁴⁵ verified this using phylogenomics and the multispecies coalescent model. An iNOS phylogenetic tree was constructed on the basis of amino-acid sequence of commonly used laboratory animals, including monkey, orangutan, pig, horse, cow, dog, mouse, rat, hamster, and rabbit (Figure 6) using the neighbor-joining method.⁴⁶ From this iNOS phyletic tree, we found that current popular laboratory animals fall into one of five orders: primates, artiodactyla, carnivora, lagomorpha, or rodenti. All seven of the species we examined are located at a homologous clade that fits with the evolutionary genetics. This result strongly supports our hypothesis that species with the same mechanism of MSC-mediated immunosuppression are more closely related in evolutionary genetics.

As the use of MSC-based therapy for the treatment of disease is becoming generally accepted by both investigators and physicians, it is urgent to decipher which are the most suitable animal models for studying the clinical application of MSCs. Of course, we cannot determine empirically the mechanisms for each and every species of laboratory animal, but of the seven species examined here, we found that the mechanisms of MSC-mediated immunosuppression can be divided into two groups, one dependent on IDO and the other relying on iNOS. These two groupings seem to be similarly defined according to relationships in iNOS evolution,^{47,48} based on our analysis of an iNOS phyletic tree. Therefore, the mechanism of MSC-mediated immunosuppression employed by a particular animal species can be estimated by its evolutionary relationship to other well-defined species.

Figure 5 BM-MSCs isolated from different species have different immunosuppression mechanism. (a) The immunosuppressive abilities of MSCs derived from different species. CFSE-stained PBMCs or Spls isolated from rat, rabbit, hamster, or pig were stimulated with concanavalin A (Con A) ($1 \mu\text{g/ml}$), or from human, monkey, or mouse were stimulated with anti-CD3 ($1 \mu\text{g/ml}$) in the presence of corresponding BM-MSCs (2.0×10^4 cells per well in 96-well plate) at a ratio of 1 : 10 (MSC: T cell) for 96 h. iNOS inhibitor L-NMMA (1 mM) or IDO inhibitor 1-MT (0.5 mM) were added into the coculture system. CFSE fluorescence intensity reduction of PBMCs or Spls was detected by flow cytometry. Data are representative of three independent experiments. (b) NO production in the MSC and activated Spls/PBMCs cocultures of different species was determined by Griess reagent



Utilization of the most appropriate animal models to predict the effects of MSC-based therapy in humans will be aided by this knowledge and lead to better and safer clinical use of MSCs.

Materials and Methods

Animals. BALB/c, C57BL/6 mice (*Mus musculus*), and Sprague–Dawley rats (*Rattus norvegicus*) were obtained from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences, Shanghai, China. New Zealand rabbits (*Oryctolagus cuniculus*) were from the Laboratory Animal Center of Chedun, Shanghai, China. Syrian hamsters (*Mesocricetus auratus*) were purchased from Vital River, Beijing, China. Rhesus macaques (*Macaca mulatta*) were from Kunming Institute of Zoology. All animals were maintained in the vivarium of Shanghai Jiao Tong University School of Medicine. The protocols used for animal experiments were approved by the Institutional Animal Care and Use Committee of the Institute of Health Sciences, Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences, and Shanghai Jiao Tong University School of Medicine.

Reagents. CFSE, indomethacin, dexamethasone, insulin, 3-isobutyl-1-methylxanthine, L-ascorbic acids, β-glycerophosphate, alizarin red, oil red O, formaldehyde solution, L-NMMA, 1-MT, and concanavalin A were purchased from

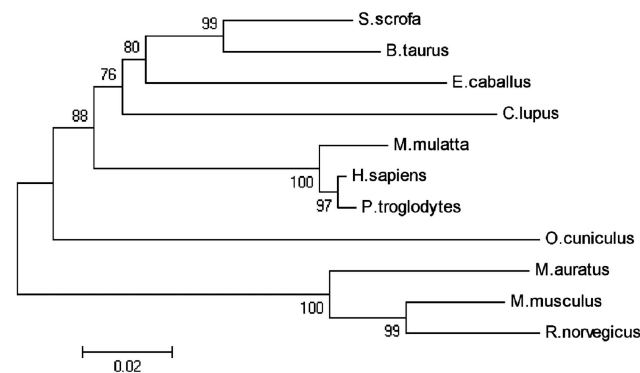


Figure 6 iNOS-based phylogenetic tree. The iNOS-coding region of amino-acid sequences of different species were used for evolutionary relationship alignment by neighbor-joining method. Numbers at nodes indicate the bootstrap support values. Domestic pig: *Sus scrofa domestica*; bovine: *Bos taurus*; horse: *Equus caballus*; domestic dog: *Canis lupus*; rhesus macaques: *Macaca mulatta*; human: *Homo sapiens*; chimpanzee: *Pan troglodytes*; New Zealand rabbits: *Oryctolagus cuniculus*; Syrian hamsters: *Mesocricetus auratus*; BALB/c, C57BL/6 mice: *Mus musculus*; Sprague–Dawley rats: *Rattus norvegicus*

Table 2 Primers for MSCs marker detection by PCR

Primer	Forward primer (5'–3')	Reverse primer (5'–3')	Primer	Forward primer (5'–3')	Reverse primer (5'–3')
Human GAPDH	GAAGGTGAAGTCCGGAGT	GAAGATGGTATGGGATTTT	Mouse CD29	TGGTCAGCAACGCATATCT	TTGTCCATCATTGGGTA AAAAC
Human CD105	ACGCTCCCTCTGGCTGTTG	GCCCTTCGAGACCTGGCTAG	Mouse CD44	CGTCCAACACCTCCCCTAT	AGCGCTGCTGACATCGT
Human CD73	CAGCGAGGACTCCAGCAAG	TATCCAACGATCCCAACT	Mouse CD45	GTTTTTCGCTACATGACTGCACA	AGGTTGTTCCAACTGACATCTTTT
Human CD29	GCATCCCTGAAAGTCCCAAG	CACTGTCCGACAGCGCACT	Rat GAPDH	GCAAGTTCAACGGCAGAC	CGCCAGTAGACTCAGCAGACA
Human CD44	GTGATGGCACCCGCTATG	ACTGTCTTCGTCTGGGATGG	Rat CD105	CGGTTGTGATCTACAGCGTG	CCCAGTCTCAGTGCCATTT
Human CD45	AACAACCACTCTGAGCCCTCT	CCCCTGGTGGCAGATCTAATA	Rat CD73	GCAGCCATCAAAGCAGACAT	AGCGGAGCCATTTCAGGTAGA
Monkey GAPDH	ACTTCAACAGCGACACCCACTC	CCCTGTTGCTGTAGCCAAATTC	Rat CD29	GCCAAACCAAGTGACATAGAGA	TCAAATAGGGTAGTCTTCAGCC
Monkey CD105	GTGGCATGGAAGTGACGG	TTGGGACGCAGGGCTAT	Rat CD44	TGGCAGCAGCAGCAGATC	CTGCACAGATAGCGTTGG
Monkey CD73	CAGCGAGGACTCCAGCAAG	ATGGCATCGTAGCGTAGGG	Rat CD45	AACAACCGACGATGGACTGG	GCCGTGAGTGGGTGAGGTC
Monkey CD29	GCACCAAGCCATTTAGC	GCCACCAAGTTTCCCCTC	Rabbit GAPDH	TTTGTGATGGGCGTGAACC	CCCTCCACAATGCCGAAGT
Monkey CD44	TCGCTACAGCATCTCTCGGAC	CATACTGGGAGGTGTTGGACG	Rabbit CD105	GCCAGCGTTGCGTCCCT	TGTTCTTCGCGGGGGTG
Monkey CD45	GGATCTCAGCAAACGGGAATA	AGCTTTTCTGTGGCACCAGT	Rabbit CD73	CTCCTTTCTCTCAAATCCAG	GTCCACGCCCTTCACTTTC
Pig GAPDH	AAGGTGCGAGTGAACGGATTCT	CATTGTATGTTGGCGGGAT	Rabbit CD29	AGAATGTCACCAACCGTAGCA	CACAAAGGAGCCAAACCCA
Pig CD105	AGTGCGACCTACAGCCTGTG	GCCAAAGTGGAGTGGGATTTT	Rabbit CD44	GGATGGCACCAGCTACA	GGAGACCCACTGCTCAGC
Pig CD73	TGTTGGTAATGAAGTCTGTG	GGAGGTGACTATGAATGGTA	Rabbit CD45	TACTCTGCCTCCGGTTG	GCTGAGTGTCTGCGTGTG
Pig CD29	GATGAATGAAATGAGGAGGAT	CCAGCAAAGTGAACCCA	Hamster β-actin	CCCATCTATGAGGGCTACGC	CAGGAAGGAGGCTGGAAAC
Pig CD44	CCACCTTCGACTACTACGC	ATTGCCCTGGGTTGTGCTTG	Hamster CD105	ACCGCACAGTACCCTAAATG	AAGTACTGCTACAATGCTGGCC
Pig CD45	AACAAGTGGATGTCTATGGCTA	AGAAGGTTCACTGGGTGGTC	Hamster CD73	ATCGTGGTGGGAGGACA	GCTGTTCAGAAGATGGGAT
Mouse GAPDH	TTCAATGGCAACGCTAAGGC	TCACCCCATTTGATGTTAGCG	Hamster CD29	CTACTCCATGAAGATGATCTGG	GCAAAAGTGAACCCAGCAT
Mouse CD105	TTGAATGGCAACCCAGCAGC	GAGCCTGACCGGAAACTGAT	Hamster CD44	GCGCACTAGATTCTCCGGT	GTATCGTAGTGGGAGTGTGG
Mouse CD73	GGCTGCTTCTCGCACTGA	CTGGTACTGGTCTCCGGC	Hamster CD45	GTTACGTTGACATCTTCCTAT	GAAAGCATTAAACCTCCTCG

Sigma-Aldrich (St. Louis, MO, USA). Purified antibodies against human CD3 and mouse CD3 were purchased from eBioscience (La Jolla, CA, USA). Anti-monkey CD3 antibody was purchased from Abcam (Cambridge, MA, USA). Ficoll was purchased from GE Healthcare (Piscataway, NJ, USA). EZ-Sep Mouse 1X was purchased from Dako Biotechnology (Shenzhen, China). Heat-inactivated fetal bovine serum (FBS), DMEM low-glucose medium, RPMI-1640 medium, trypsin-EDTA, basic fibroblast growth factor (bFGF), glutamax, and penicillin-streptomycin solution were purchased from Invitrogen (Carlsbad, CA, USA).

Cells. Human MSCs were generated from human BM according to the approved protocol by the Institutional Review Board of Robert Wood Johnson Medical School. Human BM aspirates were diluted with equal parts RPMI-1640, overlaid on Ficoll, and centrifuged at 400 × g for 30 min at 18–20 °C. Interface cells were collected, washed twice with RPMI-1640, and the cell pellet was resuspended in DMEM complete medium (DMEM low-glucose medium supplemented with 10% heat-inactivated FBS, 2 mM glutamax, 100 U/ml penicillin, and 100 μg/ml streptomycin). Cells were cultured at 37 °C in a humidified environment containing 5% CO₂. After 48 h, non-adherent cells were washed away and supplied with fresh DMEM complete medium supplemented with bFGF (5 ng/ml), with medium renewal every 3 days. Cells were collected and cryopreserved after confluence.

Monkey MSCs were obtained from rhesus monkeys BM according to the protocol by the Institutional Animal Care and Use Committee at Kunming Zoology Institute. To obtain BM from monkey, or pig, the animal was first anesthetized using amine (30–50 mg/kg), and then 2–5 ml of BM was aspirated using a 12-gauge needle and 10 ml heparinized syringe. For monkey and pig MSCs, non-adherent cells were removed after 24 h or 48 h, respectively. Cells were then purified and cultured, as for human MSCs (above). Cells were passaged using 0.25% trypsin-EDTA after reaching about 80% confluence.

BM from mouse, rat, and hamster was obtained from adult animals killed by cervical dislocation and from rabbits killed by air embolism. BM was flushed from the tibia and femur bones with 3 × penicillin/streptomycin in phosphate-buffered saline (PBS). Unpurified cells were cultured without disturbance for 24 h, then washed to remove non-adherent cells, and supplied with fresh DMEM complete medium supplemented with bFGF (5 ng/ml), with medium renewal every 3 days.

PBMCs from human, monkey, and pig were purified from fresh whole blood using ficoll density gradient centrifugation.

Mouse, rat, hamster, and rabbit Spls were obtained using EZ-Sep mouse density gradient centrifugation.

PCR reaction. The mRNA expression of specific cell surface markers was detected by reverse transcription-polymerase chain using specific primers. The primer sequences used for GAPDH, β-actin, CD105, CD73, CD29, CD44, and CD45 are listed (Table 2). Briefly, total RNA was extracted by RNAprep Pure Cell/Bacteria Kit (Tiangen Biotech, Beijing, China). First-strand cDNA synthesis was performed by 1st cDNA Synthesis Kit (Tiangen Biotech). PCR was amplified by Takara rTaq (TaKaRa Biotech, Dalian, China). Target gene size was judged by 100-bp DNA ladder on agarose gel electrophoresis (TaKaRa Biotech).

Adipogenesis and osteogenesis. Adipocyte differentiation was achieved by culturing BM-MSCs to 90% confluence in adipocyte differentiation medium consisting of 0.5 mM 3-isobutyl-1-methylxanthine, 60 μ M indomethacin, 10 nM dexamethasone, 10 μ g/ml insulin, and 2% FBS in DMEM complete medium, for 1–2 weeks, with medium renewal every 3 days. Oil red O staining solution was used to reveal lipid accumulations.

For osteoblast differentiation, BM-MSCs at 100% confluence were maintained in osteoblast differentiation medium (10 nM dexamethasone, 100 μ M L-ascorbic acids, 10 mM β -glycerophosphate I, and 2% FBS in DMEM complete medium) for 3–4 weeks. Resulting calcium deposition was stained using 2% Alizarin Red S solution.

Proliferation assay. The proliferation of Spls or PBMCs was detected using CFSE staining and flow cytometry. Freshly isolated Spls or PBMCs ($<2 \times 10^7$ cells) in 1 ml of PBS containing 0.1% bovine serum albumin were stained with 5 μ M CFSE for 10 min in a 37 °C water bath, with vortexing every 2 min. The reaction was stopped by adding 0.5 ml cold FBS. After 5 min, cells were washed with 5% FBS in PBS. Cells were then cocultured with MSCs at an MSC-to-Spl (or PBMC) ratio of 1:10. After 4 days, cells were collected and the remaining cell-associated CFSE fluorescence was analyzed by flow cytometry.

NO detection. The supernatant of MSCs and activated Spls/PBMCs cocultures was collected and stored at –20 °C. NO concentration was assessed by determining the NO_3^- and NO_2^- levels using Griess reagent (modified; Sigma-Aldrich). NaNO_2 served as a standard.

Conflict of Interest

The authors declare no conflict of interest.

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Author Contributions

JS contribute to conception and design, collection and assembly of data, data analysis and interpretation, and manuscript writing; WL, YH, and YW contributed to collection and assembly of data, data analysis and interpretation, and manuscript writing; XC, FY, KC, and GC performed data analysis and interpretation; AIR contributed to manuscript writing; WJ, GR, and LZ contributed to conception and design, data analysis and interpretation; JL, FL, and HK performed data collection; YS contributed to conception and design, manuscript writing, final approval of manuscript, and financial support.

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