



Rat mesenchymal stromal cells inhibit T cell proliferation but not cytokine production through inducible nitric oxide synthase

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Mesenchymal stromal cells (MSC) have important immunomodulatory properties, they inhibit T lymphocyte allo-activation and have been used to treat graft-versus-host disease. How MSC exert their immunosuppressive functions is not completely understood but species specific mechanisms have been implicated. In this study we have investigated the mechanisms for rat MSC mediated inhibition of T lymphocyte proliferation and secretion of inflammatory cytokines in response to allogeneic and mitogenic stimuli *in vitro*. MSC inhibited the proliferation of T cells in allogeneic mixed lymphocyte reactions and in response to mitogen with similar efficacy. The anti-proliferative effect was mediated by the induced expression of nitric oxide (NO) synthase and production of NO by MSC. This pathway was required and sufficient to fully suppress lymphocyte proliferation and depended on proximity of MSC and target cells. Expression of inducible NO synthase by MSC was induced through synergistic stimulation with tumor necrosis factor α and interferon γ secreted by activated lymphocytes. Conversely, MSC had a pronounced inhibitory effect on the secretion of these cytokines by T cells which did not depend on NO synthase activity or cell contact, but was partially reversed by addition of the cyclooxygenase (COX) inhibitor indomethacin. In conclusion, rat MSC use different mechanisms to inhibit proliferative and inflammatory responses of activated T cells. While proliferation is suppressed by production of NO, cytokine secretion appears to be impaired at least in part by COX-dependent production of prostaglandin E₂.

Keywords: mesenchymal stem cells, immunosuppression, T lymphocyte activation, mixed lymphocyte reaction, nitric oxide synthase 2, cytokines, rodent, prostaglandin E₂

INTRODUCTION

Mesenchymal stem cells have self-renewing capacity and differentiation potential for all mesodermal cell lineages (Pittenger et al., 1999). They are present within a heterogeneous cell population referred to as mesenchymal stromal cells (MSC) which are presently defined by a set of criteria based on their morphology, phenotype, and multipotency (Dominici et al., 2006). To date, MSC have been studied most thoroughly in humans and mice. They can be isolated from the bone marrow (BM) and a variety of other adult and fetal tissues (Pittenger et al., 1999; Zuk et al., 2001; in 't Anker et al., 2003, 2004; da Silva Meirelles et al., 2006; Yoshimura et al., 2007). MSC have potent modulatory effects on immune cells including T cells, B cells, natural killer cells, and dendritic cells as well as regulatory T (T_{reg}) cells (Uccelli et al., 2006; Nauta and Fibbe, 2007; Tolar et al., 2011). A range of distinct mediator molecules have been implicated (Uccelli et al., 2006; Nasef et al., 2008) but the molecular mechanisms by which MSC exert these effects are not entirely understood and the influences of tissue source, species origin, and cell culture conditions have yet to be firmly established.

Nitric oxide (NO) is a short-lived bioactive compound which is catalyzed by different tissue-specific NO synthases, of which inducible NO synthase (iNOS) encoded by the *NOS2* gene is active in macrophages, fibroblasts, and endothelial cells (Bogdan, 2001; Lukacs-Kornek et al., 2011). iNOS expression can be induced by synergistic signals of interferon (IFN) γ and tumor necrosis factor (TNF) α or Toll-like receptor (TLR) ligands (Liew et al., 1991; Muñoz-Fernández et al., 1992; Deng et al., 1993; Lorsbach et al., 1993; Lukacs-Kornek et al., 2011). NO acts as a regulator of cellular and immune functions (Bogdan, 2001) such as inhibition of T cell responses (Lejeune et al., 1994; Medot-Pirenne et al., 1999; Niedbala et al., 2006) and induction of T_{reg} cells (Niedbala et al., 2007). The iNOS pathway also has a role in the immunosuppressive potential of MSC (Sato et al., 2007). A combination of pro-inflammatory cytokines, namely IFN γ together with TNF α , interleukin (IL)1 α , or IL1 β , has been shown to trigger the expression of iNOS in murine BM-derived MSC (Ren et al., 2008). Mouse MSC (mMSC) utilize NO to arrest T cell proliferation and activation *in vitro* and *in vivo* (Oh et al., 2007; Sato et al., 2007; Ren et al., 2008).

The capacity of MSC to suppress the activation of T lymphocytes has become of interest for clinical prevention and treatment of both autoimmune diseases and graft-versus-host disease (GVHD; Dazzi and Krampera, 2011; Tolar et al., 2011). GVHD has been treated successfully with MSC infusions clinically (Le Blanc et al., 2004, 2008; Ringdén et al., 2006; Martin et al., 2010; Tolar et al., 2011) and experimentally in animal models (Yanez et al., 2006; Min et al., 2007; Tisato et al., 2007; Polchert et al., 2008; Tian et al., 2008; Joo et al., 2010). Ren et al. (2008) reported that amelioration of experimental GVHD by mMSC depended on NO production. Human MSC (hMSC), on the other hand, do not utilize NO conversion, but rather employ alternative signaling pathways such as indoleamine-2,3-dioxygenase (IDO), cyclooxygenase (COX)-2 required for synthesis of prostaglandin E₂ (PGE₂), and heme oxygenase-1 expression to inhibit T cell activation and induce expansion of T_{reg} cells (Meisel et al., 2004; Aggarwal and Pittenger, 2005; Ren et al., 2009; Mougiakakos et al., 2011).

It has been suggested that MSC are “licensed” by certain effector molecules to exert immunomodulatory functions (Dazzi and Krampera, 2011). When exposed to an inflammatory milieu, hMSC upregulated the expression of IDO and COX-2 genes and showed increased inhibitory potential in mixed lymphocyte reactions (MLR; Crop et al., 2010). In another recent paper, the immunomodulatory properties of rat MSC (rMSC) were primed by the addition of different cytokines resulting in either enhanced inhibition of proliferation or the opposite effect depending on the type of stimulatory signal (Renner et al., 2009).

In this report, we generated rMSC lines from the BM and evaluated their potential to inhibit T cell proliferation and cytokine secretion *in vitro*. We show that the regulation of immunosuppression by rMSC was more similar to mouse than to hMSC. rMSC depended on cell-to-cell contact, iNOS expression, and NO production to mediate potent anti-proliferative effects. The putative mechanism that inhibits secretion of inflammatory cytokines is distinct and does not depend on cellular contact but on a soluble factor, likely PGE₂ produced by COX-2.

MATERIALS AND METHODS

ETHICS STATEMENT

Approval for the use of organs of rats euthanized with CO₂ (license number: VIT09.1512) was obtained from our institutional veterinarian with delegated authority from the Norwegian Animal Research Authority under the Ministry of Agriculture of Norway. All experiments were conducted in compliance with institutional guidelines. All animals were sacrificed with CO₂ and every effort was made to minimize their suffering.

ANIMAL CARE

PVG strain rats express the *c* haplotype of the rat MHC (*RT1^c*, i.e., *RT1-A^c-B/D^c-CE/N/M^c*; for short, *c-c-c*). PVG-*RT1^b* strain (abbreviated PVG.7B) rats express the RT7.2 allotype of CD45, but are used interchangeably with the standard PVG strain (encoding the RT7.1 allotype) as both strains carry the *RT1^c* haplotype. The MHC-congenic PVG-*RT1^u* strain (PVG.1U) expresses the *u-u-u* MHC haplotype, the PVG-*RT1ⁿ* strain (PVG.1N) the *n-n-n* haplotype and the intra-MHC recombinant PVG-*RT1^{r23}* strain (PVG.R23) the *u-a-av1* haplotype on the PVG background.

PVG.R23, PVG.1N, PVG.1U, and PVG.7B rats were bred at the Institute of Basic Medical Sciences, University of Oslo. PVG and BN/RijHsd (BN; *RT1ⁿ*, *n-n-n*) rats were purchased from Harlan, The Netherlands¹. The animals were housed under a 12:12 h light/dark cycle with access to food and filtered drinking water *ad libitum* and were routinely screened for common pathogens following recommendations by the Federation of European Laboratory Animal Science Associations (Nicklas et al., 2002).

MATERIALS

Nylon cell strainers (70 μm mesh size) were purchased from BD Falcon, MA, USA²; GIBCO® RPMI medium 1640, OPTI-MEM® I, α-modified minimal essential medium, fetal bovine serum (FBS), penicillin and streptomycin, sodium pyruvate, 2-mercaptoethanol, trypsin and EDTA, lipopolysaccharide (LPS), polyinosinic:polycytidylic acid (poly-I:C) from Invitrogen, UK³; L-glutamine, Immobilon®-P transfer membrane from Millipore, MA, USA⁴; biotin, Brefeldin A, Concanavalin A (ConA), sodium nitrate, sodium dodecyl sulfate, 2-mercaptoethanol, glycerol, sulfanilamide, *N*-(1-Naphthyl) ethylenediamine dihydrochloride, 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE), fluorescein isothiocyanate (FITC), propidium iodide, paraformaldehyde, saponin from Quillaja bark, 1-methyl-DL-tryptophan (1-MT), N^G-monomethyl-L-arginine acetate (L-NMMA) from Sigma-Aldrich, MO, USA⁵; indomethacin (IMC; Confortid®) from Dumex-Alpha A/S, Denmark⁶; Criterion™ Precast gels from Bio-Rad Laboratories, CA, USA⁷; SuperSignal® West Pico chemiluminescent substrate from Thermo Scientific, IL, USA⁸; Amersham Hyperfilm™ ECL from GE Healthcare Ltd., UK⁹; culture flasks from Nunc, Denmark¹⁰; 96-well cell culture clusters, HTS Transwell® 96-well system with 0.4 μm polycarbonate membrane insert plates from Corning, NY, USA¹¹; recombinant rat IFNγ from Biomedical Primate Research Centre, The Netherlands¹²; recombinant rat TNFα from Pepro-Tech, UK¹³; [methyl-³H]-thymidine (³H-TTP) from Hartmann-Analytic, Germany¹⁴; MicroScint™ O solution from PerkinElmer, MA, USA¹⁵.

ANTIBODIES

Monoclonal mouse anti-rat CD25 (OX39) and anti-CD3 (G4.18) antibodies were conjugated with biotin and anti-CD4 (W3/25) antibody with FITC in our laboratory using standard methods. Supernatants of monoclonal mouse anti-rat

¹www.harlan.com

²www.bdbiosciences.com

³www.invitrogen.com

⁴www.millipore.com

⁵www.sigma-aldrich.com

⁶www.alpharma.com

⁷www.bio-rad.com

⁸www.piercenet.com

⁹www.gelifesciences.com

¹⁰www.nuncbrand.com

¹¹www.corning.com

¹²www.bprc.nl

¹³www.peprotech.com

¹⁴www.hartmann-analytic.de

¹⁵www.perkinelmer.com

CD45 (OX1), anti-RT1-B/D (pan-MHC class II; OX6), anti-CD71 (OX26), anti-CD11b (OX42), anti-CD86 (OX48), anti-CD44 (OX49), anti-RT1-A (pan-MHC class I; OX18, purified) antibodies, as well as phycoerythrin-conjugated mouse anti-rat IFN γ (DB-1), anti-CD31 (TLD-3A12), anti-CD90 (OX7), anti-CD3 (G4.18), FITC-conjugated anti-CD59 (TH9) from BD Biosciences and allophycocyanin-conjugated rat anti-mouse/rat FoxP3 (FJK-16s) from eBioscience, CA, USA¹⁶ were used for immunostaining. Phycoerythrin-conjugated donkey anti-mouse immunoglobulin (Ig) G or peridinin chlorophyll protein-conjugated Streptavidin from BD Biosciences were used as secondary antibodies for flow cytometric analysis.

Polyclonal rabbit anti-rat TNF α , goat anti-rat IFN γ , and rabbit anti-rat IL6 antibodies were from PeproTech; polyclonal rabbit anti-rat NOS2 (M-19) from Santa Cruz Biotechnology, CA, USA¹⁷; monoclonal mouse anti-GAPDH (6C5) from Millipore; horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG from Jackson ImmunoResearch, PA, USA¹⁸.

CELL LINES

The rat macrophage cell line R2 is derived from pleural macrophages induced by a silica injection in the pleural cavity of Wistar rats (Damoiseaux et al., 1994). R2 cells were maintained in complete medium comprising RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 U mL⁻¹ penicillin, 100 μ g mL⁻¹ streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate and 50 μ M 2-mercaptoethanol at 37°C in a humidified atmosphere of 5% CO₂.

MSC lines were obtained from 7- to 8-week-old female PVG.7B and PVG.1U rats as described elsewhere (Lennon and Caplan, 2006). In short, BM cells were aspirated from femurs and tibias, filtered through nylon cell strainers, and cultured in α -modified minimal essential medium supplemented with 20% FBS, 100 U mL⁻¹ penicillin, 100 μ g mL⁻¹ streptomycin, and 2 mM L-glutamine in 175 cm² culture flasks at 37°C in a humidified atmosphere of 5% CO₂. Non-adherent cells were removed after 24 h by replacement of culture medium with complete, antibiotics-free MSC medium comprising α -modified minimal essential medium supplemented with 15% FBS and 2 mM L-glutamine. Adherent cells were allowed to expand to near confluence, detached using 500 μ g mL⁻¹ trypsin and 200 μ g mL⁻¹ EDTA-4Na and reseeded at a density of approximately 400–600 cells cm⁻². MSC were used in experiments after the third passage. Supernatants from confluent cultures were frequently controlled for mycoplasma contamination by PCR as previously described (Zinöcker et al., 2011b).

CELL CULTURE

Mesenteric and cervical lymph nodes from 7- to 14-week-old male or female PVG, PVG.7B, PVG.1N, PVG.1U, PVG.R23, and BN rats were removed and filtered through nylon cell strainers. The lymphocyte population was purified by density gradient centrifugation using Lymphoprep™ 1.077 (Medinor AS, Norway)¹⁹.

Stimulator cells were irradiated (2000 cGy) using a ¹³⁷Cs source (Gammacell® 3000; MDS Nordion, ON, Canada)²⁰ to inhibit mitosis. MLR were performed in a total volume of 200 μ L complete MLR medium comprising RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 U mL⁻¹ penicillin, 100 μ g mL⁻¹ streptomycin, 2 mM L-glutamine, and 50 μ M 2-mercaptoethanol using round bottom 96-well plates. Equal numbers (2×10^5) of responder and stimulator cells were mixed and incubated for 4 days at 37°C in a humidified atmosphere of 5% CO₂. ConA was used at 5 μ g mL⁻¹ final concentration for mitogenic stimulation of 2×10^5 responder cells unless specified otherwise. MSC were harvested from culture, washed twice (500 g for 6 min) in phosphate-buffered saline (PBS), resuspended in MLR medium and seeded at least 2 h before lymphocytes were added to allow attachment.

For stimulation experiments, cell-free supernatants were centrifuged at 400 \times g for 10 min before transfer of equal volumes to MSC culture. For transwell experiments, MSC were seeded either in 0.4 μ m polycarbonate membrane inserts or in the reservoirs of 96-well flat-bottom receiver plates. Responder cells were added to the bottom reservoirs and co-incubated for 3 days.

RADIONUCLIDE INCORPORATION ASSAY

DNA synthesis during mitogen stimulation or mixed lymphocyte culture was assessed after 20 h pulsing with 1 μ Ci ³H-TTP before termination of the culture. Cells were harvested on glass fiber filters using a Filtermate 196 cell harvester (Packard Bioscience Co., CT, USA)²¹ and radioactivity was measured using a Wallac 1450 MicroBeta® TriLux (PerkinElmer) microplate scintillation counter. Relative inhibition of the culture was calculated by the following equation:

$$\text{inhibition} = 1 - \left(\frac{\text{proliferation count of the sample}}{\text{mean proliferation count of the positive control}} \right)$$

CFSE DILUTION ASSAY

Responder cells were stained with CFSE prior to *in vitro* culture as previously described (Zinöcker et al., 2011b). Briefly, cells were resuspended in OPTI-MEM at 2×10^6 mL⁻¹ and incubated with 0.5 μ M CFSE for 10 min at 37°C. Stained cells were then washed (400 g for 8 min) in MLR medium, incubated once more for 5 min at 37°C, washed twice and resuspended in MLR medium.

At the termination of MLR and ConA cultures, cells were harvested and washed in PBS before immunostaining and flow cytometric analysis. Fifty micromolar propidium iodide was added before flow cytometric analysis to exclude non-viable cells. The percentage of dividing cells was determined as the fraction of cells that had undergone one or several cell divisions (CFSE^{lo}) relative to the total number of CFSE⁺ cells including cells that had not undergone cell divisions (CFSE^{hi}).

IMMUNOSTAINING AND FLOW CYTOMETRIC ANALYSIS

All MSC lines were tested for surface marker expression by flow cytometry. Cells were labeled with anti-CD11b, -CD31, -CD44, -CD45, -CD59, -CD71, -CD86, -CD90, anti-class I, and anti-class II

¹⁶www.ebioscience.com

¹⁷www.scbt.com

¹⁸www.jacksonimmuno.com

¹⁹www.medinor.no

²⁰www.mds.nordion.com

²¹www.packardbioscience.com

MHC monoclonal antibodies (cf. *Antibodies*). Monoclonal mouse IgG₁ and IgG_{2a} were used as isotype controls.

For intracellular IFN γ staining, Brefeldin A (10 μ g mL⁻¹) was added 4 h prior to termination of ConA-stimulated LNC cultures to inhibit protein secretion. Cells from triplicate or quadruplicate wells were pooled, stained with anti-CD3 antibody, fixed with 4% paraformaldehyde in PBS, permeabilized with 0.5% saponin in water and stained with anti-IFN γ antibody.

For intracellular FoxP3 staining, ConA-stimulated LNC were harvested after 3 days and stained with anti-CD3, -CD4, -CD25 antibodies. Immunostained cells were subsequently treated with fixation/permeabilization buffer (eBioscience) and stained with anti-FoxP3 antibody following the manufacturer's protocol. Cells were analyzed on a FACSCalibur™ flow cytometer (BD Biosciences) using CellQuest™ software (BD Biosciences). FACS data were further analyzed using FlowJo™ software (Treestar, OR, USA)²².

WESTERN BLOT

Cells were pooled from triplicates and lysed for 30 min on ice with buffer consisting of 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% (w/v) Triton® X-100, 1 mM phenylmethanesulfonyl fluoride in isopropanol, 1 mM Na₃VO₄ and proteinase inhibitor cocktail (all from Sigma-Aldrich). Lysates were centrifuged at 10,000 \times g for 10 min to remove debris, and supernatants were resuspended in standard sample buffer containing 2% (w/v) sodium dodecyl sulfate and 2.5% (v/v) 2-mercaptoethanol. Samples were then heated at 95°C for 5 min, allowed to cool at ambient temperature and loaded onto polyacrylamide gels. Electrophoresis was run at 80–200 V. Proteins were transferred onto a polyvinylidene fluoride membrane in a TE 70 semi-dry transfer unit (GE Healthcare) using a current of 100 mA for 65 min. Western blotting was performed using anti-rat NOS2 (iNOS) and anti-rat GAPDH antibodies followed by secondary staining with horseradish peroxidase-conjugated IgG. Chemiluminescent substrate was used to visualize the immunoreactive proteins by horseradish peroxidase detection.

NO QUANTIFICATION

The nitrite concentration in the medium was measured as an indicator of NO production by virtue of the Griess reaction (Beda and Nedospasov, 2005). Fifty microliters of cell-free supernatant from MLR or ConA cultures was mixed with equal volumes of 1% (w/v) sulfanilamide in 5% phosphoric acid and 0.1% (w/v) naphthylethylenediamine dihydrochloride in water. Absorbance of the reaction at 540 nm was measured using a Labsystems Multiskan® bichromatic plate reader (Titertek Instruments, AL, USA)²³ and concentrations were calculated based on a standard curve of twofold dilutions of sodium nitrate which was assayed in parallel.

CYTOKINE ASSAYS

For cytokine measurements, supernatants from ConA cultures were collected after 2–3 days, pooled from triplicate or quadruplicate wells, centrifuged at 1,500 \times g for 4 min to remove cellular

debris and stored at –80°C or –20°C until analysis. Samples were thawed at 37°C in a water bath and analyzed using the Bio-Plex™ Rat Cytokine 9-Plex A Panel (Bio-Rad) according to the manufacturer's protocol. Concentrations were determined based on a standard curve using defined reference samples which were assayed (Luminex xMAP® Technology; Bio-Rad) in parallel.

STATISTICAL ANALYSIS

Normal distribution of data was assumed and tested by Shapiro–Wilk's test. The paired Student's *t* test (two-tailed) was used to evaluate the probability of differences between group means. Statistical analysis was performed using SPSS® software version 18.0.1 (SPSS, IL, USA)²⁴.

RESULTS

GENERATION OF MSC LINES FROM RAT BM

We produced MSC lines from the BM of PVG.7B and PVG.1U MHC-congenic rats. The cells showed adherence to plastic and spindle-shaped fibroblast-like morphology in culture (data not shown). They expressed MHC class I, CD44, CD59, CD71, and CD90 surface markers, but lacked CD11b, CD31, CD45, CD86, and MHC class II expression (**Figure 1**). Their potential to develop into adipocytes and osteocytes was confirmed by *in vitro* differentiation assays (Zinöcker et al., manuscript submitted). Together, this data was in accordance with the current definition of the MSC phenotype (Dominici et al., 2006; Harting et al., 2008).

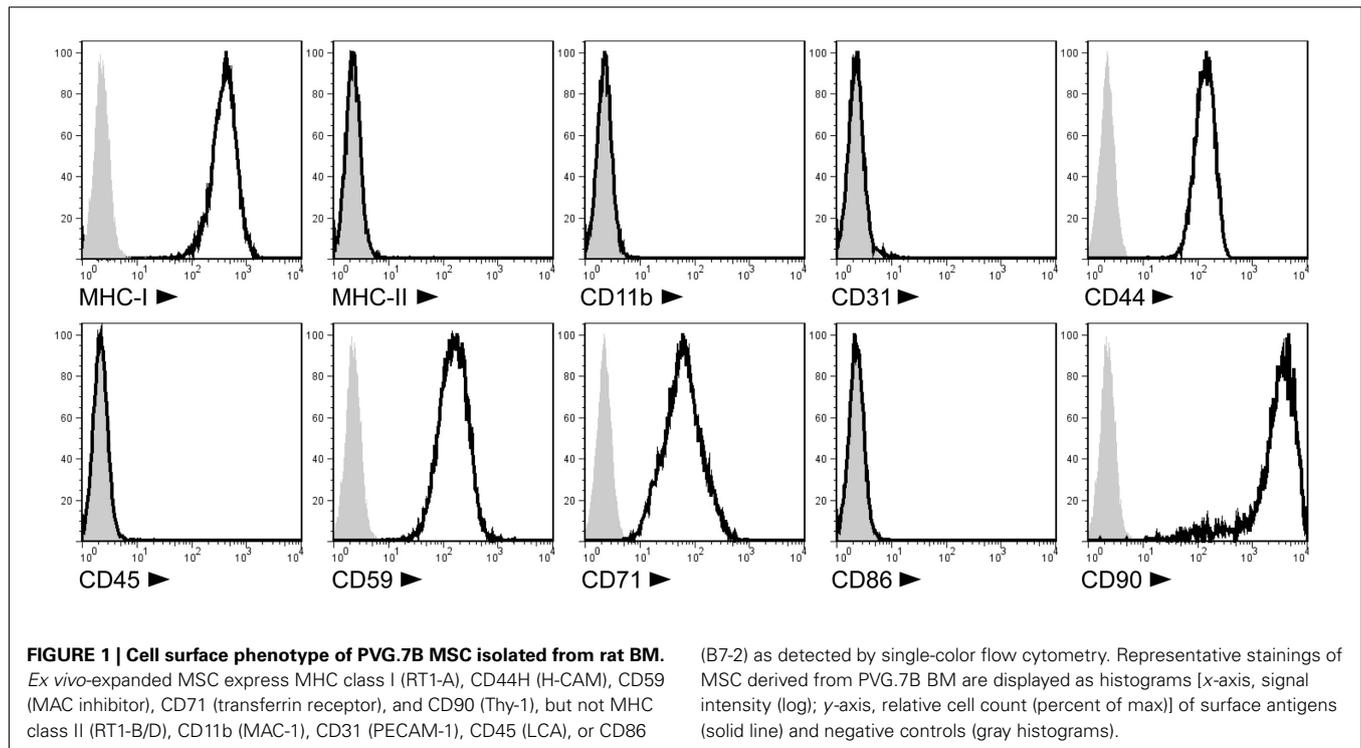
RAT BM–MSC INHIBIT ALLO-ANTIGEN AND MITOGEN-INDUCED T CELL PROLIFERATION *IN VITRO* INDEPENDENT OF MHC HAPLOTYPE

We tested the ability of MSC to inhibit lymphocyte proliferation induced by ConA or in allogeneic mixed lymphocyte cultures. ConA stimulation induced proliferation of LNC from PVG.7B (*RT1^c*) rats as assessed by CFSE dilution and radio-labeled nucleotide incorporation. Proliferation was fully inhibited in the presence of PVG.7B MSC at a cell ratio of 1 MSC per 10 LNC (1:10); significant suppression was also observed at a ratio of 1:100 (**Figure 2A**). The inhibitory effect was not dependent on the MHC combination of MSC and responder cells as allogeneic PVG.1U (*RT1^u*) and syngeneic PVG.7B MSC suppressed ConA-induced proliferation of PVG.7B responder cells equally well (**Figure 3A**). Similarly, both PVG.1U and PVG.7B MSC inhibited antigen-induced proliferation of both PVG.1U and PVG.7B responder cells in allogeneic MLR with equal efficiency (**Figure 3B** and data not shown). This suggested that the inhibitory capacity of MSC did not depend on the MHC-allotype, in line with previous findings (Krampera et al., 2003; Le Blanc et al., 2003). Proliferation of MSC alone was negligible and below the negative control (LNC proliferation in the absence of a stimulus), which was consistently less than 10% of the positive control (data not shown). Irradiation (2000 cGy) of MSC did not reduce their inhibitory capacity significantly in ConA- and MLR-induced lymphocyte cultures (data not shown), suggesting that this property is not dependent on their ability to proliferate. Cell-free supernatant from MSC culture had no suppressive effect (data not shown).

²²www.treestar.com

²³www.titertek-berthold.com

²⁴www.spss.com



T CELL PROLIFERATION IN THE PRESENCE OF MSC IS RESTORED BY ADDITION OF THE iNOS INHIBITOR L-NMMA

We tested inhibitors of different signaling pathways which have been implicated in immunosuppression by MSC (Meisel et al., 2004; Aggarwal and Pittenger, 2005; Sato et al., 2007) and found that the addition of L-NMMA, an inhibitor of iNOS, at the start of MLR or ConA stimulation (Figure 2 and data not shown) restored proliferation to normal levels. Conversely, addition of 1-MT, an IDO inhibitor, or IMC, a COX inhibitor, had no effect. The presence of all three inhibitors in lymphocyte culture had no additional effect compared to L-NMMA alone (Figure 2B). L-NMMA fully reversed the proliferative arrest of both CD4⁺ and CD8⁺ T cells which were equally affected by MSC inhibition (data not shown). Together, these data indicated that rMSC use iNOS to suppress T cell proliferation *in vitro*.

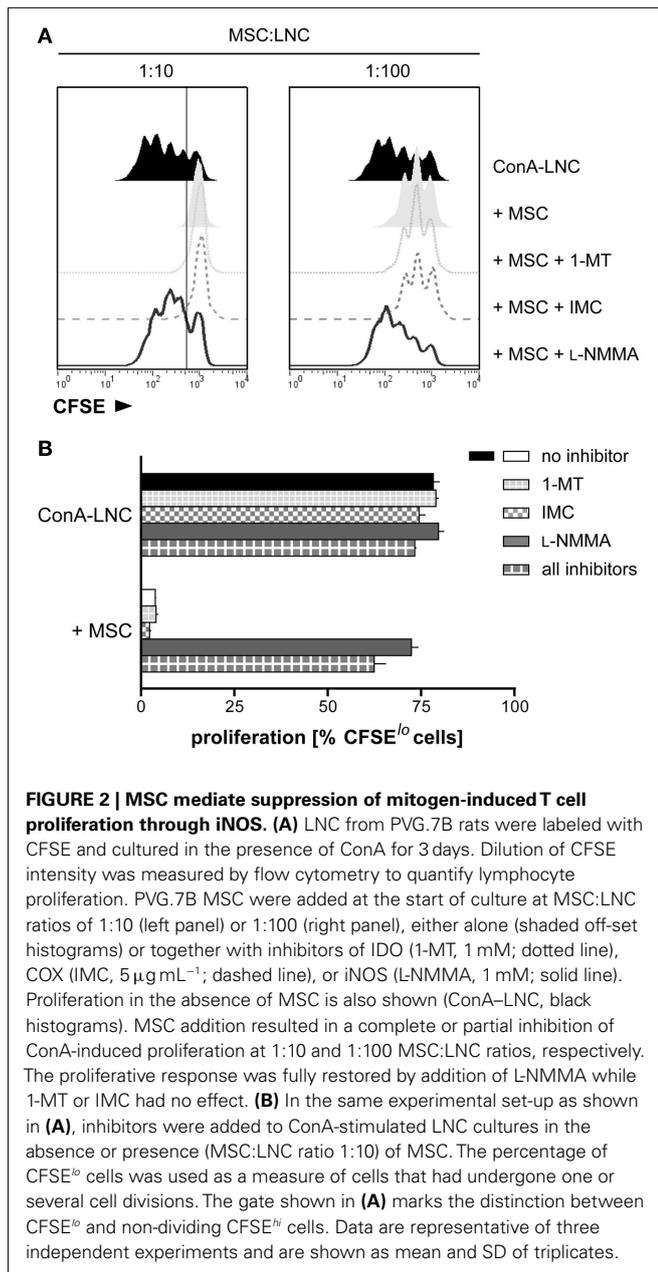
MSC EXPRESS iNOS AND PRODUCE NO IN RESPONSE TO LYMPHOCYTE ACTIVATION

Our findings suggested that the production of NO through iNOS was responsible for the suppression of T cell proliferation by rMSC. To test this hypothesis further, we measured the expression of iNOS by Western blot. iNOS was only detected in ConA cultures upon addition of MSC (Figure 4A) and correlated with the number of MSC present. iNOS was not detected in cultures of MSC alone, which suggested that its expression was induced in co-cultures with activated T lymphocytes. We also measured the concentration of nitrate as a proxy of NO (Beda and Nedospasov, 2005) and found that NO levels correlated with the observed iNOS levels. Neither MSC nor ConA-activated LNC cultures produced significant levels of NO (Figure 4B and data not shown). Notably, addition of L-NMMA did not affect iNOS protein levels (data not

shown) but completely abrogated NO production (Figure 4B). We obtained identical results from MLR:MSC co-cultures (unpublished observations). The upregulation of iNOS in MSC co-cultures with stimulated lymphocytes correlated with the observed dose-dependent inhibition of proliferation (as shown in Figures 2 and 3) and thus provided further evidence that inducible NO production represents a key mechanism for the suppressive capacity of rMSC.

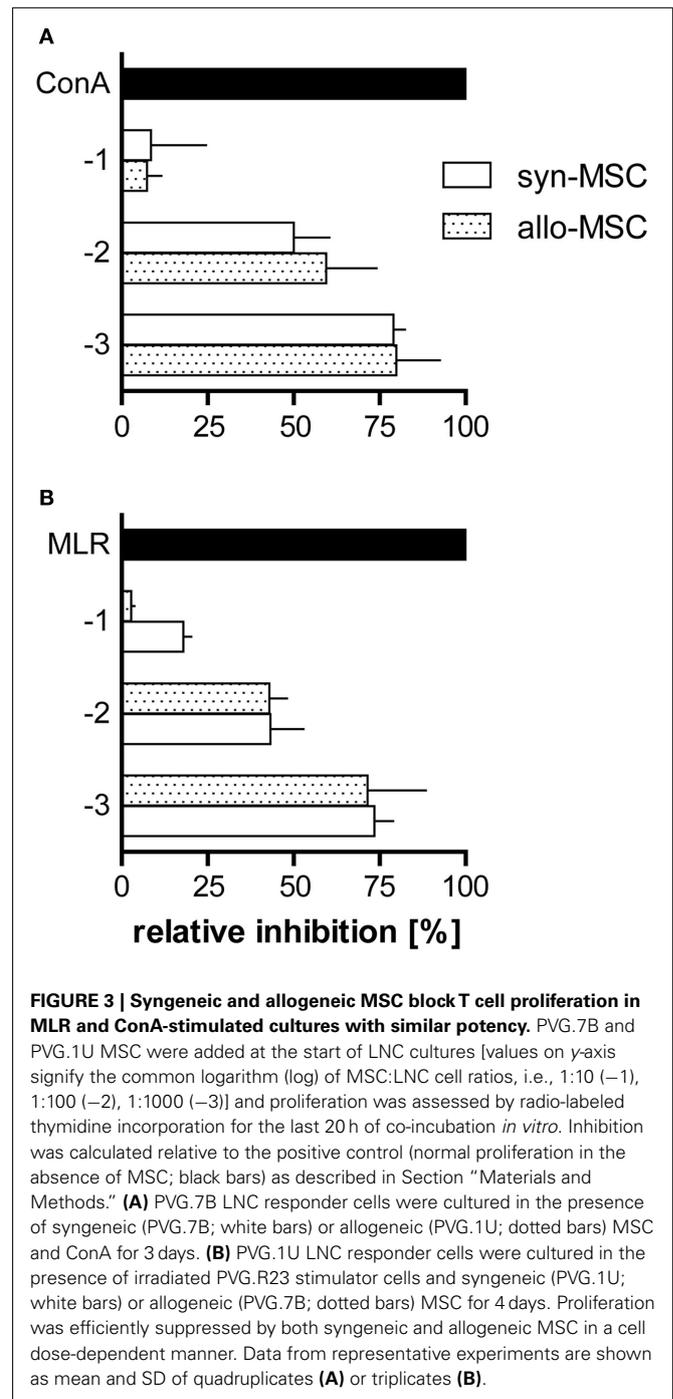
IMMUNOSTIMULATORY CYTOKINES INDUCE iNOS EXPRESSION IN MSC

It has been proposed that MSC depend on “licensing” in order to assume immunosuppressive functions, e.g., by stimulation through IFN γ or a TLR3 ligand (Krampera et al., 2006; Waterman et al., 2010; Dazzi and Krampera, 2011). Furthermore, it has been shown that iNOS expression is induced in response to synergistic stimulation by pro-inflammatory cytokines in mMSC (IFN γ together with either TNF or IL1 signals; Ren et al., 2008; Ren et al., 2009), and we therefore tested whether this was the case also for rMSC. Different combinations of IFN γ , TNF α , and TLR agonists induced iNOS expression in rMSC as judged by Western blot analysis. Incubation with TNF α for 24 h was sufficient to induce iNOS expression and increase NO concentrations in fresh MSC cultures (Figure 5A). Conversely, addition of IFN γ did not activate iNOS at the concentrations tested (titrations of 15 up to 5000 U mL⁻¹, data not shown) but potentiated the effect of TNF α resulting in significantly higher levels of both iNOS expression and NO compared with addition of TNF α alone (Figure 5A). LPS, a TLR4 agonist, or poly-I:C, a TLR3 agonist, resulted neither in iNOS expression nor NO production by MSC, in contrast to rat macrophages used as positive control (Figure 5A; Figure A1 in



Appendix). LPS synergized with IFN γ in inducing iNOS expression in MSC, albeit with variable potency, while simultaneous stimulation with poly-I:C and IFN γ had no effect (Figure 5A, Figure A1 in Appendix).

Supernatants from stimulated LNC cultures (allogeneic MLR or ConA culture) also led to a potent induction of iNOS (Figure 5B and data not shown) in MSC. The concentrations of NO in the culture medium were concordant with the observed protein expression levels. Induction of iNOS and NO production was inhibited by addition of anti-IFN γ antibody and, more potently, anti-TNF α antibody either alone or in combination (Figure 5B). These data indicate that the inflammatory cytokine TNF α has a key role in priming MSC for their immunosuppressive function, and that IFN γ potentiates this effect.



MSC-MEDIATED INHIBITION OF CYTOKINE SECRETION BY ACTIVATED T CELLS IS NOT DEPENDENT ON iNOS ACTIVITY BUT ON COX-MEDIATED PRODUCTION OF PROSTAGLANDIN

Next, we tested the influence of MSC on the cytokine secretion patterns of ConA-stimulated lymphocytes. Although iNOS was important for MSC-mediated inhibition of T cell proliferation, this factor could not explain the inhibitory effect on IFN γ production as evaluated by intracellular flow cytometry following addition of L-NMMA to the LNC–MSC co-cultures

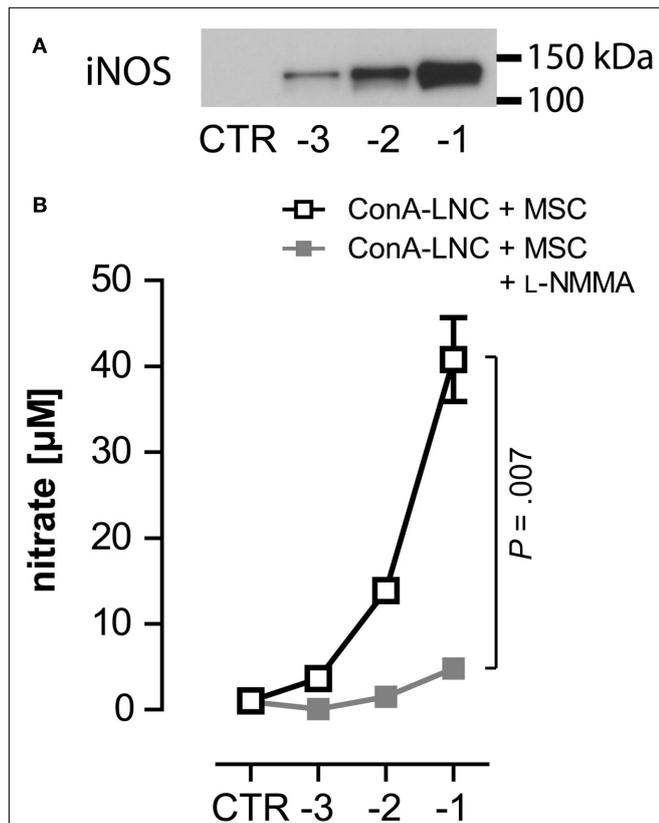


FIGURE 4 | MSC express iNOS and produce NO in response to lymphocyte stimulation. (A) 1×10^6 ConA-stimulated PVG LNC were cultured in the absence (CTR) or presence of syngeneic PVG.7B MSC [log (MSC:LNC)] and expression of iNOS was analyzed after 24 h. iNOS was detected in co-cultures of ConA-LNC with MSC and correlated with the numbers of MSC present. **(B)** In the same set-up as shown in **(A)** the amount of nitrate (shown as the mean and the SD of triplicates) as a proxy for NO concentration was determined in the absence (\square) or presence (\blacksquare) of L-NMMA. Addition of L-NMMA abolished NO levels produced in MSC:LNC co-cultures. NO was not detected in the absence of MSC (CTR). Data are representative of three independent experiments.

(Figures 6A,B). We further measured cytokine profiles by multiplex analysis and found that MSC constitutively secreted IL6 and vascular endothelial growth factor (VEGF) but not other cytokines analyzed (data not shown). The addition of MSC resulted in an accumulation of IL6 and VEGF also in ConA-stimulated LNC–MSC co-cultures (Figure 6C) as reported previously (Djouad et al., 2007). Addition of neutralizing concentrations ($2 \mu\text{g mL}^{-1}$) of anti-rat IL6 antibody to the co-culture failed to reverse inhibition (data not shown), in contrast to previous studies of human and mouse MSC (Di Nicola et al., 2002; Djouad et al., 2007; Najjar et al., 2009). LNC secreted significant amounts of the cytokines IL18, TNF α , and in particular IFN γ in response to ConA, which were markedly reduced when MSC were present. The inhibitory effect was proportionate to the numbers of MSC added (Figure 6C and data not shown). Addition of recombinant rat IFN γ (1000 U mL^{-1}) to the co-culture failed to reverse the suppressive effect (data not shown). Expression levels of IL4 and IL10, cytokines which are typically considered as

anti-inflammatory, were not increased in co-culture supernatants (data not shown).

Addition of L-NMMA had no effect on MSC-mediated modulation of cytokine secretion in co-culture experiments, and the same was true for the IDO antagonist 1-MT. By contrast, addition of the COX inhibitor indomethacin resulted in a striking reversal of inhibition of IFN γ and TNF α secretion at a MSC:LNC ratio of 1:100, but not at the highest ratio of 1:10 (Figure 6C). This result suggested that MSC can inhibit T cell mediated secretion of inflammatory cytokines by COX-dependent synthesis of PGE $_2$.

INHIBITION OF PROLIFERATION BUT NOT CYTOKINE PRODUCTION IS DEPENDENT ON CO-LOCALIZATION OF MSC AND T CELLS

The immunosuppressive effect of hMSC depends on soluble factors such as IDO, PGE $_2$, hepatocyte growth factor and transforming growth factor β 1 (Di Nicola et al., 2002; Meisel et al., 2004; Aggarwal and Pittenger, 2005) and does not require cell contact (Hoogduijn et al., 2010) although there have been reports that co-localization of MSC with lymphocytes augmented inhibition (Krampera et al., 2003). mMSC require proximity for the effective inhibition of T cells by short-range activity of NO (Ren et al., 2008). Therefore, we examined whether inhibition of T cell effector function by MSC in the rat depended on cell-to-cell contact or if other soluble factors might be important. Physical separation of MSC from LNC using transwell membrane inserts restored ConA-stimulated proliferation which was inhibited in co-cultures where LNC and MSC were in contact (Figure 7A), indicating that suppression of proliferation required close proximity of MSC and target cells. In marked contrast, the cytokine secretion profiles in these cultures were altered irrespective of cell-to-cell contact (Figure 7B). IL6 and VEGF were increased to similar levels when MSC and LNC were either co-localized or separated (data not shown). Inflammatory cytokines were significantly reduced in the presence of MSC in either experimental set-up (Figure 7B). These latter data showed that inhibition of cytokine expression required a soluble factor, likely PGE $_2$, and provided further evidence that this property of rMSC is not dependent on close cellular contact.

T_{REG} CELL NUMBERS ARE REDUCED IN STIMULATED LYMPHOCYTE CO-CULTURES WITH MSC

We also analyzed the effect of MSC on the CD4 $^+$ CD25 hi FoxP3 $^+$ T_{reg} cell population using mitogen-induced lymphocyte cultures. The proportion of T_{reg} cells was significantly diminished in the CD4 $^+$ T cell population by addition of MSC at the start of the culture (Figure 8). This finding is in contrast to a number of previous studies which have demonstrated the induction of T_{reg} cells by MSC in the human system (Aggarwal and Pittenger, 2005; Di Ianni et al., 2008; English et al., 2009; Ghannam et al., 2010; Mougiakakos et al., 2011). It should be noted that the functional potential of these T_{reg}-like cells has not been tested in the present study.

DISCUSSION

Herein we show that rat BM-derived MSC up-regulate iNOS in response to TNF α and IFN γ secreted by activated lymphocytes and produce NO, which exerts a potent inhibitory effect on the proliferative T cell response to mitogen or allogeneic stimuli *in vitro*.

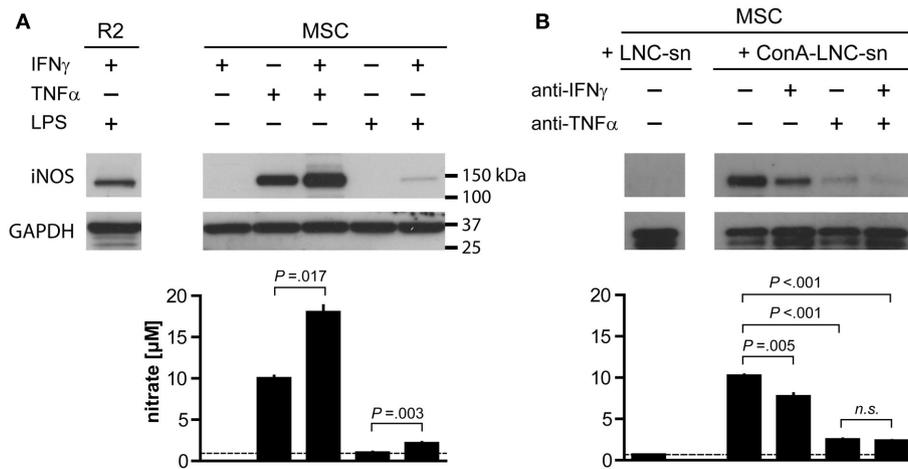


FIGURE 5 | Tumor necrosis factor α and IFN γ synergistically induce iNOS expression and NO production by MSC. 1×10^4 MSC (PVG.7B) were stimulated for 24 h with combinations of IFN γ , TNF α , and a TLR agonist **(A)** or by medium supernatants from ConA-activated LNC (PVG.7B or PVG.1U) cultures **(B)**. Pooled triplicates and quadruplicates, respectively, were analyzed for iNOS expression by Western blot with GAPDH assayed as a loading control. Nitrate concentrations were determined in the culture supernatants of the same wells (bottom panels). **(A)** MSC produced iNOS (130 kDa) in response to TNF α (25 ng mL $^{-1}$) alone or together with IFN γ (100 U mL $^{-1}$) or by a combination of IFN γ and LPS (100 ng mL $^{-1}$). Nitrate concentration levels correlated with the observed levels of iNOS protein expression. The dotted line indicates baseline NO production without

cytokines added. The R2 macrophage cell line was used as positive control for iNOS expression. **(B)** Supernatants from ConA-stimulated (ConA-LNC-sn) and unstimulated LNC cultures as a control (LNC-sn; baseline) were added to MSC cultures. Neutralizing antibodies (each 10 μ g mL $^{-1}$ final concentration) against either IFN γ or TNF α or both (indicated) were added at the start of culture and incubated for 24 h. iNOS was induced by addition of supernatant from stimulated LNC cultures and was blocked by IFN γ -specific antibody and, more potently, by TNF α -specific antibody alone or in combination. Nitrate concentrations correlated with the observed iNOS expression levels. Data are representative of four independent experiments. Nitrate concentration data are shown as the mean and the SD of triplicates or quadruplicates.

The present study supports an important but not exclusive role of iNOS in the immunosuppressive function of MSC in rodents (Oh et al., 2007; Sato et al., 2007; Ren et al., 2008). COX-dependent PGE $_2$ is apparently also involved, in the inhibition of IFN γ and TNF α secretion by activated T cells.

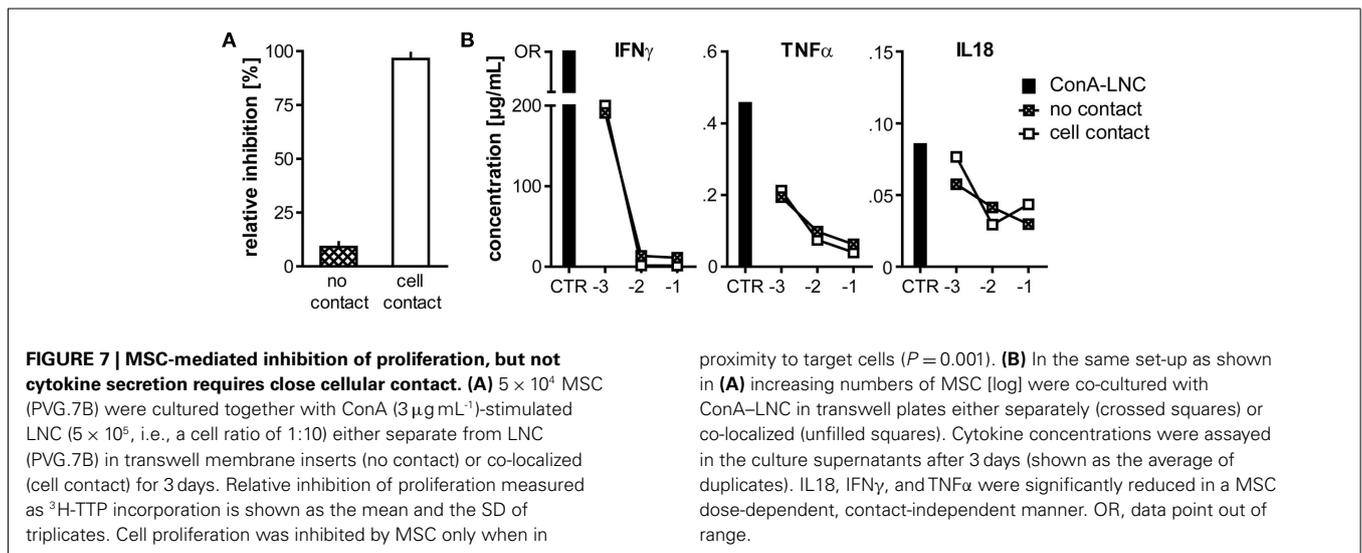
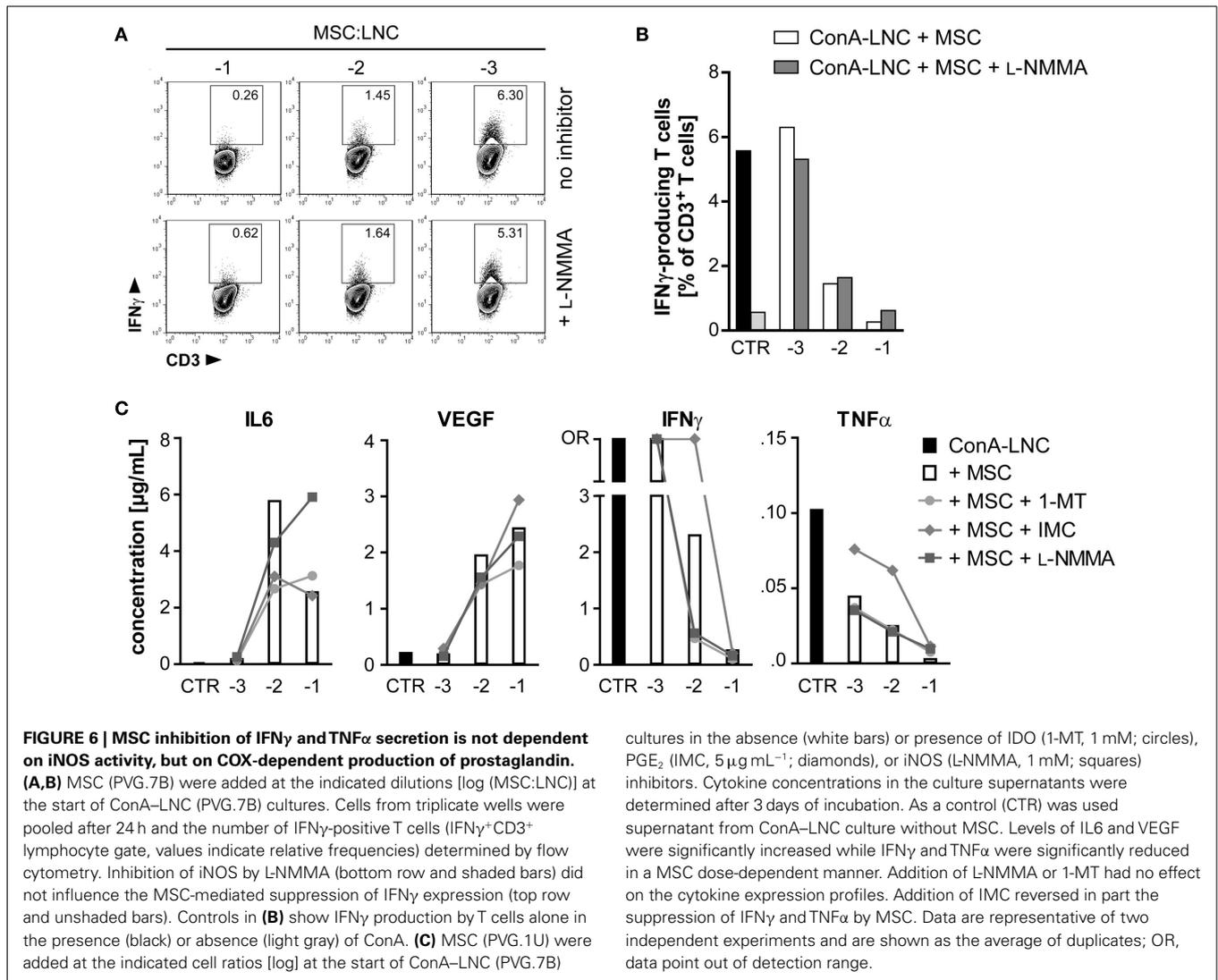
To our knowledge, the only study that has investigated the immunosuppressive function of rMSC via iNOS to date (Chabannes et al., 2007) showed an effect in combination with heme oxygenase-1, but not a critical role for iNOS *per se*. Furthermore, that study showed that stimulation with recombinant IFN γ alone resulted in detectable expression of iNOS, which is in contrast to our findings showing that TNF α is sufficient and IFN γ is not required. This difference could be related to variations in cell isolation protocols, culture conditions, or between different rat strains. The MSC line generated from LEW.1A BM by Chabannes et al. (2007) were applied before the fourth passage and a minority of myeloid cells present in this population (3.2% of the cells expressed CD45) could account for the reported detection of iNOS expression in response to IFN γ .

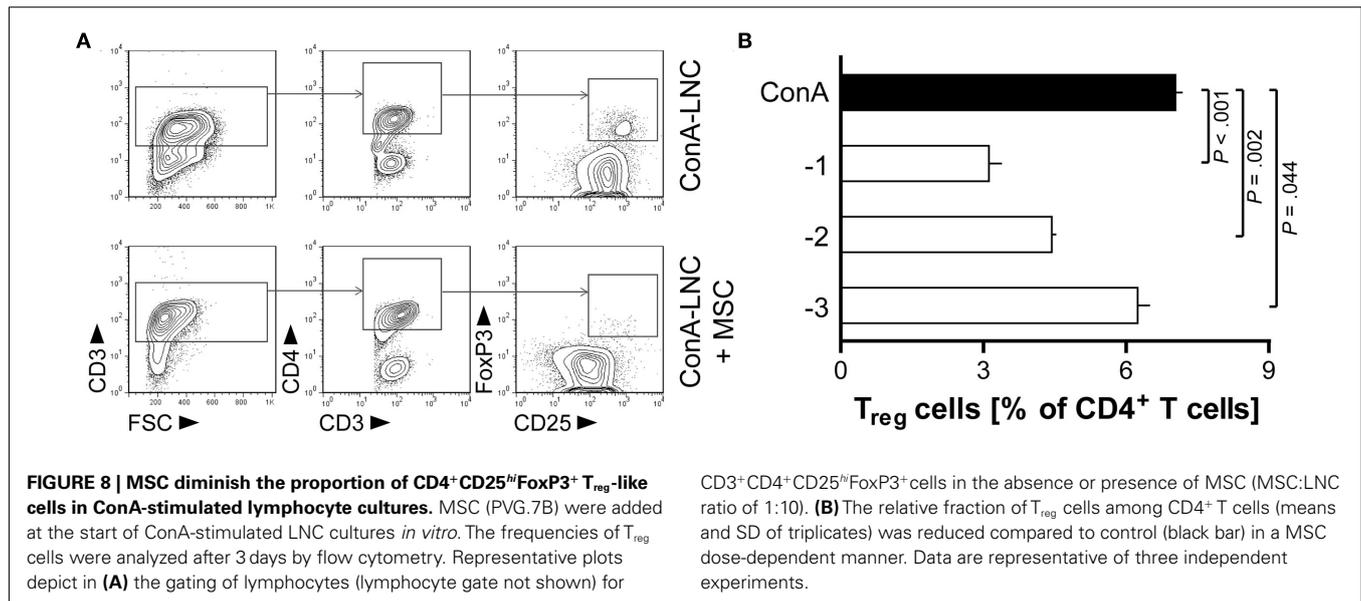
Tumor necrosis factor α , a potent mediator of immune stimulation, induced iNOS expression in rMSC *in vitro* without a requirement for auxiliary signals, and neutralizing the TNF α signal in the supernatant from stimulated lymphocyte cultures abolished it, underlining the importance of this cytokine in MSC modulation of T cell activation. The pro-inflammatory cytokine IFN γ has also been ascribed an important role in the inhibition of T cell responses mediated by MSC (Krampera et al., 2006; Ryan et al., 2007). IFN γ levels were markedly reduced after addition of MSC

to LNC cultures and attempts to restore T cell proliferation by addition of exogenous IFN γ were unsuccessful. Although IFN γ did not by itself induce iNOS expression in MSC it did show a synergistic effect in combination with TNF α or certain TLR ligands (LPS, but not poly-I:C) in support of studies performed in the mouse (Oh et al., 2007; Ren et al., 2008, 2009). This could explain why blocking the IFN γ signal by addition of anti-IFN γ antibody resulted in a significant reduction of iNOS expression in co-culture experiments as others also have shown (Oh et al., 2007; Ren et al., 2008).

Our data are in agreement with reports that have argued for “licensing” of MSC to acquire suppressor functionality (Waterman et al., 2010; Dazzi and Krampera, 2011). Collectively, our results support a model for the regulation of suppressor functions by rat BM stromal cells where MSC are primed by inflammatory signals, e.g., immunostimulatory cytokines, primarily TNF α , to induce iNOS expression leading to the accumulation of NO and in turn the inhibition of proliferation of activated T cells in the immediate proximity. In addition, MSC effectively shut off the generation of inflammatory cytokines by activated T cells (Ren et al., 2008) via a soluble factor dependent on COX activity, likely PGE $_2$.

The mechanisms employed to achieve immunosuppression are not identical in different species. In mMSC (Ren et al., 2008), as in rMSC, iNOS expression seems critically important for the inhibition of T cell proliferation. Our data imply that the cytokine requirement of MSC to activate this inhibitory pathway is not identical in rats (TNF α alone is sufficient) and mice (IFN γ and





any of the cytokines TNF α , IL1 α , or IL1 β are required). Ren et al. (2009) demonstrated that other inhibitory mechanisms, namely the secretion of IDO, but not iNOS, were employed by monkey and human BM-derived MSC in this context.

MSC-mediated inhibition of secretion of IFN γ and TNF α by activated T cells was dissociated from iNOS activity and NO, as demonstrated by transwell cultures and biochemical inhibition of iNOS. Our data are in contrast with a study of mMSC, which suggested that iNOS is involved in inhibiting IFN γ production by T cells stimulated with anti-CD3/CD28 beads in the presence of IL12 and anti-IL4 antibody (Oh et al., 2007). Our study identifies PGE₂ as a candidate soluble factor responsible for the dampening of inflammatory cytokine production by activated lymphocytes, in agreement with a previous study of hMSC (Aggarwal and Pittenger, 2005). Taken together, it can be concluded that rMSC utilize different pathways to regulate proliferation and cytokine production by T cells. The species-specific mechanisms regulating the suppression of immune cell activation and effector functions through BM stromal progenitor cells should be investigated further, because this research might have important implications for the use of MSC or related cell types in the clinical treatment of GVHD, autoimmune diseases or other syndromes of undesired T cell activity.

In a separate series of experiments, we have tested the therapeutic potential of the MSC lines presented here in a model of experimental GVHD induced by donor lymphocyte infusions after MHC-mismatched allogeneic stem cell transplantation (Zinöcker et al., 2011a). Despite their marked inhibitory potential *in vitro*, repeated systemic injections of MSC did not improve GVHD in these experiments even after prestimulation with IFN γ and TNF α to boost the iNOS pathway in MSC (Zinöcker et al., manuscript submitted). This suggests that their immunosuppressive efficacy is limited with respect to GVHD-related morbidity and mortality.

We show here that rMSC suppress proliferation *in vitro* only when in close proximity to stimulated T cells through short-range

activity of NO. The route of administration may determine the effectiveness of treatment if co-localization of MSC is required for the efficient suppression of alloreactive immune cells *in vivo*. We have previously observed by *in vivo* imaging that MSC which expressed enhanced green fluorescence protein transgenically accumulated in the lungs shortly (within minutes) after intravenous injection but did not detect these cells at that site or in other organs after several days *in vivo* and *post mortem* (unpublished observations). Failure to migrate to sites of allopriming and alloreactivity in GVHD is a plausible explanation for the observed lack of efficiency of MSC therapy.

Besides NO synthases, arginase 1 is an important enzyme that regulates L-arginine metabolism and production of NO and has the ability to inhibit T cell proliferation (Grohmann and Bronte, 2010). We did not address a potential role of arginase 1 in the suppression of activated T cells by rMSC in this study. If these cells make use of other, potentially redundant molecular pathways to inhibit T cells or other immune cell types, the strength of NO-dependent immunosuppression observed in this study would suggest that such alternative mechanisms are of minor importance regarding MSC-mediated inhibition of T cell proliferation in the rat species.

MSC have been shown to induce different subtypes of T_{reg} cells (Aggarwal and Pittenger, 2005; Di Ianni et al., 2008; English et al., 2009; Ghannam et al., 2010; Mougiakakos et al., 2011) as potential immunomodulatory mechanisms operating *in vivo* (Hoogduijn et al., 2010). Our finding that CD4⁺CD25^{hi}FOXP3⁺ T_{reg} cells were reduced after ConA-stimulation of T cells in the presence of rMSC was in marked contrast to the studies with hMSC and makes it less likely that T_{reg} cells are involved in the observed inhibition of proliferation or cytokine secretion of activated rat T cells *in vitro*. Further experiments could clarify the functional characteristics of this putative T_{reg} cell type.

Recently, Turley and co-workers presented findings which showed that iNOS represents a central pathway employed by both fibroblastic reticular and lymphoid endothelial cells to regulate

T cell activation in the lymph nodes of mice (Lukacs-Kornek et al., 2011). The observed inhibitory effects depended on IFN γ , TNF and direct cell contact (Lukacs-Kornek et al., 2011) and correlate well with our observations for rMSC, which displayed very similar cytokine requirements and inhibitory effects. We therefore speculate that the enzymatic conversion of NO is a common feature of stromal cells in rodents (Jones et al., 2007), which, in combination with PGE $_2$, control T cell expansion at sites of priming of the adaptive immune system (Lukacs-Kornek et al., 2011) and subsequent inflammation (Nombela-Arrieta et al., 2011).

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APPENDIX

