

Stem Cells and Development

Stem Cells and Development: <http://mc.manuscriptcentral.com/scd>

Licensing by inflammatory cytokines abolishes heterogeneity of immunosuppressive function of mesenchymal stem cell population

Journal:	<i>Stem Cells and Development</i>
Manuscript ID:	SCD-2014-0581.R2
Manuscript Type:	Original Research Report
Date Submitted by the Author:	n/a
Complete List of Authors:	Szabó, Enikő; Biological Research Centre, Hungarian Academy of Sciences, Institute of Genetics Fajka-Boja, Roberta; Biological Research Centre, Hungarian Academy of Sciences, Institute of Genetics Kriston-Pál, Éva; Biological Research Centre, Hungarian Academy of Sciences, Institute of Genetics Hornung, Ákos; Biological Research Centre, Hungarian Academy of Sciences, Institute of Genetics Makra, Ildikó; Biological Research Centre, Hungarian Academy of Sciences, Institute of Genetics Kudlik, Gyöngyi; National Blood Service, Stem Cell Biology Unit Uher, Ferenc; National Blood Service, Stem Cell Biology Unit Katona, Róbert; Biological Research Centre, Hungarian Academy of Sciences, Institute of Genetics Monostori, Éva; Biological Research Centre, Hungarian Academy of Sciences, Institute of Genetics Czibula, Ágnes; Biological Research Center, Institute of Genetics
Keyword:	MSC
Abstract:	When mesenchymal stem cells (MSC) are used for therapy of immunological pathologies, MSCs get into an inflammatory environment altering the effectiveness of the treatment. To establish the impact of environmental inflammatory factors on MSC's immunofunction in the mirror of intrinsic heterogeneity of mouse MSC population, individual MSC clones were generated and characterized. Adipogenic but not osteogenic differentiation and pro-angiogenic activity of five independent MSC cell lines were similar. Regarding osteogenic differentiation, clones MSC3 and MSC6 exhibited poorer capacity than MSC2, MSC4 and MSC5. To study the immunosuppressive heterogeneity, in vitro and in vivo experiments have been carried out using T-cell proliferation assay and delayed-type hypersensitivity (DTH) response, respectively. Remarkable difference was found between the clones in their ability to inhibit T-cell proliferation in the following order: MSC2≥MSC5>MSC4>MSC3>>MSC6. Nevertheless, the differences between the immunosuppressive activities of the individual clones disappeared upon pre-treatment of the cells with pro-inflammatory cytokines, a procedure called licensing. Stimulation of all clones with IFN-γ

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	and TNF- α resulted in elevation of their inhibitory capability to similar level. Nitric oxide (NO) and prostaglandin E2 (PGE2) were identified as major mediators of immunofunction of the MSC clones. The above findings were also supported by in vivo results. Without licensing, MSC2 inhibited, while MSC6 did not affect DTH response. In contrast, pre-stimulation of MSC6 with inflammatory cytokines resulted in strong suppression by this clone as well. Here we have showed that MSC population is functionally heterogeneous in term of immunosuppressive function, however, this variability is largely reduced under pro-inflammatory conditions.

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14 Enikő Szabó*, Roberta Fajka-Boja*, Éva Kriston-Pál, Ákos Hornung, Ildikó Makra, Gyöngyi
15 Kudlik¹, Ferenc Uher¹, Róbert László Katona, Éva Monostori, Ágnes Czibula[#]
16
17

18
19
20 Institute of Genetics, Biological Research Centre, Hungarian Academy of Sciences, Szeged,
21 Hungary
22

23
24 ¹ Stem Cell Biology Unit, National Blood Service, Budapest, Hungary
25

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28 Running title: Heterogeneity of immunological function of MSCs
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30
31 *Authors contributed equally to the paper
32

33 # Corresponding author:
34

35 Ágnes Czibula
36

37 Institute of Genetics, Biological Research Centre, Hungarian Academy of Sciences
38

39 62 Temesvári krt, Szeged, 6726 Hungary
40

41 Tel: 36-62-599-684, Fax: 36-62-433-503
42

43 E-mail: czibula.agnes@brc.mta.hu
44
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60

Authors' informations:

Enikő Szabó, Roberta Fajka-Boja, Éva Kriston-Pál, Ákos Hornung, Ildikó Makra, Róbert László

Katona, Éva Monostori:

Institute of Genetics, Biological Research Centre, Hungarian Academy of Sciences

62 Temesvári krt, Szeged, 6726 Hungary

Tel: 36-62-599-684, Fax: 30-62-433-503

E-mail addresses:

ES: szabo.eniko@brc.mta.hu

RFB: fajka_boja.roberta@brc.mta.hu

EKP: kriston_pal.eva@brc.mta.hu

AH: hornung.akos@brc.mta.hu

IM: mildicko@gmail.com

RLK: katona.robert@brc.mta.hu

EM: monostori.eva@brc.mta.hu

Gyöngyi Kudlik and Ferenc Uher

Stem Cell Biology Unit, National Blood Service

64 Diószegi út, Budapest, 1113 Hungary

Tel: 36-1- 372-4151, Fax: 36-1-372 4352

E-mail addresses:

GK: gyongyi0888@gmail.com

FU: uher.ferenc@gmail.com

Abstract

When mesenchymal stem cells (MSC) are used for therapy of immunological pathologies, MSCs get into an inflammatory environment altering the effectiveness of the treatment. To establish the impact of environmental inflammatory factors on MSC's immunofunction in the mirror of intrinsic heterogeneity of mouse MSC population, individual MSC clones were generated and characterized. Adipogenic but not osteogenic differentiation and pro-angiogenic activity of five independent MSC cell lines were similar. Regarding osteogenic differentiation, clones MSC3 and MSC6 exhibited poorer capacity than MSC2, MSC4 and MSC5. To study the immunosuppressive heterogeneity, *in vitro* and *in vivo* experiments have been carried out using T-cell proliferation assay and delayed-type hypersensitivity (DTH) response, respectively. Remarkable difference was found between the clones in their ability to inhibit T-cell proliferation in the following order: MSC2 \geq MSC5>MSC4>MSC3>>MSC6. Nevertheless, the differences between the immunosuppressive activities of the individual clones disappeared upon pre-treatment of the cells with pro-inflammatory cytokines, a procedure called licensing. Stimulation of all clones with IFN- γ and TNF- α resulted in elevation of their inhibitory capability to similar level. Nitric oxide (NO) and prostaglandin E2 (PGE2) were identified as major mediators of immunofunction of the MSC clones. The above findings were also supported by *in vivo* results. Without licensing, MSC2 inhibited, while MSC6 did not affect DTH response. In contrast, pre-stimulation of MSC6 with inflammatory cytokines resulted in strong suppression by this clone as well. Here we have showed that MSC population is functionally heterogeneous in term of immunosuppressive function, however, this variability is largely reduced under pro-inflammatory conditions.

Introduction

Mesenchymal stem or stromal cells are adult multipotent fibroblast-like progenitor cells present in the bone marrow and virtually in all other organs [1]. The major criteria to define MSCs are the plastic adherence in standard culturing conditions, *in vitro* differentiation capacity toward mesodermal lineages *i.e.*, osteoblasts, chondroblasts and adipocytes and the expression of specific set of immunophenotypic markers [2]. Moreover, a powerful immunosuppressive activity also characterizes MSCs, particularly those isolated from the bone marrow or adipose tissue [3]. Due to the ability of differentiation into various tissues and immunomodulatory function MSCs have been suggested as a potential tool in regenerative and immunosuppressive medicine. Therefore, the safety and efficacy of using MSCs for the treatment of various pathological conditions have recently been a matter of intensive investigation [4,5]. Therapy of severe refractory acute graft-versus-host disease (GVHD) [6] has already received conditional approval in several countries. Moreover, treatment of myocardial infarction is one of the most successful clinical improvements being at phase II clinical trial [7,8]. Benefits of MSC therapy originate from that the MSCs promote tissue regeneration via differentiation and engraftment into the injured recipient tissue and/or production of wide panel of bioactive molecules supporting angiogenesis, stimulating endogenous stem cell recruitment and differentiation, modulating immune response by diminishing inflammation [9]. Although growing body of data has been accumulated suggesting clonal heterogeneity of MSCs, it is still not well established, how the heterogeneity of MSC population affects the therapeutical application.

The heterogeneity of MSCs has been demonstrated regarding morphology, cell surface marker expression, differentiation potential and transcriptome [10-12]. It was first proposed by Friedenstein and his colleagues who described variable osteogenic potential of bone marrow-

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9 derived fibroblastoid colonies *in vivo* [13]. This study was followed by more than a dozen studies
10 showing that MSC clones displayed various capacity of multilineage differentiation *in vitro* and
11 *in vivo* (reviewed in [10]). On the other hand, immunosuppressive functions of single cell-derived
12 MSC populations have been improperly compared. The few studies available resulted in
13 controversial data showing either similar or different immunomodulatory capacities of single
14 cell-derived MSC lines [14-16]. Recent findings have provided evidence that the
15 immunosuppressive function of these cells is modulated by an inflammatory microenvironment
16 [15,17]. Whether licensing by pro-inflammatory cytokines affects the immunosuppressive
17 function at clonal level has not yet been revealed.

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26 The current work aims to explore the heterogeneity of mouse bone marrow derived MSC
27 population with a special focus on the consequence of the heterogeneity to the immune function.
28 Differentiation properties and pro-angiogenic activities are characterized at clonal level. Our
29 results show that the analyzed 5 MSC clones are similar in immunophenotype, adipogenic
30 differentiation and pro-angiogenic activity. Two clones, MSC3 and MSC6 show poorer capacity
31 to differentiate toward osteogenic direction. Most importantly, remarkable differences are
32 observed in immunosuppressive capabilities between the individual clones both *in vitro* and *in*
33 *vivo*. However, the variability in the immune function disappears when MSCs are licensed by
34 inflammatory cytokines indicating that the functions of individual cells are largely dependent on
35 their surrounding environment.
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Materials and Methods

Cell culture

Mesenchymal stem cells were isolated from bone marrow of one C57BL/6 mouse and characterized as described previously [18,19]. The cells were cultured in DMEM (Gibco®) supplemented with 10% fetal bovine serum (FBS) (Gibco®), penicillin-streptomycin (Sigma-Aldrich) and 2 mM L-glutamine (Gibco®) (complete medium, CM) in a humidified incubator with 5% CO₂ at 37°C.

MSC culture at passage 10 was cloned with limiting dilution in DMEM/F-12 (Gibco®) supplemented with 10% FBS and 5% horse serum (Gibco®) by plating 1 cell/well in a 96-well culture plate (Orange Scientific). Five monoclonal MSC cultures labeled as MSC2-6 were randomly selected under a CKX41 inverted light microscope (Olympus Holding Europa GmbH) and further cultured up to 5-10 passages.

Flow cytometry

Cells (5×10^5 cells/sample) were labeled for 30 min at 4°C with various antibodies: anti-mouse Sca-1 labeled with phycoerythrin (PE), CD44-PE, biotin-conjugated CD119 (BD Pharmingen) followed by streptavidin-PE (Sigma-Aldrich). Mouse Mesenchymal Stem Cell Marker Antibody Panel (BD Pharmingen) was used to analyze CD11b, CD45, CD29 and CD106 according to manufacturer's instructions. The proper isotype matched immunoglobulins were used as controls. Cell analysis was performed by FACSCalibur flow cytometer (Becton Dickinson). Evaluation of the data was carried out with CellQuest™ software (Becton Dickinson).

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9 *Detection of adipogenic and osteogenic differentiation*

10 Adipogenic medium consisted of DMEM/F-12 supplemented with 10% FBS, penicillin-
11 streptomycin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich), 10 μ M
12 dexamethasone (Sigma-Aldrich), 66 μ M indomethacin (Sigma-Aldrich), 2 μ L/mL insulin (insulin
13 lispro injection, 100 U/mL, Humalog[®]) [20]. MSCs were maintained in adipogenic medium for 3
14 days, detached with 0.05 % trypsin-EDTA, fixed with 4% paraformaldehyde (Sigma-Aldrich)
15 and lipid droplets were stained with fluorescent AdipoRed[™] according to the manufacturer's
16 instructions (Lonza). Fluorescent lipid droplets were detected with Axioskop 2 Mot fluorescence
17 microscope (Carl Zeiss) using EC Plan-Neofluar 20x/0.5 objective (Carl Zeiss). For quantitation
18 of lipid accumulation in adipocytes, the cells (5×10^5 cells/sample) were labeled with
19 AdipoRed[™] as described above and quantified by FACSCalibur flow cytometer using
20 CellQuest[™] software.
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31 Osteogenic medium was composed of CM and 10 mM β -glycerophosphate (Sigma-
32 Aldrich), 50 μ g/mL L-ascorbic acid 2-phosphate (Sigma-Aldrich) and 0.1 μ M hydrocortisone
33 (Sigma-Aldrich). MSCs were cultured in osteogenic medium for 14 days then fixed with 8%
34 formaldehyde and stained with Alizarin Red S (Sigma-Aldrich) solution (pH 4.1) to detect
35 calcium deposition. Photomicrographs were taken with Olympus CKX41 inverted light
36 microscope and Olympus Camedia C-5060 camera (Olympus Holding Europa GmbH).
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45 *In vitro pre-vascular structure assay*

46 Co-culture of MSCs and mouse heart endothelioma cells, H5V (kindly provided by Vizler
47 C of HAS, BRC, Hungary) [21] was initiated in a ratio of 1 : 1 in a 24-well plate at a cell density
48 of 2×10^4 cells/well. Pre-vascular structure formation was evaluated as follows: 5 randomly
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9 selected non-overlapping areas of co-plated cells were examined by Olympus Cell-R
10 fluorescence microscope (Olympus Holding Europa GmbH) using UPlanSApo 4x/0.16 objective.
11 The lengths of pre-vascular structures were quantified by measuring with CellR Imaging
12 Software (Olympus Holding Europa GmbH) after 3 days of cell culture.
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18 *T-cell proliferation assay*

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20 First, MSCs were seeded on a 96-well plate at cell density of 5×10^3 cells/100 μ L CM in a
21 well. Mesenteric, inguinal and brachial lymph nodes from Balb/C mice were dissected. Lymph
22 nodes were disrupted mechanically to release most of the leukocytes. Leukocytes were harvested
23 by centrifugation and resuspended in phosphate buffered saline (PBS) containing 5% FBS at a
24 final concentration of 2×10^6 cells/mL. Carboxyfluorescein diacetate succinimidyl ester (CFSE,
25 CellTrace™ CFSE Cell Proliferation Kit, Life Technologies) was added to leukocytes at a final
26 concentration of 2.5 μ M and cells were incubated in dark for 5 minutes at room temperature.
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28 Leukocytes were washed twice with fresh RPMI culture medium (Gibco®) then 2×10^5 cells
29 were added to the pre-seeded MSCs in 100 μ L of RPMI containing 10% FBS, 50 μ M β -
30 mercaptoethanol (Sigma-Aldrich) and 5 μ g/mL Concanavalin A (ConA, Sigma-Aldrich). In
31 blocking experiments, nitric oxide synthase (NOS2), prostaglandin-endoperoxide synthase 2
32 (cyclooxygenase 2) (PTGS2) or indolamine-2,3-dioxygenase (IDO) inhibitors, 1 mM N^G-methyl-
33 L-arginine acetate salt, L-NMMA (Sigma-Aldrich), 10 μ M, indomethacin (Sigma-Aldrich) or
34 1 mM 1-methyl-L-tryptophan (Sigma-Aldrich), respectively were added to MSC/leukocyte co-
35 cultures. After 72 h of incubation, non-adherent leukocytes were collected, washed with PBS,
36 settled by centrifugation and resuspended in PBS supplemented with 1% FBS, 0.1% sodium-
37 azide (FACS-buffer) and 10 μ g/mL propidium-iodide. Cell proliferation was measured by flow
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9 cytometry. Evaluation of the data was carried out using CellQuest™ software (Becton
10 Dickinson) and ModFit LT™ Version 2.0. software (Verity Software House, Inc). T-cell
11 proliferation was evaluated as proliferating fraction (PF) calculated as follows: PF % = number of
12 proliferating T-cells / total number of T-cells × 100.
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16 In some experiments, MSCs were pre-treated with a mixture of 100 ng/mL mouse
17 recombinant IFN-γ (R&D Systems) and 50 ng/mL TNF-α (kindly provided by Duda E of HAS,
18 BRC Hungary) [22] for 24 h then washed twice with CM and T-cell proliferation was tested as
19 described above.
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24 25 26 *Western blot analysis*

27 Mesenchymal stromal cells were lysed (10^7 MSCs/mL) in RIPA buffer containing 10 mM
28 Tris•HCl and 150 mM NaCl (Molar Chemicals), 1% Triton X (Sigma-Aldrich), 0.5% sodium
29 deoxycholate (Reanal), 0.1% SDS (Sigma-Aldrich), 2 mM EDTA (Reanal) and 1 mM PMSF
30 (Sigma-Aldrich). The lysates were analyzed by Western blotting after running the samples on a
31 10% SDS-polyacrylamide gel then electroblotted onto a nitrocellulose membrane (Whatman®
32 Protran®). After blocking with 3% cold fish gelatin (Sigma-Aldrich) in Tris buffered saline (TBS)
33 and 0.05% Tween® 20 (Sigma-Aldrich), the membranes were incubated with rabbit anti-Gal-1
34 (produced in our laboratory, [19]) then HRP-conjugated anti-rabbit immunoglobulin (Dako).
35 Rabbit anti-β-actin antibody (Abcam) was used as a loading control. Immunoreactive proteins
36 were visualized with Amersham™ ECL Prime Western Blotting Detection Reagent (GE
37 Healthcare Life Sciences) and analyzed using EC3 Imaging System furnished with a CCD Chemi
38 410 Camera (Ultra-Violet Products). As molecular mass standard, Page Ruler™ Prestained
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Protein Ladder (Fermentas) was used. Density of the bands was measured by VisionWorks[®]LS Image Acquisition and Analysis Software (Ultra-Violet Products).

Quantitative real-time PCR

MSCs (10^5 cells/well) were plated in 35 mm diameter culture dishes in CM. After 24 h, fresh CM was added with or without 100 ng/mL IFN- γ and 50 ng/mL TNF- α then further incubated for 24 h. Finally, total RNA was extracted using Nucleospin RNA II isolation kit (MACHEREY-NAGEL GmbH) according to manufacturer's instruction. RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc.) was used to obtain cDNA from 2 μ g of total RNA/reaction. Quantitative PCR (qPCR) was performed using TaqMan[®] Gene Expression Master Mix (Life Technologies) for *Ptgs2*, *Nos2* and *Ido1* and AccuPower[®] 2X Greenstar qPCR Master Mix (Bioneer) for angiotensin-1 (*Angpt1*), vascular endothelial growth factor A (*Vegfa*), transforming growth factor beta 1 (*Tgfb1*) and galectin-1 (*Lgals1*) in RotoGene3000 instrument (Corbett Life Science). Relative gene expression normalized to glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was presented as $2^{-(Ct_{Gapdh}-Ct_{GOI})}$. TaqMan[®] Gene Expression Assays were commercially available for *Ptgs2* (Mm00478374_m1), *Nos2* (Mm01309902_m1), *Ido1* (Mm00492586_m1*) and *Gapdh* (Mm99999915_g1). Primers for qPCR were designed using Universal Probe Library Assay Design program (Roche Applied Science) for *Gapdh*, *Angpt1*, *Lgals1*, *Tgfb1* and *Vegfa*. The following primer sequences were used: *Gapdh* fwd: ttgatgttagtgggtctcg, rev: agcttgcatcaacgggaag; *Angpt1* fwd: eggatttctctccagaaac, rev: tccgacttcatatttccacaa; *Lgals1* fwd: ctctcgggtggagtctctg, rev:

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9 gggttgagattcaggttctg; *Tgfb1* fwd: tggagcaacatgtggaact, rev: cagcagccggttaccag; *Vegfa* fwd:
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11 aaaaacgaaagcgcaagaaa, rev: ttctccgctctgaacaagg.

12 13 14 *Induction of Delayed Type Hypersensitivity (DTH) response*

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16 Ovalbumin (1 mg/mL in PBS, Sigma-Aldrich) was emulsified at a 1 : 1 ratio in complete
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18 Freund's adjuvant (Sigma-Aldrich). Male C57BL/6 mice (6- to 8-weeks-old) were immunized by
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20 subcutan injection of 100 µg ovalbumin/animal. Subcutan injection of PBS with complete
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22 Freund's adjuvant served as a control. Cells (10⁶ MSCs/animal) or PBS were injected
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24 intraperitoneally in 200 µL volume. After 4 days, DTH was initiated by challenging with 250 µg
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26 heat-aggregated ovalbumin in 12.5 µL PBS or PBS alone injected into the right or left hind
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28 footpad, respectively. After 48 h, ovalbumin-induced footpad thickness was measured using a
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30 caliper and calculated as follows: (T_{48 hours} - U_{48 hours}) - (T_{0 hours} - U_{0 hours}), where T and U are
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32 thickness of ovalbumin treated and ovalbumin untreated footpads, respectively. All animal
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34 studies were approved by the Animal Care and Use Committee of the National Medical Center
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36 (Budapest, Hungary) in accordance with the national and international law and regulations of
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38 animal experiments.

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39 40 41 42 *Statistical analysis*

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44 Statistical analysis was performed using one-way analysis of variance test (ANOVA) in
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46 GraphPad Prism Version 6.03 (GraphPad Software). When significant ($p < 0.05$) by ANOVA,
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48 pairwise comparisons of experimental groups were carried out using Fisher's least significant
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50 difference (LSD) method as a *post hoc* test. For statistical analysis of qPCR data non-parametric
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9 Kruskal-Wallis test followed by Mann-Whitney test was used. Differences were considered
10 statistically significant with a p value less than 0.05 (set at *p < 0.05, **p < 0.01, ***p < 0.001).
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16 Results

18 Characterization of monoclonal MSC cell lines

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20 Each clone expressed uniformly Sca-1, CD29, CD44, CD106 and CD119 (Fig. 1) at similar
21 degree and none of the clones expressed lineage specific markers CD11b and CD45 (Fig. 1).
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24 *In vitro* osteoblast differentiation was assayed with Alizarin Red S staining showing that MSC2,
25 MSC4 and MSC5 exhibited strong, MSC3 and MSC6 weak *in vitro* osteoblast differentiation
26 (Fig. 2A). During adipocyte differentiation, all MSC clones accumulated comparable amount of
27 neutral lipids into intracellular lipid droplets (Fig. 2B, C), indicating that these cells had similar
28 adipogenic differentiation potential.
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35 *In vitro* pro-angiogenic activity of MSC clones

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37 We have previously reported that MSCs and H5V endothelial cells spontaneously
38 arranged into vessel-like structures within 3 days of co-culture [19]. Moreover, deficiency in
39 galectin-1, a pro-angiogenic lectin in MSCs resulted in reduced formation of pre-vascular
40 structures [19]. To determine whether individual MSC clones supported similarly or differently
41 the shaping of these structures, *in vitro* pro-angiogenic assays were performed with the
42 monoclonal MSC cultures. While neither MSC clones nor H5V endothelial cells formed pre-
43 vascular structures alone, co-culturing any of the clones with H5V resulted in arrangement into
44 pre-vascular structures (Fig. 3A) in similar extent (Fig. 3B).
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9 Analysis of pro-angiogenic factor expression by qPCR showed that all clones expressed similar
10 level of *Angpt1*, *Vegfa* and *Lgals1* mRNAs (Fig. 3C). Results of qPCR experiments (Fig. 3C)
11 were validated by Western blot analysis of galectin-1 protein (Supplementary Fig. S1)
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14 15 16 MSC clones vary in in vitro immunosuppressive activity 17

18 Immunomodulatory properties of MSC clones were assessed *in vitro* by analyzing their
19 effect on Con A-activated T-cell proliferation. Proliferation of T-cells was robustly diminished
20 in the presence of MSC2, MSC4 and MSC5 while poorly inhibited by MSC3 and MSC6 (Fig.
21 4A, B). Differences were significant and the order of their inhibitory capacity was as follows:
22 MSC2 \geq MSC5 > MSC4 > MSC3 \gg MSC6 (Fig. 4B).
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25 Our aim was to determine whether variability between the clones regarding their inhibitory
26 activity was reflected in the expression levels of immunomodulatory factors. Therefore, the
27 expression levels of mRNAs encoding NOS2, PTGS2 responsible for the production of anti-
28 inflammatory mediators, NO and PGE2, respectively and IDO1 creating a tryptophan deprived
29 environment were analyzed. Relative *Nos2* mRNA level well coincided with anti-proliferative
30 function of the clones as it was expressed at higher levels in MSC2, MSC5 (significant) and
31 MSC4 (not significant) than in MSC3 and was undetectable in MSC6 (Fig. 5A). Expression of
32 *Ptgs2* did not show any remarkable difference between the clones (Fig. 5B). The two enzymes,
33 NOS2 and PTGS2 seemed to be important in the inhibitory function of the most
34 immunosuppressive clone MSC2. The inhibition of T-cell proliferation by MSC2 was blocked
35 entirely with L-NMMA and partially with indomethacin, inhibitors of NOS2 and PTGS2,
36 respectively (Fig. 6, Unstimulated MSC). In contrast, the presence of these inhibitors did not
37 affect the activity of the least immunosuppressive clone MSC6 (Fig. 6, Unstimulated MSC).
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9 Expression of other immunomodulatory factors such as *Tgfb1* (Supplementary Fig. S2) and
10 *Lgals1* (Fig. 3C) were similar and *Ido1* mRNA expression couldn't be detected in any of the
11 clones (Supplementary Fig. S3A).
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14 To model the *in vivo* inflammatory environment in which MSCs act as anti-inflammatory
15 cells, the monoclonal cell lines were pre-treated with Th1 type cytokines, IFN- γ and TNF- α and
16 the effect of the licenced MSC on T-cell proliferation was analyzed. Licensing by pro-
17 inflammatory cytokines, MSC3 and MSC6 clones, which exerted poor inhibitory activity,
18 blocked the T-cell proliferation at similar extent to the clones with strong inhibition. In contrast,
19 licensing was not or barely able to further enhance immunosuppressive activity of MSC2, MSC4
20 and MSC5 (Fig. 4C). Expression of *Nos2* gene largely increased in all clones to an equal level
21 upon IFN- γ and TNF- α treatment. Nevertheless, the degree of elevation was the most remarkable
22 in clones with poor inhibitory activity (Fig. 5A). On the other hand, *Ptgs2* expression was also
23 upregulated in all clones, although much less than that of *Nos2* gene (Fig. 5B). Furthermore,
24 unstimulated MSC clones didn't express *Ido1*, unless they were pre-activated with IFN- γ and
25 TNF- α . Pro-inflammatory cytokine treatment triggered *Ido1* mRNA expression as well
26 (Supplementary Fig. S3A).
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39 To determine the potential role of anti-inflammatory mediators in the inhibition of T-cell
40 proliferation under inflammatory conditions, anti-proliferative activities of the clones were
41 blocked with inhibitors. Immunosuppressive activity of licenced MSC2 and MSC6 were assayed
42 in the presence of L-NMMA and confirmed the crucial role of NO. On the other hand, the
43 presence of indomethacin slightly affected MSC6 but not MSC2 activity (Fig. 6, MSC pre-treated
44 with IFN- γ + TNF- α) suggesting a less pronounced function of PGE2. In addition, neither MSC2
45 nor MSC6 activities were influenced by IDO1 inhibitor 1-MT, independently from IFN- γ and
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9 TNF- α -activation (Supplementary Fig. S3B) indicating that IDO1 did not participate in the
10 immunosuppression by these clones.
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12 13 14 Ovalbumin-induced Delayed Type Hypersensitivity (DTH) model supports in vitro findings 15

16 The effect of the most and least immunosuppressive MSC2 and MSC6 clones,
17 respectively, was tested in ovalbumin-induced DTH response in mice. MSC2 significantly
18 prevented the thickening of footpad while MSC6 injection resulted in no change compared to the
19 control (treatment without MSCs) (Fig. 7). Nevertheless, when pre-treated with IFN- γ and TNF- α
20 prior to injection, MSC6 diminished DTH reaction similarly to non-treated MSC2 clone while
21 immunosuppressive activity of MSC2 was not affected by licensing (data not shown).
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30 **Discussion**

31 It has been proposed that the heterogeneity of MSCs may be partially responsible for the
32 divergent outcomes of MSC-based clinical trials [11]. Several studies reported that various MSC
33 clones differed in differentiation potential into mesenchymal lineages [10]. Other functional
34 properties, such as pro-angiogenic effect has not yet been extensively investigated at clonal level.
35 In addition, the data regarding heterogeneity in immunosuppressive properties of MSC
36 population is insufficient and meets discrepancy. While Xu *et al.* argued that single cell-derived
37 MSC populations possessed uniform immunomodulating capacities [14] others demonstrated
38 large differences in immunosuppression at clonal level [15,16].
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46 In the present study, clonal diversity of murine bone marrow MSCs was examined with a special
47 focus on the *in vitro* and *in vivo* immunosuppression. Moreover, *in vitro* osteoblast and adipocyte
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9 differentiation and pro-angiogenic effect of the MSC clones were also compared as a part of the
10 characterization of the clones.

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12 In according to previous results regarding variable osteogenic differentiation of single cell-
13 derived human or murine MSCs [15,23-25] we found that the clones exhibited different
14 osteoblast potential *in vitro*. On the other hand, the analyzed clones similarly differentiated
15 toward adipocytes. It has recently been described that MSCs produce wide array of factors
16 influencing endothelial cell growth and angiogenesis [26] and thereby inducing capillary-like
17 structure formation *in vitro* [27,28]. We previously demonstrated that MSCs spontaneously
18 formed interconnected pre-vascular structures on a galectin-1 dependent fashion when co-
19 cultured with H5V endothelial cells [19]. However, only one paper analyzed angiogenic activity
20 of MSCs at clonal level [29]. Burns *et al.* reported that two telomerase-immortalized human MSC
21 (hMSC-TERT20) clones distinctly formed cord-like structures due to their different extracellular
22 matrix protein production and the key role of galectin-1 had been proven in *in vitro* and *in vivo*
23 angiogenesis in serum-deprived condition in spite of equivalent expression of two major pro-
24 angiogenic factors (VEGFA, ANGPT1) [29]. We analyzed the *in vitro* pro-angiogenic activity of
25 MSC2-6 clones in a pre-vascular structure assay in which MSCs were co-cultured with H5V
26 cells. The clones expressed comparable level of pro-angiogenic factors, including *Vegfa*, *Angpt1*
27 and *Lgals1* and accordingly, formed similar pre-vascular structures. The controversy between our
28 and Burns's results may derive from the different experimental conditions, mainly that human
29 MSCs were genetically modified and cultured under serum depriving condition.

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47 The major aspect of this work has been the comparison of the immunomodulatory activity
48 of MSCs at clonal level. The isolated MSC clones differently influenced the proliferation of Con
49 A-activated T-cells ranging from 30% to 70% of inhibition. In this term, MSC2, MSC4 and
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9 MSC5 strongly inhibited T-cell proliferation while inhibitory activities of MSC3 and MSC6 were
10 significantly weaker. Our results were in accordance with those published by others [15,16]
11 reporting difference between the immunosuppressive activities of single cell-derived MSC
12 clones. The diversity in the inhibition of T-cell proliferation could be explained by the different
13 expression of immunomodulatory gene *Nos2*, responsible for the generation of a crucial
14 mediator, NO. Elevated level of *Nos2* expression was found in naïve MSC2, MSC4 and MSC5,
15 the clones representing high inhibitory activity. Whereas it was expressed at a lower level or was
16 undetectable in MSC3 and MSC6, respectively, the clones slightly inhibiting T-cell proliferation.
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18 The central role of NO in inhibition of T-cell proliferation was also confirmed using NOS2
19 inhibitor, L-NMMA which blocked the inhibitory effect of the most immunosuppressive clone,
20 MSC2. These results were in good agreement with previous experimental data [15,16,30].
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22 Expression of *Ptgs2* playing role in PGE2 synthesis showed no significant difference among the
23 clones. Studies examining the function of PGE2 in immunosuppression by murine MSCs
24 produced controversial data namely PGE2 had either important [31] minor [30] or no role [14-
25 16]. Yet, the importance of PGE2 was unambiguously shown here by using an inhibitor of
26 PTGS2, indomethacin. Interestingly, blocking NOS2 resulted in full inhibition of T-cell anti-
27 proliferative effect of MSC2, the most immunosuppressive clone while indomethacin only
28 incompletely reversed T-cell growth. Our recent result [32] might serve an explanation for this
29 phenomenon; PGE2 secretion by MSC seems to be promoted by a NO-dependent signaling
30 pathway, since L-NMMA inhibits not only NO production but also partially diminishes PGE2
31 secretion, resulting in complete recovery of T-cell proliferation.
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48 Recently, a growing body of data supports the idea that the biological functions of MSCs
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9 Activation, termed as licensing [33] is triggered by pro-inflammatory cytokines released from
10 immune cells at the site of inflammation [15,34], albeit the polarization toward anti-inflammatory
11 phenotype may depend on the concentration and duration of the stimulating agent or the type of
12 the factor itself [33]. The site of inflammation abounds in pro-inflammatory mediators, including
13 IFN- γ and TNF- α [35]. For priming of MSC's immunosuppressive function, the combination of
14 IFN- γ and TNF- α or IL-1 α/β was recommended [15]. The production of numerous cytokines and
15 chemokines involved in MSCs' T-cell inhibitory machinery in mouse or in human (IL-6, IL-8,
16 IL-10, HGF, PD-L1, PGE2, NO and IDO1) are induced by priming MSCs with IFN- γ and TNF- α
17 [15,31,36,37]. In the present study, the combination of IFN- γ and TNF- α was applied for the
18 priming of MSC inhibitory activity to model an inflammatory milieu. The pre-treatment of MSC
19 clones with IFN- γ and TNF- α equated the inhibition of T-cell proliferation hence clones, MSC3
20 and MSC6, inhibiting poorly the T-cell proliferation, acquired similarly strong inhibitory
21 activities to those of MSC2, MSC4 and MSC5. This result indicated that stimulation of MSC
22 clones with IFN- γ and TNF- α enhanced the immunosuppressive effect of otherwise non-
23 functional single cell-derived MSC cultures. Indeed, licensing MSC clones resulted in a
24 remarkable upregulation of *Nos2* and *Ido1* and various moderate increase of *Ptgs2* gene
25 expression in every MSC clone. Examining the role of inflammatory cytokines we found that the
26 NOS2 expression was essential since its inhibitor L-NMMA reversed T-cell proliferation in
27 licensed MSC2 and MSC6 clones. Inhibition of PGE2 synthase reversed only partially the
28 suppression by MSC6 but did not affect that by MSC2. These results might allow the conclusion
29 that MSC population has an intrinsic heterogeneity regarding the mediators of the
30 immunosuppressive function, such as PTGS2.
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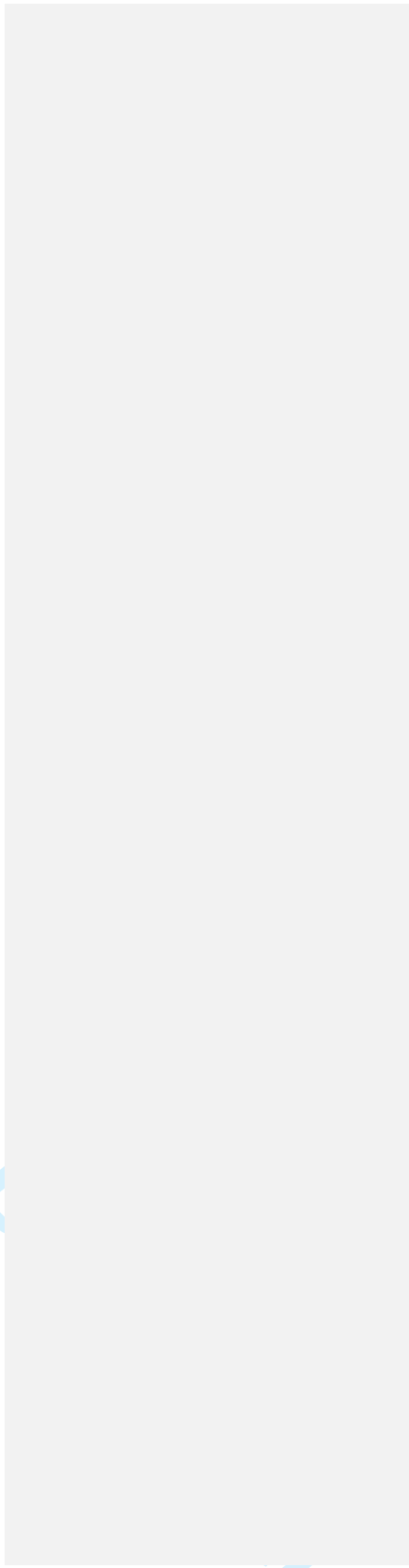
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9 Induction of *Ido1* mRNA expression under inflammatory conditions was the most striking since
10 naïve clones did not express the gene at all whereas remarkable increase occurred upon IFN- γ
11 and TNF- α treatment. In spite of the high gene expression of *Ido1*, IDO1 inhibitor, 1-MT did not
12 affect activities of licensed MSC clones. Although IDO1 is an important factor in human MSC
13 [38], its function in mouse MSCs is inconsistent. Even though *Ido1* mRNA expression is
14 inducible with pro-inflammatory cytokines, the IDO1 protein expression is undetectable in mouse
15 MSC [39]. One possible explanation was served by Hucke *et al.* showing that an accelerated
16 proteosomal degradation of IDO protein occurred upon NO production [40]. Accordingly,
17 chemical inhibition of IDO1 in mouse MSCs did not affect MSC function on T-cell proliferation.
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28 Experimental animal models clearly revealed that MSCs could be effective cell-based
29 therapies in immunological pathologies [17,18,41-43]. Whether *in vivo* immunoregulatory
30 property of MSCs was reflected at clonal level the effects of the most and least
31 immunosuppressive clones, MSC2 and MSC6, respectively, were examined in ovalbumin-
32 induced delayed-type hypersensitivity (DTH) model. The *in vitro* results were validated *in vivo*,
33 since MSC2 diminished whereas MSC6 did not affect DTH response when naïve MSC clones
34 were applied. In contrast, pre-treatment of MSC6 with licensing cytokines IFN- γ and TNF- α
35 prior to intraperitoneal injection resulted in similar blocking effect of MSC6 to that of MSC2
36 indicating the increasing immunosuppressive function of an otherwise poorly acting clone. Our
37 results were supported by the data obtained in GVHD model since pre-activation of MSCs
38 resulted in more effective prevention of the disease [17].
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48 Taken together, our experiments clearly show that murine MSC population possesses
49 intrinsic heterogeneity regarding the immunosuppressive function of single cell-derived MSC
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clones. Variations between the clones can be at least partly, explained by the different expression of immunoregulatory factors. In this sense, NO is the key mediator while PGE2 is involved and IDO1 has no role in the suppressive activity of MSC clones. An important novel message of this work is that variability in the immune function of the different MSC clones can be abolished by licensing the cells by inflammatory cytokines prior to application for immunological assays or therapy of immune disorders.



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Acknowledgements

The authors would like to thank Mrs. Andrea Gercsó for the excellent technical assistance, Edit Kotogány and Nóra Fehér for flow cytometric analysis. Csaba Vizler, Ernő Duda and János Haskó are acknowledged for the generous gifts of H5V cell line and recombinant TNF- α and helping us with the animals, respectively. This work was supported by TÁMOP-4.2.2.A-11/1/KONV-2012-0035. Roberta Fajka-Boja has been supported by János Bolyai Research Fellowship of the Hungarian Academy of Sciences.

Author Disclosure Statement

The authors declare no competing financial interests.

Figure legend

FIG. 1. Expression of cell surface markers of monoclonal MSC lines. MSC clones 2-6 were labeled with antibodies to CD11b, CD45, Sca-1, CD29, CD44, CD106 or CD119 (black lines). The negative controls were isotype matching immunoglobulins (grey shadowed area). Samples were then analyzed with flow cytometry.

FIG. 2. *In vitro* adipogenic and osteogenic differentiation of MSC clones. Cells were induced to differentiate toward osteoblastic (A) and adipocytic (B) lineage. In osteogenic cultures calcium accumulations were stained with Alizarin Red S on the 14th day (A). Adipogenic cultures were fixed and stained with AdipoRed™ on the 3rd day (B) and lipid accumulation was quantified by flow cytometry (C). The pictures are representatives of 3 independent experiments. Scale bar = 50 μm (A and B)

FIG. 3. Monoclonal MSC lines exhibit similar pro-angiogenic activity. MSCs were co-cultured with or without H5V in 1 : 1 ratio for 3 days. Pre-vascular structures were visualized with Olympus Cell-R fluorescence microscope using UPlanSApo 4x/0.16 objective (representative images selected from three independent experiments) (A). Lengths of the structures were measured in 5 randomly selected non-overlapping areas of co-plated cells using CellR Imaging Software. Averages and deviations were calculated using data of three independent experiments (B). Expressions of pro-angiogenic factors, *Angpt1*, *Vegfa* and *Lgals1*, were determined from total mRNA extract prepared from MSC2-6 with qPCR. *Gapdh* expression served as internal control. Results of three independent experiments were used to draw the graphs (C).

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4 of CFSE labeled T-cells under different conditions are presented (representative
5 experiment) (A). Freshly isolated, CFSE-labeled lymph node cells were co-cultured
6 with or without MSC clones at 40 to 1 ratio in the presence of Con A. The day before
7 co-culture with T-cells MSC were plated without (B) or with 100 ng/mL IFN- γ and
8 50 ng/mL TNF- α for 24 hours (C). T-cell proliferation was evaluated after 72 hours
9 by flow cytometry and data were analyzed by ModFit LTTM software. The data shown
10 on (B) and (C) are the means of 5 independent experiments. Each experiment was
11 carried out in triplicates (n = 15). n.s. = not significant, p > 0.05, * p < 0.05; **
12 p < 0.01; *** p < 0.001.
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27 FIG. 5. Expression levels of genes affecting immunosuppressive function of MSCs.
28 Total RNA was extracted from cells cultured for 24 h in the absence or presence of
29 100 ng/mL IFN- γ and 50 ng/mL TNF- α and expression levels of *Nos2* (A), *Ptgs2* (B)
30 were analyzed by qPCR. *Gapdh* served as a housekeeping gene. The results are the
31 averages of 2 independent experiments +S.E.M.
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41 FIG. 6. The role of NO and PGE2 in the inhibition of T-cell proliferation by MSC2
42 and MSC6. MSCs were plated on 96-well plates, left unstimulated or pre-activated
43 with 100 ng/mL IFN- γ and 50 ng/mL TNF- α for 24 hours. After 24 hours, MSCs were
44 washed with DMEM, co-cultured with freshly isolated, CFSE-labeled lymph node
45 cells in 1 to 40 ratio with or without 1mM L-NMMA or 10 μ M indomethacin (Indo).
46 Proliferation of T-cells was stimulated by Con A. After 72 hours of co-culture of
47 MSCs and T-cells, proliferation of T-cells was assessed by cytofluorometry and data
48 were analyzed by ModFit LTTM software. The figure shows the percent of
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3 proliferating T-cells.. Con A-activated T-cells cultured with or without L-NMMA or
4 indomethacin in the absence of MSCs served as positive controls. The data are the
5 representative of three independent experiments + SD. The experiments were
6 performed in triplicate. n.s. = not significant $p > 0.05$; * $p < 0.05$; ** $p < 0.01$;
7 *** $p < 0.001$.
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16 FIG. 7. Effect of intraperitoneal injection of MSC2 and MSC6 in ovalbumin-induced
17 delayed type hypersensitivity (DTH) model. Hypersensitivity was triggered in male
18 C57BL/6 mice with subcutaneous administration of ovalbumin. At the same time,
19 mice were injected intraperitoneally with 10^6 MSC2 or 10^6 MSC6 untreated or
20 pretreated with IFN- γ and TNF- α in PBS or with PBS alone. After 4 days, heat
21 aggregated ovalbumin was injected into the right and PBS in the left hind footpad.
22 After 48 hours, ovalbumin-induced footpad thickness increment was measured. RFI,
23 relative footpad increment, was calculated as follows: $RFI = (\text{treated}_{48\text{ h}} -$
24 $\text{untreated}_{48\text{ h}}) - (\text{treated}_{0\text{ h}} - \text{untreated}_{0\text{ h}})$. The data are the means of two independent
25 experiments. One-way ANOVA, Fisher's LSD multiple comparison test was used for
26 statistical analysis, n.s. $p > 0.05$, * $p < 0.05$; ** $p < 0.01$. Black lines mark the average
27 footpad thickness increment in each experimental group.
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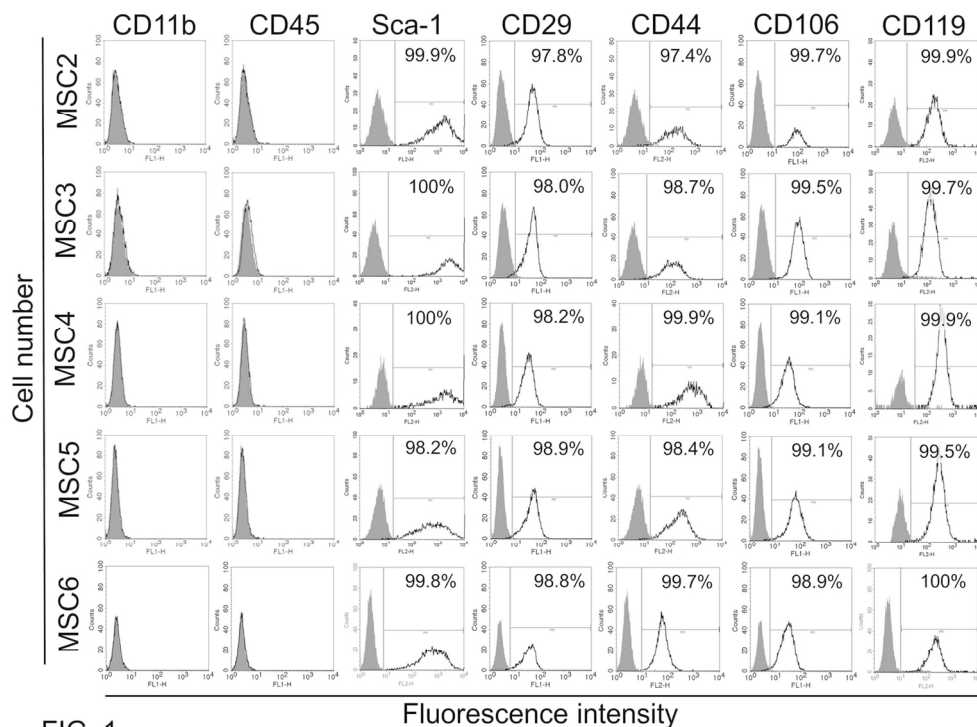


FIG. 1

138x103mm (300 x 300 DPI)

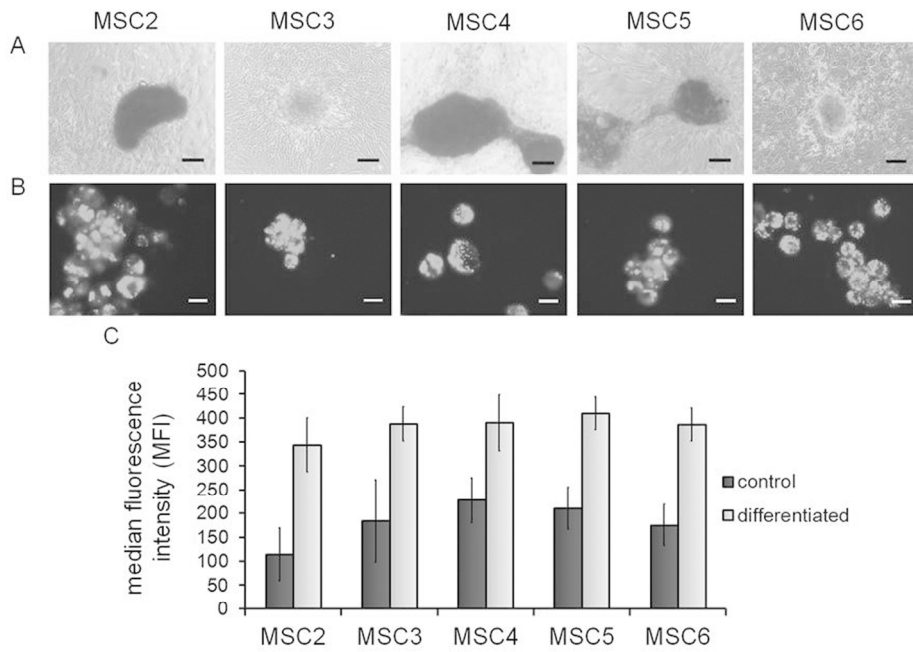


FIG. 2

138x103mm (300 x 300 DPI)

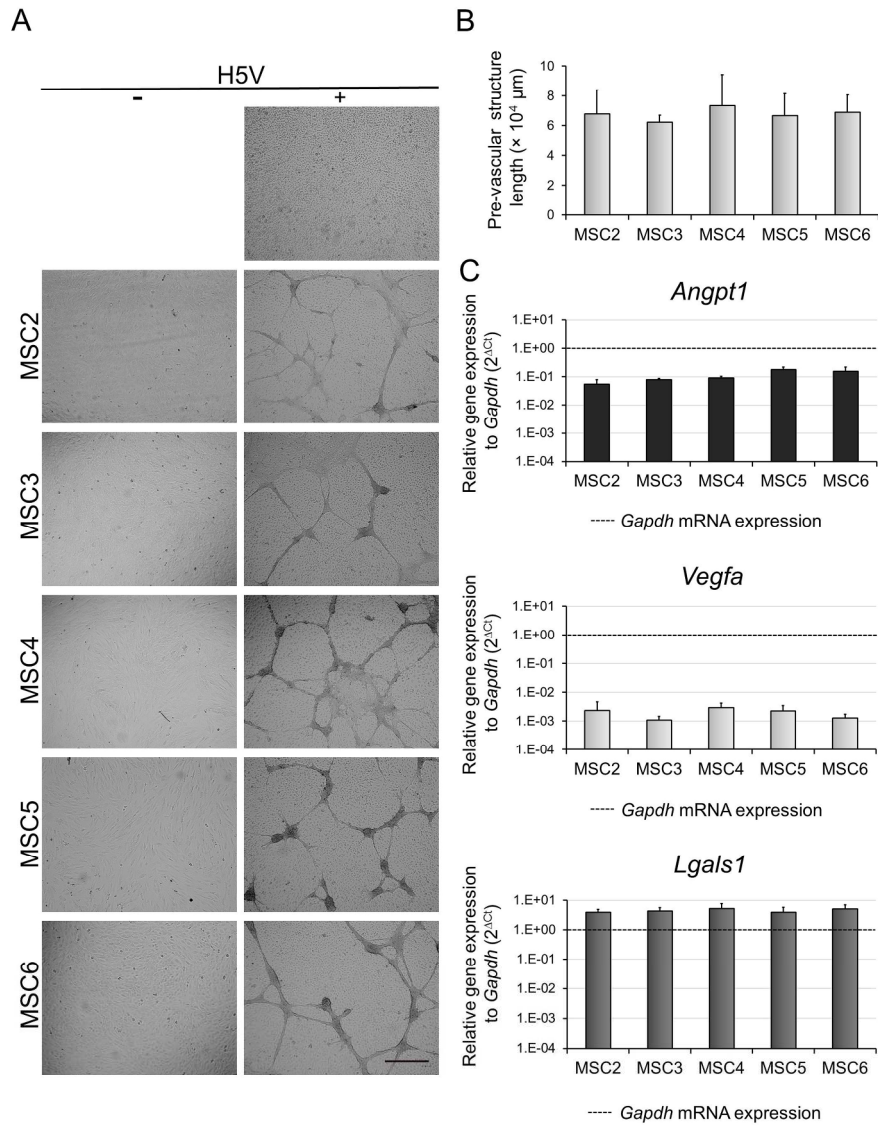


FIG. 3

246x328mm (300 x 300 DPI)

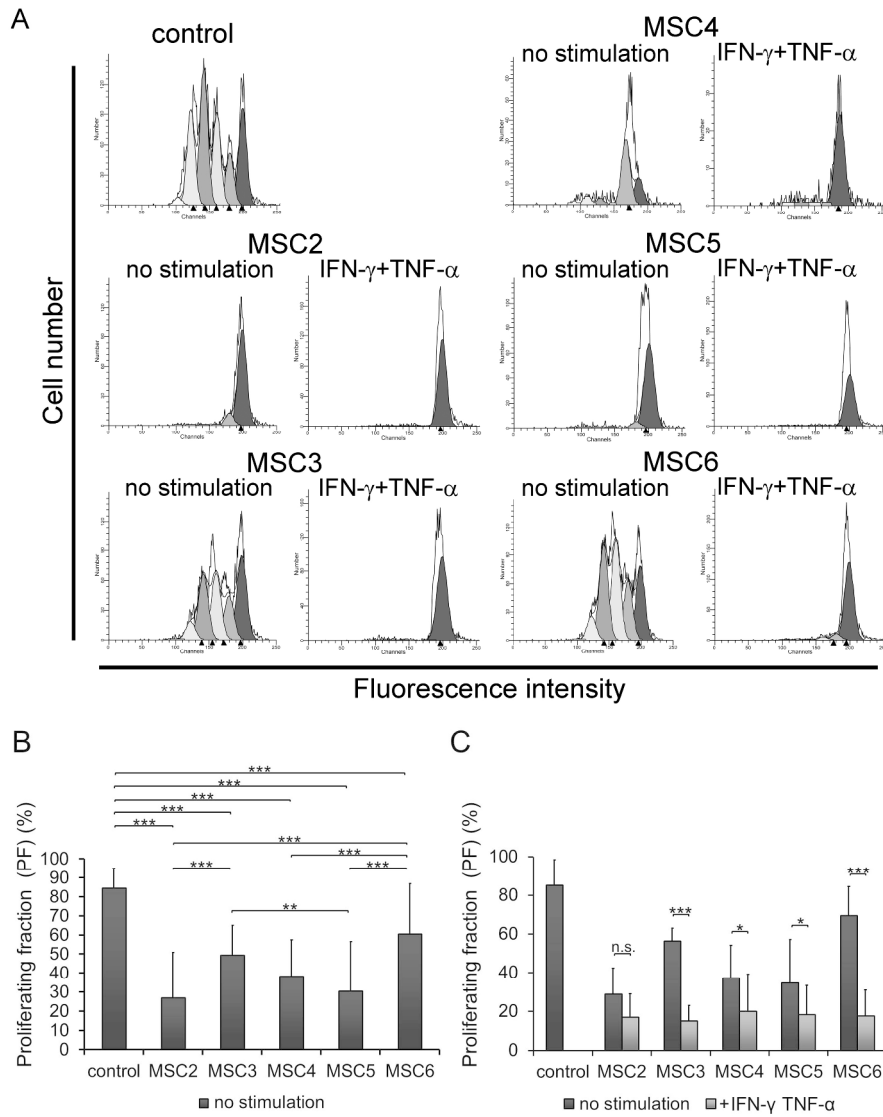


FIG. 4

246x329mm (300 x 300 DPI)

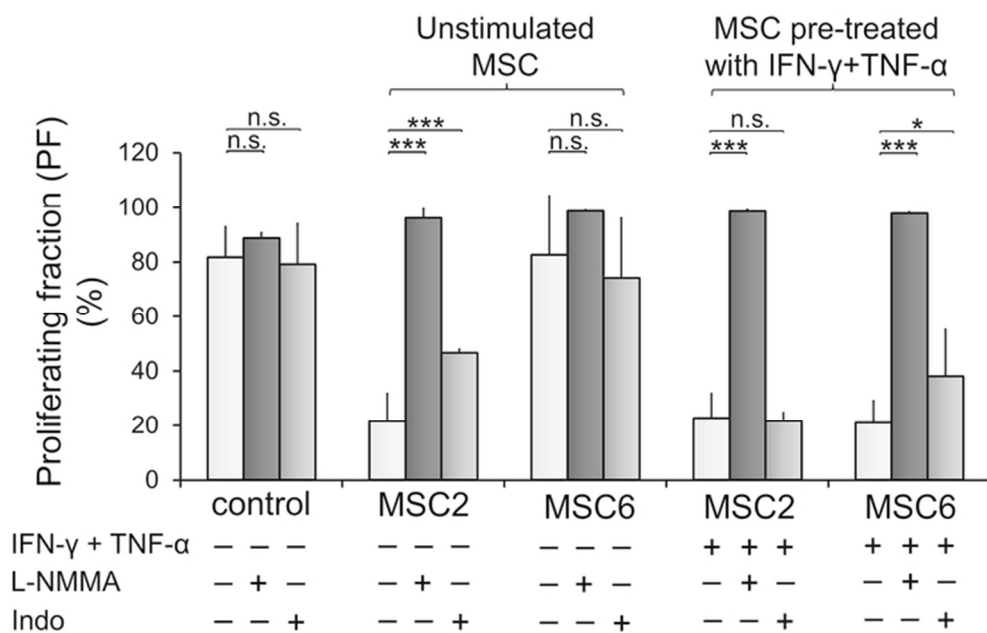


FIG. 6

63x47mm (300 x 300 DPI)

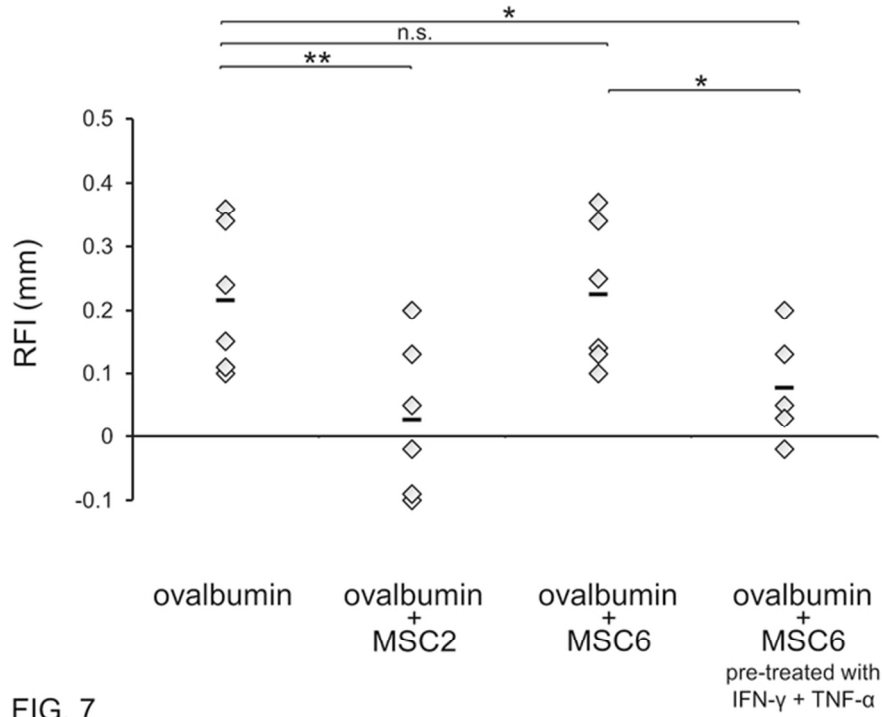
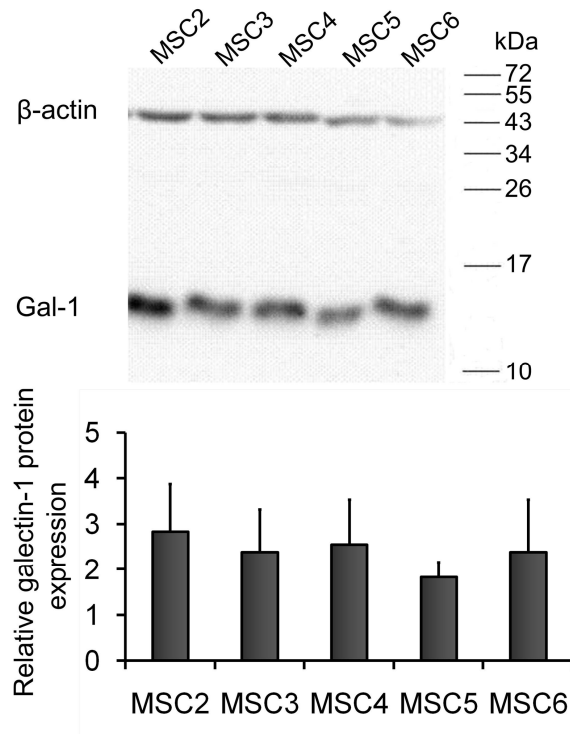


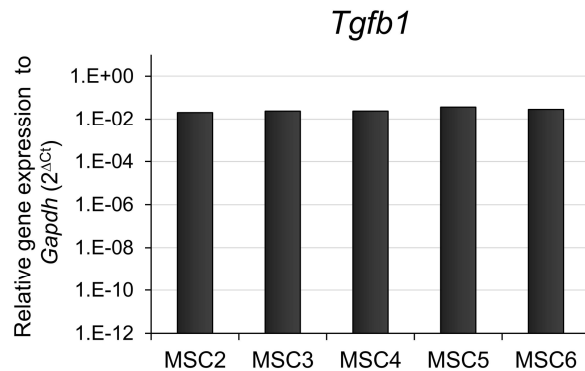
FIG. 7

63x47mm (300 x 300 DPI)

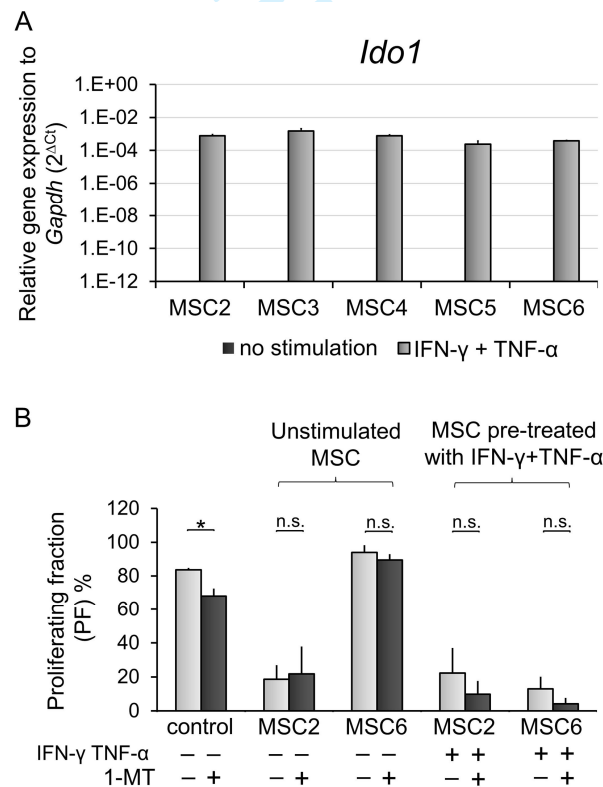
Supplementary Data



SUPPLEMENTARY FIG. S1. MSC clones express similar amount of galectin-1 (Gal-1). MSCs were lysed in RIPA buffer, and 30 μ g protein of the lysates/sample were analyzed by Western blot after running the samples on a 10% SDS-polyacrylamide gel. Polyclonal rabbit anti-Gal-1 and HRP-conjugated anti-rabbit immunoglobulins were applied for Gal-1 detection. Rabbit anti- β -actin was used as loading control. Density of the bands was determined by VisionWorks[®]LS Image Acquisition and Analysis Software (Ultra-Violet Products).



SUPPLEMENTARY FIG. S2. *Tgfb1* mRNA expression levels of MSC clones under normal culture conditions. After 2 days of culture, total RNA was isolated from cells and analyzed by qPCR. Data were normalized to the expression level of housekeeping gene *Gapdh*.



SUPPLEMENTARY FIG. S3. The expression and role of IDO1 in the inhibition of unstimulated and pre-activated MSC2 and MSC6. (A) MSCs were treated with

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3 100 ng/mL IFN- γ and 50 ng/mL TNF- α for 24 hours or left unstimulated. After 24
4 hours cytokine treatment total RNA was isolated and *Ido1* gene expression analyzed
5 by qPCR. *Gapdh* expression served as an internal control. (B) MSCs were plated on a
6 96-well culture plate, pre-treated with 100 ng/mL IFN- γ plus 50 ng/mL TNF- α for 24
7 hours or left unstimulated. After 24 hours, MSCs were washed with medium, co-
8 cultured with freshly isolated, CFSE-labeled lymph node cells in 1 to 40 ratio with or
9 without 1 mM 1-MT. T-cells were activated with Con A. After 72 hours of co-culture,
10 proliferation of T-cells was assessed by flow cytometry and data were analyzed by
11 ModFit LT™ Version 2.0. software. The figure shows the percent of proliferating T-
12 cells. Con A-activated T-cells alone with or without 1 mM 1-MT served as positive
13 controls. The data are the representative experiment + SD, which was carried out in
14 triplicate. n.s. = not significant $p > 0.05$
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