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ORIGINAL RESEARCH REPORT

Decidual Stromal Cells Promote Regulatory T Cells and Suppress Alloreactivity in a Cell Contact-Dependent Manner

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Acute graft-versus-host disease (GvHD) is a severe adverse event after stem cell transplantation. Bone marrow-derived mesenchymal stromal cells (BM-MSCs) have been used to treat GvHD, but decidual stromal cells (DSCs) isolated from term fetal membrane have advantages compared with BM-MSCs, including increased allosuppression, unlimited supply, and high expression of integrins. We introduced the use of DSCs in patients with steroid refractory aGvHD. In this study, we investigated factors of importance in the reduction of alloreactivity by DSCs. We found that DSCs need to have cell–cell contact in order to mediate suppression in mixed lymphocyte reactions (MLRs). This contact dependency is consistent with an increased frequency of $CD4^+$ $CD25^{high}FOXP3^+$ regulatory T cells (Tregs) and an augmented intensity of CD25 expression in $CD4^+$ T cells. Blocking of the activity of indoleamine-2,3-dioxygenase (IDO), prostaglandin E_2, PD-L1, and IFN-γ impaired the antiproliferative ability of the DSCs in MLRs. Neutralization of IDO also reduced the frequency of Tregs. In contrast to BM-MSCs, pretreatment of DSCs with high concentrations of IFN-γ (100 U/mL) reduced their ability to suppress alloreactivity, but stimulation of DSCs with MLR supernatants containing low levels of IFN-γ had no effect on the suppressive capacity in MLR. To conclude, DSCs differ in several aspects from MSCs and need to be close to alloreactive lymphocytes to mediate a suppressive effect and increase the frequency of Tregs. Thus, DSCs may not only use paracrine factors for systemic immunosuppression, but also more specifically target T cells locally in affected tissues.

Introduction

In order to prevent allogeneic responses against fetal tissue during pregnancy, the maternal immune system should develop tolerance to paternal-derived antigens [1]. Cell types of both maternal and fetal origin have been proposed to induce this tolerance through several mechanisms [2]. The site for the interaction between maternal immune cells and fetal cells is the placenta and the decidua. Stromal cells can be isolated from term fetal membrane and the adjacent decidua and are easily accessible and expanded [3,4]. The isolation requires minimum ethical consideration, as the placenta is discarded after delivery. Decidual stromal cells (DSCs) may therefore have promising potential in cellular therapy, which may require large quantities of cells.

Allogeneic hematopoietic stem cell transplantation (HSCT) is already established as an effective therapy for hematological malignancies and congenital disorders [5,6]. However, this treatment is associated with adverse events such as graft-versus-host disease (GvHD). Severe steroid-refractory acute GvHD is associated with a very high mortality rate [7]. Screening of cell types originating from different anatomical structures in the fetal–maternal interface has revealed that third-party HLA-mismatched DSCs are more suppressive in alloantigen-stimulated lymphocyte cultures in vitro than in third-party bone marrow-derived mesenchymal stromal cells (BM-MSCs) [4,8,9], which have been previously used to treat acute GvHD [10–12]. In addition, DSCs have a higher expression of the negative co-stimulatory marker programmed cell death 1 ligand (PD-L1) and integrins such as α4 (CD49d), intercellular adhesion molecule-1 (ICAM-1), and β1 (CD29) [4,13,14]. These cells, however, do not have the same multipotent differentiation capabilities as BM-MSCs and other cells isolated from the fetal membranes [3,15–17].

The mechanisms for maintaining fetal–maternal tolerance resemble the immunomodulatory traits of BM-MSCs quite well. BM-MSCs can inhibit proliferation of target cells that are in direct physical contact with them through engagement of programmed cell death 1 (PD-1) or through production of indoleamine-2,3-dioxygenase (IDO) in the presence of IFN-γ [18–20]. BM-MSCs also constitutively produce molecules

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such as transforming growth factor (TGF)-β, interleukin (IL)-10, HLA-G5, prostaglandin E2 (PGE_2), and galectins, which give them the ability to avoid rejection and to suppress allostimulated lymphocytes [21–27].

The fetal membranes and cells originating from the fetal membranes have shown great potential in regenerative medicine for a number of applications, including burn treatment, corneal reconstruction, reduction of lung fibrosis, and alveolar restitution [28–31]. We introduced the use of DSCs in patients with severe acute GvHD, and the overall response rate was 75% [32]. To further improve the efficacy of this treatment, the immunomodulatory properties of DSCs should be examined in greater detail. Here, we have investigated the inhibitory mechanisms mediated by DSCs in allogeneic settings. We have identified several factors involved in the suppressive effect that distinguish these cells from BM-MSCs.

Methods

Isolation of DSCs from term fetal membrane

Term placentas were taken from caesarean sections after obtaining informed consent. DSCs from nine donors were isolated and used separately in the experiments. The ages of five donors with known identity were 25, 26, 29, 33, and 40 years. Ethical approval was obtained from the institutional ethical review board (2009/418-31/4). The method for isolating the cells has been previously described in detail [4,32]. Briefly, the fetal membrane was dissected from the placenta and washed several times in HBSS (Invitrogen) and phosphate-buffered saline (PBS; Thermo Fisher Scientific) before being incubated with trypsin/EDTA (Thermo Fisher Scientific) in three consecutive steps. The trypsin digests were pooled and washed in DMEM (Thermo Fisher Scientific) containing 10% fetal calf serum (Thermo Fisher Scientific), penicillin (100 U per mL, Thermo Fisher Scientific), streptomycin (100 μg per mL; Thermo Fisher Scientific) (herein referred to as complete DMEM), and plated in T175 flasks (Nunc A/S). After trypsination, the fetal membranes were washed in complete DMEM and cut into small pieces, and these were spread out and incubated at 37°C in T175 flasks. The tissue explants were removed from the flasks when colonies of fibroblast-like cells appeared. Thus, the cells were isolated by two different methods to optimize the number of cells, but both the trypsin digests and the tissue explants generated homogenous populations of cells of maternal origin and with the same phenotypes. The cells were harvested with trypsin/EDTA when the cells were approximately 90% confluent. The DSCs were expanded further to passage 2 or 3 and thereafter used in the experiments. To illustrate from which part of the placenta the DSCs are isolated, a schematic drawing of the term placenta and adjacent decidua is shown in Supplementary Fig. S1(Supplementary Data are available online at www.liebertpub.com/scd).

Isolation of BM-MSCs has been previously described [4].

Mixed lymphocyte reaction

Peripheral blood mononuclear cells (PBMCs) were isolated from buffycoats using Lymphoprep™ gradient separation according to the instructions of the manufacturer (Axis-Shield). Responder PBMCs from at least five different donors were used in the experiments to ensure reliability, considering the biological variability among the PBMC donors. Responder PBMCs (10^6 cells per mL) from one donor and irradiated DSCs from one donor (30 Gy, 10^5 cells per mL) were mixed with an irradiated stimulator pool of PBMCs (30 Gy, 10^6 cells per mL) from at least six donors. DSCs or BM-MSCs were added either directly to the mixed lymphocyte reaction (MLR) or in a transwell (0.4 μm pore size) (BD labware). For flow cytometry and for transwell experiments, the cells were incubated in a 12-well tissue culture plate (BD labware) for 5 days in 37°C, 5% CO_2, in RPMI with 5% AB serum, PEST (see previous section), and 2 mM L-Glu (complete RPMI; Invitrogen). On day 5, the cells were thoroughly resuspended, and 200 μL of cell suspension was transferred in triplicate to a 96-well tissue culture plate (BD labware), to which 1 μCi of ^{3}H-thymidine (PerkinElmer) was added in each well. The remaining cells were used for flow cytometry. For neutralization experiments, the MLRs were performed in 96-well plates. Then, 16–18 h after ^{3}H-thymidine addition (day 6), the cells were harvested using Harvester 96 (Tomtec), and the beta radiation was measured with a Trilux 1450 MicroBeta microplate scintillation counter (Wallac Sverige AB).

Neutralization

Antibodies were added to the MLRs to block IL-10 (1 μg/mL), IL-17 (10 μg/mL), TGF-β (10 μg/mL), IFN-γ (1 μg/mL), (all from R&D Systems), HLA-G5 (10 μg/mL), PD-L1 (10 μg/mL), and (from BioLegend). Isotype control antibodies (IgG1, -2a, or -2b; R&D Systems) were added at the same concentrations. IDO, PGE_2, and galectin-1 were blocked with 1-methyl-DL-tryptophan (1 mM), indomethacin (200 μM; Sigma-Aldrich), and thiodigalactoside (1 μg/mL; Peprotech), respectively.

Flow cytometry

A five-color panel was used to analyze the MLR with added DSCs or BM-MSCs. The cells were harvested from the culture plates to falcon tubes by thoroughly resuspending with a pipette. They were washed twice in PBS and stained in 100 μL PBS with antibodies to CD3, CD4, CD8, and CD25 (BD Biosciences). For intracellular staining, FOXP3 fixation/permeabilization concentrate and diluent, and permeabilization buffer from eBioscience were used. After cell-surface staining, the cells were washed once in PBS, followed by incubation in fixation/permeabilization buffer for 20 min. After being washed twice in permeabilization buffer, the cells were incubated with anti-FOXP3 antibody for 30 min in permeabilization buffer (eBiosciences). They were washed twice in permeabilization buffer and once in PBS before flow cytometry analysis (Gallios; Beckman Coulter) (FACSAria, BD). "Fluorescence minus one" controls were used when analyzing the data using FlowJo (Tree Star) software.

A three-color panel was used for characterization of the DSCs. The cells were stained with antibodies to CD45, CD14, CD31, CD34, CD90, CD105, CD86, CD44, CD73, CD11a, SSEA-3, SSEA-4, CD49d, CD29 (BD), CXCR4, EPCAM, VCAM, ICAM, PD-L1, PD-L2, HLA-G (BioLegend), HLA-I, and HLA-II (Dako).

Pretreatment of fetal membrane cells

An MLR culture with 30×10^6 responder cells in 30 mL of complete RPMI medium was cultivated for 6 days in a

75-cm^2 tissue culture flask (Nunc) with a screw cap facing upward. On day 6, the contents were centrifuged at 500 *g* for 10 min, and the supernatants were frozen until use. As pretreatment, a subconfluent culture of DSCs was allowed to grow for 48 h in 15 mL of complete DMEM and 15 mL of MLR supernatant. As a control, DSCs were grown in 15 mL of complete DMEM and 15 mL of complete RPMI medium. Addition of 100 U/mL IFN-γ (R&D Systems) to a subconfluent flask of DSCs was also done 48 h before addition to an MLR. As a control, cells cultivated without IFN-γ were used.

Test of multipotent differentiation capabilities

The method for testing multipotential differentialtion capabilities has been described in detail elsewhere [33]. Briefly, for differentiation to osteoblasts, the cells were cultured in DMEM with 0.1 μM dexamethasone and 0.05 mM ascorbic acid-2-phosphate. For differentiation to adipocytes, we used DMEM with 1 μM dexamethasone, 0.2 mM indomethacin, and 0.01 mg/mL insulin. For differentiation to chondrocytes, the cells were pelleted and cultured in DMEM supplemented with 0.1 μM dexamethasone, 1 mM Na-pyruvate, 0.17 mM ascorbic acid-2phospate, 0.35 mM Proline, 6.25 μg/mL ITSS, 5.33 μg/mL linoleic acid, 1.25 mg/mL BSA, and 0.01 μg/mL TGF-β-3. To view lipid vacuoles in adipose-differentiated cells, ORO staining was used. Differentiation to osteoblasts was viewed by coloring minerals with Alizarin red S (0.5% w/v in dH_2O, pH 4). As negative and positive controls, DSCs were cultured in complete DMEM, and BM-MSCs were cultured in the induction medium, respectively. Apart from everyday chemicals, all substances were obtained from Sigma-Aldrich. Chondrocyte differentiation was analyzed by RNA expression of aggrecan. The pellets from the cultivations were lysed using RLT buffer (Qiagen). RNA from the lysate was isolated using the RNeasy mini kit (Qiagen), and cDNA was synthesized from the RNA using the High-Capacity cDNA Reverse Transpcription kit (Applied Biosystems). Real-time quantitative-PCR (RQ-PCR) was performed on the ABI 7500 Sequence Detection System (Applied Biosystems) using TaqMan technology. The reactions included 1 × TaqMan Universal RQ-PCR Master Mix (Applied Biosystems), cDNA sample, 1 × primer- and probe-mix of aggrecan (*ACAN*) (cat. No. 4331182. Life technologies). *ABL* was used as internal reference genes. All samples were run in triplicate. Relative quantification of aggrecan expression was calculated according to the ΔCt method. The formula used was $\Delta Ct = 2^{(CtABL - CtAggrecan)}$. The following primer and probe sequences for ABL were used. *ABL*-Sense: 5′- TGG AGA TAA CAC TCT AAG CAT AAC TAA AGG T -3′, *ABL*-AntiSense: 5′- GAT GTA GTT GCT TGG GAC CCA -3′, *ABL*-Probe: 5′-6-FAM- CCC TTC AGC GGC CAG TAG CAT CTG A -TAMRA-3′.

Elisa

To measure the concentration of TGF-β in DSC culture supernatants, the DuoSet kit (R&D Systems) was used according to the manufacturer's instructions.

Determination of cell origin by PCR and capillary electrophoresis

The origin of the DSCs was determined by examining microsatellite polymorphism. To obtain fetal cells as a control for determination of origin of the DSCs, amniotic epithelial cells were isolated from the amnion. Briefly, amniotic membrane was carefully separated from the chorionic plate of the placenta and was thereafter incubated in trypsin/EDTA to digest amnion epithelial cells according to a previously published protocol [34]. Alternatively, cord blood was collected. DNA was extracted from DSCs, cord blood, amniotic epithelial cells, and blood samples from donating mothers using the EZ1 DNA Blood 350-μL kit and an EZ1 Advanced XL instrument (Qiagen). Microsatellites were amplified using a PTC 200 thermal cycler (SDS Scandinavian Diagnostic Services). PCR products were processed with capillary electrophoresis (ABI 3130XL Genetic Analyzer; Applied Biosystems).

Fluorescence microscopy

DSCs were cultured on chamber slides (Nunc). DSCs and BM-MSCs were fixed with acetone/methanol or 1% formaldehyde. Fresh cells were permeabilized with 0.2% Triton-X 100 (Sigma). The cells were stained with a sheep anti-human antibody to IDO (Thermo Fisher Scientific) as primary antibody and an FITC-labeled goat anti-sheep antibody as secondary antibody (Jackson ImmunoResearch). The cells were viewed with an Olympus BX-51 fluorescence microscope, and images were taken with an Olympus XC30 camera. The data were collected and edited using CellSens standard software (Olympus). Trypsinated DSCs were also examined for IDO with FACS using the same staining protocol as for FOXP3.

Statistical analysis

Statistics were applied using GraphPad Prism software (GraphPad Software) to identify trends and statistically significant outcomes. We used the Wilcoxon matched-pair signed-rank test. All outcomes with *P*-values of < 0.05 were considered statistically significant.

Results

Phenotypic characteristics of stromal cells isolated from term fetal membrane

The characterization and morphology of the cells has been previously published and can be found in detail in previous publications by Karlsson et al. [4] and Ringdén et al. [32]. Briefly, flow cytometry showed that the DSCs were positive for HLA class I and CD markers 29, 44, 49d, 54 (ICAM), 73, 90, 105, 273 (PD-L2), and 274 (PD-L1), and negative for SSEA-3, SSEA-4, HLA class II, HLA-G5, and CD markers 11a, 14, 31, 34, 45, 86, 106 (VCAM), 184 (CXCR4), and 326 (EpCAM). Microsatellite polymorphism analysis revealed that all the DSCs isolated from term fetal membrane for use in the experiments were of maternal origin. The DSCs showed differentiation to adipocytes but not chondrocytes. All positive controls with BM-MSCs showed differentiation to osteoblasts, adipocytes, and chondrocytes. One representative experiment of bone and adipocyte differentiation for both DSCs and BM-MSCs is shown in Supplementary Fig. S1. For analysis of differentiation to cartilage, RQ-PCR was used to detect RNA expression for aggrecan. No expression of aggrecan was observed in any of the DSC donors, except very low levels for one donor

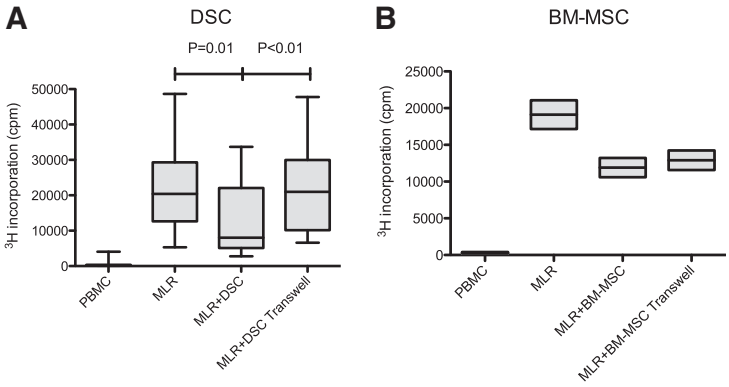

FIG. 1. Decidual stromal cells (DSCs) suppress alloreactivity in a contact-dependent manner. **(A)** Proliferation in mixed lymphocyte reactions (MLRs) was significantly reduced by co-culture with third-party DSCs ($n=14$). When DSCs were placed in a transwell, disallowing cell-to-cell contact between the responder peripheral blood mononuclear cells (PBMCs) and the DSCs, the antiproliferative effect was significantly reduced. (DSCs from $n=7$ placental donors) **(B)**. When the DSCs were replaced with bone marrow-derived mesenchymal stromal cells (BM-MSCs), the proliferation was reduced both in the regular setting and in transwells ($n=2$).

(aggrecan/*ABL* ratio $=2.3585\times10^{-5}$). The same aggrecan/*ABL* ratio for the positive control (BM-MSC) was 12.2.

Stromal cells from term fetal membrane reduce proliferation in MLRs in a contact-dependent fashion

Proliferative responses of MLRs were measured in the presence or absence of DSCs (responder PBMC to DSC ratio: 10:1; $n=14$). The proliferation of responder PBMCs in the MLR was significantly reduced when they were co-cultured with DSCs, as compared with the control MLR (median 61% reduction, $P=0.01$) (Fig. 1A). When DSCs were physically separated from the MLR in a transwell system, we found that the suppressive effect was almost completely abolished ($P<0.01$). Furthermore, the proliferation was not reduced if the DSCs in the transwell were cultured with an equal amount of irradiated PBMCs from multiple donors during the whole incubation period ($n=2$). As previously shown, BM-MSCs suppressed proliferation of responder PBMCs both when added directly to the MLR and when added in a transwell, compared with the control MLR (Fig. 1B) ($n=2$).

Several factors are involved in the immune regulation by DSCs

Next, we examined the importance of different immunomodulatory factors for the DSC-mediated suppression. When IDO was neutralized with the competitive inhibitor methyl-DL-tryptophan (1-MT), the regular modulation of proliferation of the alloantigen-stimulated PBMCs by DSCs was almost completely absent (Fig. 2A). The proliferation in the MLR with DSCs was increased by a median of 105% in the presence of 1-MT ($P<0.005$, $n=11$) compared with cultures without 1-MT. A significant increase in proliferation compared with the negative control was also observed when we added neutralizing agents directed against PGE_2 (median 26%, $P<0.02$, $n=12$), PD-L1 (24%, $P<0.05$, $n=7$), and IFN-γ (101%, $P<0.001$, $n=11$) (Fig. 2B–D). However, the presence of neutralizing antibodies to IL-10 ($n=8$), IL-17 ($n=4$), TGF-β ($n=12$), HLA-G5 ($n=10$), and galectin-1 ($n=4$) did not modulate the effect of DSCs on lymphocyte proliferation (Fig. 2E–H and data not shown). Thus, neutralization of TGF-β had no effect on proliferation, but we found that TGF-β is constitutively produced by DSCs (median 727 pg/mL (range 14–844) after subtracting the medium control, $n=3$). The number of experiments performed varies for each neutralizing substance due to the fact that the neutralizing substances were included in the experiment layout at different time points in the ongoing series of experiments.

Since IFN-γ was found to be of importance for the DSC-mediated suppression, we investigated whether the suppressive effect can be potentiated by pretreating the cells with IFN-γ. Unexpectedly, when the DSCs were stimulated with a high-dose IFN-γ (100 U/mL) before addition to the MLR, there was an increase in proliferation compared with MLR with untreated DSCs (44.8% median increase, $P=0.0002$, $n=16$) (Fig. 3A). In contrast, pretreatment of BM-MSCs with the same concentration of IFN-γ potentiated the suppressive effect compared with untreated BM-MSCs (median decrease of 177%, $P=0.03$, $n=7$) (Fig. 3B).

To investigate whether physiological levels of cytokines that are released during an alloresponse affect the suppression of the MLR by DSCs, the DSCs were cultivated with MLR supernatants for 48 h before incubation in the MLR. As previously shown, the median IFN-γ concentration in MLR supernatants is around 400 pg/mL [4], corresponding to 8 U IFN-γ/mL. There was no effect on proliferation of responder PBMCs whether or not the DSCs had been pretreated MLR supernatants, either in the regular setting or in transwell systems ($n=7$) (Fig. 3C).

Immunofluorescence microscopy showed that DSCs constitutively express IDO. The expression was not noticeably increased if the DSCs were pretreated with 10- or 100 U IFN-γ per mL for 48 h (Fig. 3D–F) ($n=4$). This finding was also confirmed with flow cytometry when analyzing DSCs from three different placental donors ($n=3$) using the same primary and secondary antibodies as for the immunofluorescence microscopy. The mean fluorescence intensity (MFI) for untreated DSCs was 481 (range 452–561), and it was 561 (range 556–598) for DSCs treated with 100 U IFN-γ per mL. The MFI for the negative control was 306 (range 286–322) for untreated cells and 356 (range 339–357) for treated cells (data not shown).

DSCs modulate the expression of CD25 and FOXP3 in alloantigen-stimulated $CD4^+$ T lymphocytes

The frequency of total $CD25^+$ cells within the $CD3^+$ population, of which the majority are presumably recently activated T cells, increased when PBMCs were stimulated with allo-antigens (median 49.7% (range 18.1–64.1), $n=13$) (Fig. 4A). However, the frequency was reduced when DSCs

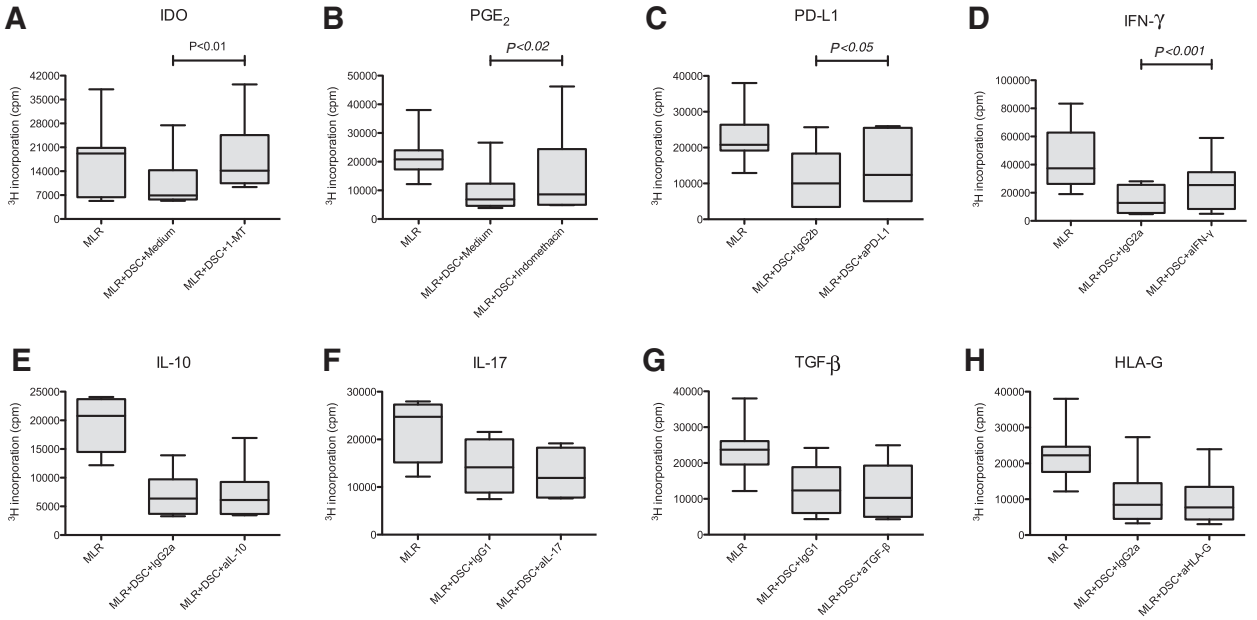

FIG. 2. Indoleamine 2,3-dioxygenase (IDO), prostaglandin E_2 (PGE_2), programmed death ligand 1 (PD-L1), and IFN-γ are involved in the DSC-mediated suppression. The proliferation of responder PBMCs in the MLR was reduced if DSCs were added to the culture, but neutralization of **(A)** IDO ($n=11$), **(B)** PGE_2 ($n=12$), **(C)** PD-L1 ($n=7$), and **(D)** IFN-γ ($n=11$) significantly reduced the suppressive effect. Neutralizing antibodies to **(E)** IL-10 ($n=8$), **(F)** IL-17 ($n=4$), **(G)** TGF-β ($n=12$), and **(H)** HLA-G5 ($n=10$) did not affect the proliferation of PBMCs in MLRs with DSCs. (DSCs from $n=7$ placental donors)

were present [35.2% (range 13.0–60.7), $P<0.07$]. When the DSCs were added in a transwell system, there was no significant reduction in CD25-expressing $CD3^+$ T cells compared with the control MLR [43.5% (range 13.0–67.1)]. However, the MFI of CD25 among the $CD3^+CD4^+$ cells was highly increased in the presence of DSCs compared with the MLR control and the transwell setting ($P=0.001$, $n=11$) (Fig. 4B).

In line with the findings that the intensity of CD25 expression in $CD4^+$ T cells was increased in the presence of DSCs, we found that the frequency of $CD25^{high}FOXP3^+$ cells in the $CD3^+CD4^+$ cell population [referred to as regulatory T cells (Tregs)] was higher when DSCs were added to the MLR cultures [$n=11$, median frequency 6.8% (range 4.0–10.0)] than in the control MLR [median 2.7% (range 1.8–6.2)](Fig. 4C, E, F). In a setting that did not allow cell-to-cell contact between DSCs and lymphocytes, the proportion of Tregs was significantly lower than when cell-to-cell contact was allowed [median 4.9% (range 2.2–7.2)] (Fig. 4C, E–G). Thus, these data indicate that DSCs reduce the proportion of activated T cells and partly promote the expansion and/or induction of Tregs in a contact-dependent manner. The induction of Tregs by DSCs is, however, not entirely dependent on cell–cell contact as the frequency of Tregs was increased in the transwell setting compared with control MLR (Fig. 4C). When we repeated the experiments using BM-MSCs, we observed no contact-dependent effect, as Treg frequencies were increased both when BM-MSCs were added directly to the MLR and when they were placed in a transwell ($n=2$) (Fig. 4H–J).

Since IDO appeared to be critical for the modulation of proliferation as described earlier, the expression of CD25 and FOXP3 was investigated when the DSCs were incubated with 1-MT. Interestingly, we found that IDO inhibition significantly decreased the frequency of Tregs in MLR cultures with DSCs ($n=7$, $P=0.03$) (Fig. 4D). One representative experiment is shown in Fig. 4K and L. Addition of 1-MT to MLR cultures without DSCs had no effect on the proportion of Tregs (data not shown).

Discussion

Severe steroid-refractory acute GvHD is a major complication after HSCT, and new interventions are needed. There are therapeutics and diagnostic tools in early-phase trials, some of which may pave the way for reduced mortality from GvHD in the future [35,36]. BM-MSCs have immunosuppressive properties in vitro, and they have been successfully used to treat acute GvHD [10–12]. However, it is of interest to find new cell types with better ability to quench acute inflammatory conditions. Since the developing fetus is in a milieu in which tolerance is an absolute requirement for success, we thought it would be logical to search for a cell type with the required immunomodulatory attributes at the fetal–maternal interface. In a previous study, we isolated stromal cells from different compartments of the fetal–maternal interface and found that decidual cells isolated from term fetal membrane are most suitable for immune suppression and cell-surface expression [4]. In the present study, we examined the properties of these cells in greater detail. We found that the cells we isolated from term fetal membrane were of maternal origin only. The fetal membranes, amnion, and chorion are intimately associated with the decidua, which suggests that the cells may originate from decidua parietalis and/or decidua capsularis. Cells originating from the fetal membranes, the placenta, and the

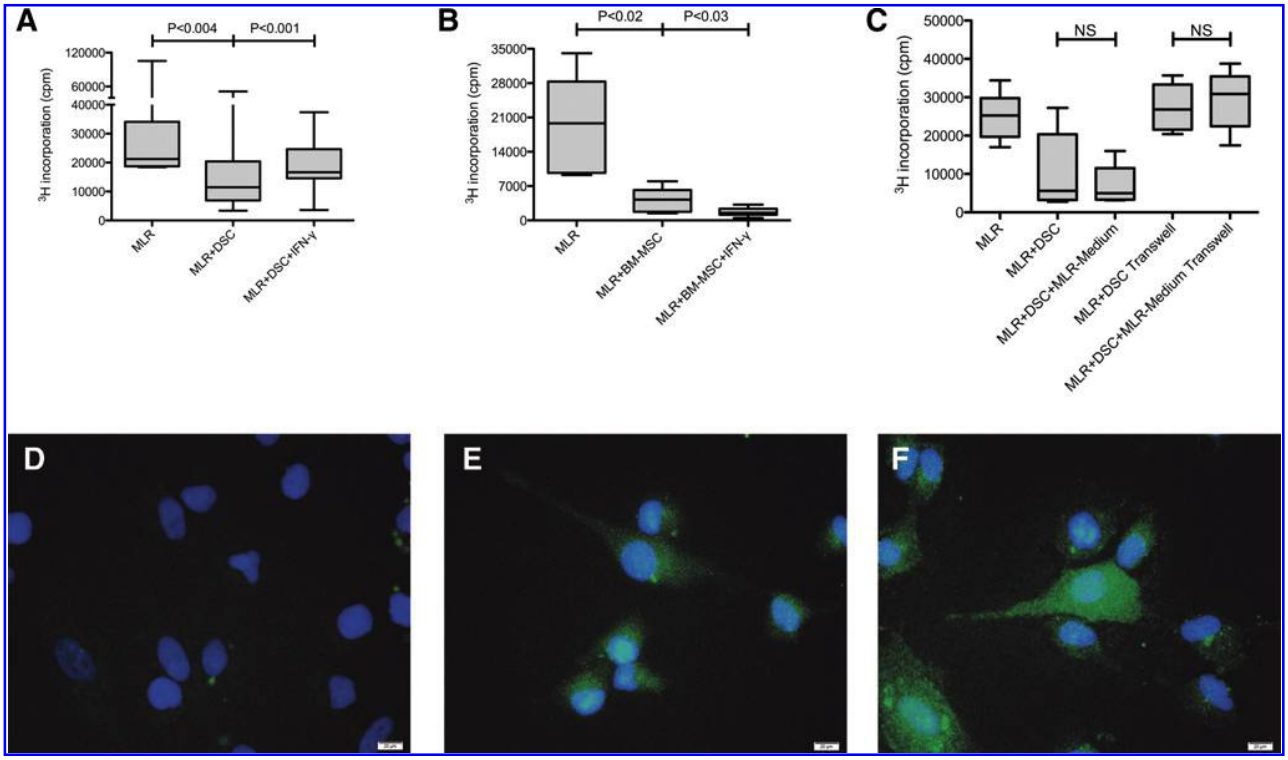

FIG. 3. Effect of inflammatory cytokines on DSC-mediated immune regulation **(A)** Pretreatment of the DSCs with IFN-γ (100 U/mL, 48 h) increased the proliferation of responder PBMCs in the MLR compared with untreated DSCs ($n=16$). **(B)** Bone marrow-derived mesenchymal stromal cells (BM-MSCs) pretreated with IFN-γ had a higher antiproliferative capacity in MLRs than untreated cells ($n=7$). **(C)** When the DSCs were incubated with MLR supernatants 48 h before co-culture with the MLR, there was no difference between treated and untreated DSCs, either in a regular setting or in transwells ($n=7$). Immunofluorescence microscopic images of unstimulated DSCs **(E)**, or DSCs pretreated with 100 U/mL IFN-γ, stained with 4′,6-diamidino-2-phenylindole (DAPI, *blue*), and treated consecutively with goat anti-human IDO antibody and FITC-conjugated sheep anti-goat antibody (*green*) **(F)**. The negative control **(D)** was stained with DAPI and secondary antibody alone. Original magnification ×40; bar represents 20 μm. Gamma is increased by the same amount in all microscopy pictures. (DSCs from $n=7$ placental donors). Color images available online at www.liebertpub.com/scd

decidua have similar immunomodulatory properties, as shown by work from our group and that of others [4,9,13,15,37,38]. Since both maternal and fetal cells are present in the fetal membranes, determination of the origin of cells is an important step in the characterization.

Our results show that the ability of DSCs to suppress proliferation in the MLR is contact dependent. This contrasts with BM-MSCs, which suppress MLRs independently of cell-to-cell contact (Fig. 1B). This finding has also been reported by others [19,39]. Cells isolated from amnion also suppress T cells' contact independently, as shown by Magatti et al. [37]. In the clinical setting, contact-dependent immune modulation could be advantageous, as a local immunosuppression may not affect the overall mechanisms of defense against infection.

T-cell proliferation can be inhibited by the activation of IDO, which leads to depletion of tryptophan and production of kynurenine. IDO can be expressed by both the decidua and the fetal syncytiotrophoblasts and is important for prevention of rejection of the fetal allograft [40,41]. We found that inhibition of IDO in an MLR with DSCs almost entirely eliminated their antiproliferative effect. We also found that neutralization of IFN-γ increased proliferation, which is in accordance with the fact that this cytokine has been reported to be an inducer of IDO activity [27]. Therefore, we assumed that the observed effect on proliferation after blocking of IFN-γ was due to a lack of induction of IDO. However, we found that DSCs constitutively express IDO, that IFN-γ had no notable effect on induction of IDO, and that high levels of IFN-γ reduced the immunosuppressive capacity of DSCs. As reported by others, BM-MSCs do not constitutively express IDO [20,42], but it is highly up-regulated when the BM-MSCs are treated with IFN-γ. This difference might partially elucidate why the immunosuppressive capacity of BM-MSC is increased when pretreated with high levels of IFN-γ [43], whereas DSCs fail to potentiate allosuppression by the same treatment. IFN-γ is a potent regulator of IDO gene expression, but the regulation of IDO is complex and differs between cell types [44]. The fact that DSCs produces IDO in the absence of IFN-γ is intriguing.

In a previous study, we found that IFN-γ did not induce expression of HLA class II in DSCs [4], which contrasts with the situation in BM-MSCs where IFN-γ up-regulates expression of HLA class II [45]. This implies that the reduced suppression of alloreactivity after high IFN-γ treatment of DSCs is not due to an increased presentation of alloantigens.

One may speculate that the fact that high concentrations of IFN-γ diminish immunosuppression is an evolutionary trait of

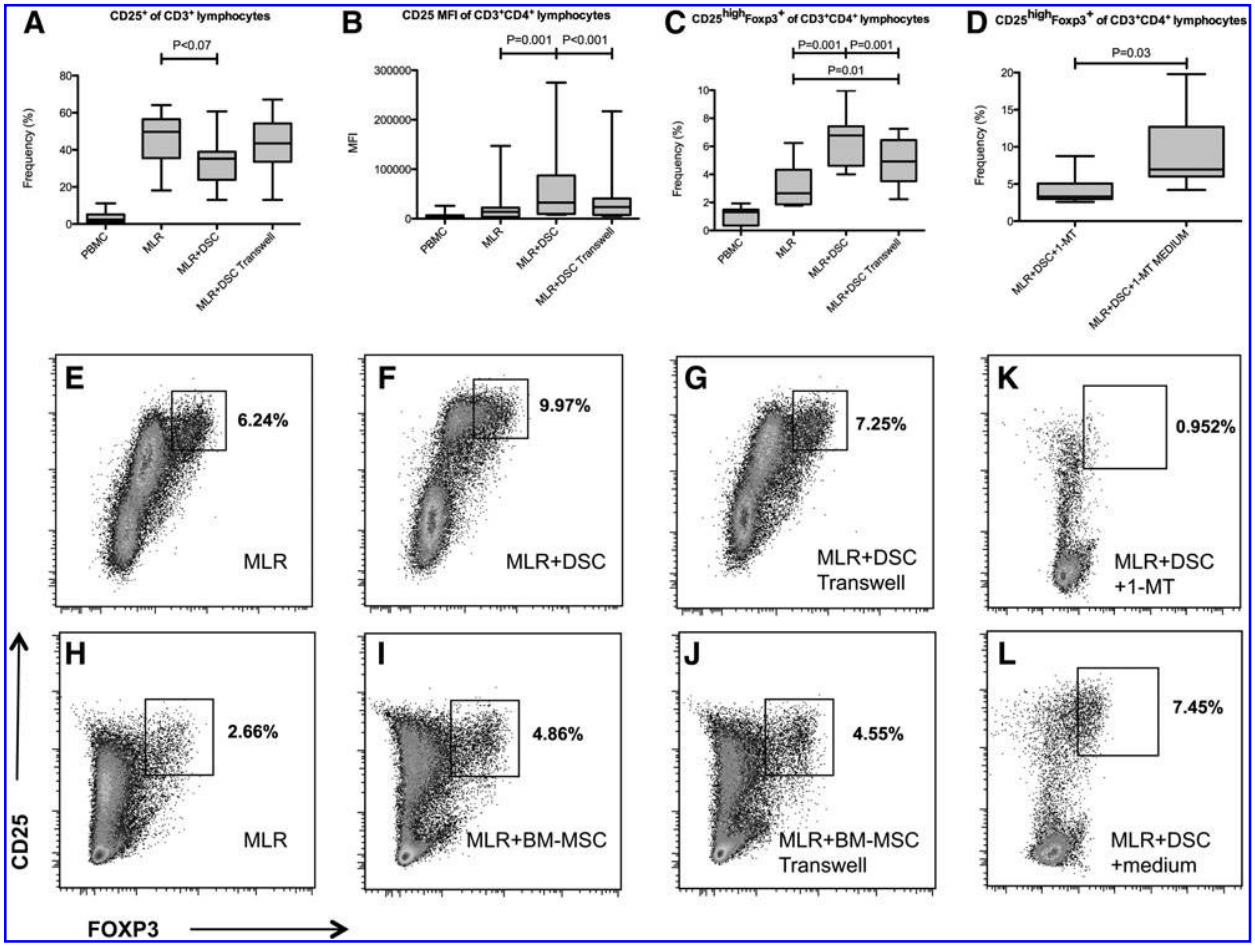

FIG. 4. DSCs promote $CD4^+CD25^+FOXP3^+$ regulatory T-cell expansion in a contact-dependent manner. **(A)** Flow cytometry showed that the proportion of total $CD25^+$ cells in the $CD3^+$ cell population was reduced when DSCs were added to the MLR ($n=13$), but only when cell-to-cell contact was allowed. **(B)** The mean fluorescence intensity (MFI) of CD25 expression in the $CD3^+CD4^+$ cell population increased when DSCs were present compared with both regular MLR and the transwell setting ($n=11$). **(C)** The frequency of $CD25^{high}FOXP3^+$ cells in the $CD3^+CD4^+$ cell population increased in both in a cell-to-cell contact-dependent and independent manner when DSCs were added to the culture ($n=11$). **(D)** The induction of Tregs by DSCs is dependent on IDO. When 1-MT are added to the MLR with DSCs, the frequency of $CD25^{high}FOXP3^+$ Tregs is significantly reduced compared with control ($n=7$). **(E–L)** Representative dot plots gated for lymphocytes, CD3 and CD4, showing the differences in CD25 and FOXP3 expression under the conditions indicated. **(K, L)** Representative plots from one experiment. The expression of CD25 and FOXP3 after addition of 1-methyl-DL-tryptophan (1-MT) to MLRs with DSCs to block activity of indoleamine-2,3-dioxygenase (IDO) was reduced **(K)** compared with if 1-MT was absent **(L)**. (DSCs from $n=7$ placental donors)

stromal cells in the fetal–maternal interface to allow T-cell effector functions during microbial invasion to protect the baby from infection. When the DSCs were pretreated with MLR supernatants before co-culture in MLR, which presumably contains biological concentrations of various cytokines produced in an allogeneic setting, the suppression was sustained.

The reason for the DSC-mediated suppression being reduced when IFN-γ is blocked is not clear. However, we have previously shown that IFN-γ up-regulates the expression of various different molecules in DSCs, including ICAM-1 (CD54) and PD-L1 [4]. ICAM-1 has shown to be important for BM-MSC-mediated suppression [46]. Since DSCs promote their suppressive effect via contact-dependent mechanisms, it is possible that high ICAM-1 expression is of significance in our setting. It is already established that engagement of PD-1 by PD-L1 leads to suppression of T-cell activation [47]. DSCs have previously been shown to inhibit cytokine production by $CD4^+$ cells through the PD-L1/PD-1 pathway [13]. However, the origin of the isolated cells in that study was not determined, and the isolation protocol differs from ours. Nagamatsu et al. [13] showed that PD-L1 expression is enhanced when the cells are treated with IFN-γ, which we also described in our previous report [4]. We noted a small but still statistically significant effect on proliferative responses when antibodies blocked PD-L1. We have previously shown that DSCs have higher expression of PD-L1 than BM-MSCs. Since DSCs have a higher expression of PD-L1 and suppress lymphocytes in a contact-dependent manner, it is likely that suppression of T cells through PD-1 is of importance [18].

Inhibition of PGE_2 also increased proliferation in the MLR with DSCs. PGE_2 plays an important role in implantation

and suppression of alloreactivity during the first trimester, as well as during initiation of parturition [48]. There is also evidence to suggest that PGE_2 inhibits production of IFN-γ and promotes development of regulatory T cells [49], which is in line with the results we have presented. In addition, PGE_2 has also been described as an inducer of IDO [50], and neutralization of PGE_2 may increase alloreactivity in the MLRs due to a decreased production of IDO in the DSCs.

We have previously shown that the concentration of IL-10 is increased in the MLR when DSCs are present, but that DSCs themselves do not produce IL-10 [4]. The present study shows that TGF-β is produced by DSCs. We attempted to neutralize both IL-10 and TGF-β, but neutralization of these cytokines did not affect the proliferation in MLRs. This does not exclude the possibility that these factors are important for suppression of alloreactivity in vivo. For example, neutralization of IL-10 and TGF-β does not abrogate Treg-mediated suppression in vitro [51,52]. Still, IL-10 in vivo knockout models show the importance of IL-10 for the suppression of inflammatory bowel disease, a condition with a similar pathophysiology to that of GvHD [53].

The frequency of $CD25^{high}FOXP3^+$ regulatory T cells in the $CD3^+CD4^+$ population was significantly increased when DSCs were added to the MLR. Physical contact between the DSCs and responder T cells induced higher frequencies of Tregs compared with cultures in which responder cells and DSCs were separated. This indicates that proximity is important but that there also seems to be a contact-independent mechanism for Treg induction. DSCs constitutively expresses TGF-β that may induce Tregs. In this context, it is also interesting that the proportion of CD25-expressing $CD3^+$ T cells was reduced by the presence of DSCs, but only if cell contact was allowed. The majority of the CD25-expressing T cells after in vitro stimulation are recently activated cells [54], and our data suggest that DSCs suppress activation of effector T cells, and promote Treg expansion and/or induction. This is further supported by the finding that T cells with high-intensity expression of CD25 were expanded by DSCs. Interestingly, the in vivo concentration of cells with high expression of CD25 was reported to be higher in decidua parietalis than in decidua basalis and peripheral blood of pregnant women [55,56]. As shown in those studies, the induction of CD25 expression is elevated in certain areas of the fetal–maternal interface. Mjösberg et al. have further shown that first-trimester decidua is enriched in $CD25^{high}Foxp3^+$ Tregs and that these cells had a higher proliferative activity compared with circulating Tregs and also compared with decidual $CD25^{dim}$ T cells [57], suggesting that Tregs at the fetal–maternal interface have a great expansion potential. It is, therefore, likely that DSCs, and/or other cells from the placenta and fetal membranes with similar characteristics, have a capacity to promote Treg proliferation.

IDO-producing dendritic cells can induce generation of Tregs [58]. There is also supporting evidence suggesting that if IDO is neutralized in an inflammatory environment, the Treg phenotype can be destabilized and the cells may switch toward a Th17 phenotype [59]. Tregs themselves may also be important for production of IDO; a few studies have suggested that Tregs stimulate dendritic cells to produce IDO [60,61]. Our data suggest that IDO may have a substantial role in the suppression of alloreactivity by DSCs. Inhibition of IDO did not only reduce the ability of DSCs to suppress alloreactivity, as previously discussed, but we also showed that inhibition of IDO reduced the frequency of Tregs. The association between IDO and regulatory T cells has also been shown in a cancer model [62].

In conclusion, we have shown that DSCs suppress alloreactivity and increase the frequency of Tregs in a contact-dependent manner. Several factors, such as IDO, PGE_2, PD-L1, and IFN-γ, are important for the suppression. DSCs constitutively express IDO, and this enzyme appears to be important for the induction of Tregs in our system. High concentrations of IFN-γ impair the suppressive function of DSCs, whereas levels of cytokines that presumably are more biologically relevant in allogeneic settings do not. Several of these findings further distinguish DSCs from BM-MSCs. Thus, the many advantages of DSCs make them a potential candidate for adoptive cell therapy against inflammatory conditions. We are currently investigating the effect of these cells in patients with GvHD and other inflammatory disorders, and are examining immunological effects after treatment, such as frequencies of Tregs and other cell subsets, as well as systemic levels of cytokines and growth factors.

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Author Disclosure Statement

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