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Xeno-immunosuppressive properties of human decidual stromal cells in mouse models of alloreactivity *in vitro* and *in vivo*

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Abstract

Background aims. Human decidual stromal cells (*h*DSCs) may cure acute graft-versus-host disease (GVHD) in humans. We evaluated immunosuppression by xenogenic *h*DSCs in mice, both *in vitro* and *in vivo*. *Methods.* *h*DSCs inhibited mouse lymphocyte proliferation in allo- and xeno-stimulation assays in mixed lymphocyte culture (MLC) and after mitogenic stimulation. The immunosuppressive effect of *h*DSCs was dose-dependent and strain-independent. Trans-well experiments showed that *h*DSCs needed cell-to-cell contact to be immunosuppressive. In a GVHD model, Balb/c mice were transplanted with bone marrow and splenocytes from C57BL/6 a donor. Varying doses of *h*DSCs (10^5–10^6 per mouse) were infused at different time points. Recipient mice showed lower GVHD scores than untreated mice, but they did not have consistently improved survival. Histopathological investigation of liver, gastrointestinal tract and skin of animals with GVHD did not show any significant improvement from *h*DSC infusion. *Results.* *h*DSCs were transduced with immunosuppressive genes including those encoding interleukin-10, prostaglandin-E2 receptor, indoleamine dioxygenase, interferon-γ and programmed death ligand-1. Transduced and untransduced *h*DSCs showed similar effects *in vitro* and *in vivo*. At a dose of 10^6 *h*DSCs per mouse, the majority of recipients died of embolism. *Conclusions.* *h*DSCs inhibit allo-reactivity, xeno-reactivity and mitogen-induced stimulation in mouse lymphocytes. Although the GVHD score was reduced by *h*DSC infusion, survival and GVHD histopathology were not improved. One reason for failure was fatal embolism.

Key Words: *cell therapy, decidual stromal cells, graft-versus-host disease, mesenchymal stromal cells, mouse model*

Introduction

Graft-versus-host disease (GVHD) is a severe and life-threatening complication after allogeneic hematopoietic stem cell transplantation (A-HSCT). It is an inflammatory condition in which allo-reactive donor T cells attack recipient tissues [1] and is most prominent in the skin, gastrointestinal tract and liver. Without preventive or therapeutic intervention, almost all A-HSCT patients will have acute or chronic GVHD [2]. Despite advances in the development of new immunosuppressive drugs and novel therapeutic methods, GVHD is still a major threat [3]. In severe cases of GVHD in which there is no response to standard treatments, the prognosis is poor and survival is low [3,4].

Several reports have suggested that mesenchymal stromal cells (MSCs) have an immunosuppressive effect both *in vitro* and *in vivo* [4–6]. It has been shown both in humans and mice that MSCs can inhibit T-cell proliferation in mixed lymphocyte culture (MLC) as well as after non-specific mitogenic stimulation [6–8]. Considering the immunomodulatory effects of MSCs [7,9], we used bone marrow (BM) MSCs to treat steroid-resistant GVHD for the first time [4,10]. Subsequently, MSC therapy was extended also to other immunological and inflammatory disorders [11].

Despite the fact that the first cases responded dramatically, with complete reversal of acute GVHD, subsequent observations showed that some patients do not respond at all [4,10]. MSCs with similar cell-surface markers and function can be isolated from various tissues (eg, BM, adipose tissue and umbilical cord) [12,13]. The main common characteristic of MSCs from different sources is their immune-modulatory properties [13,14]. We recently

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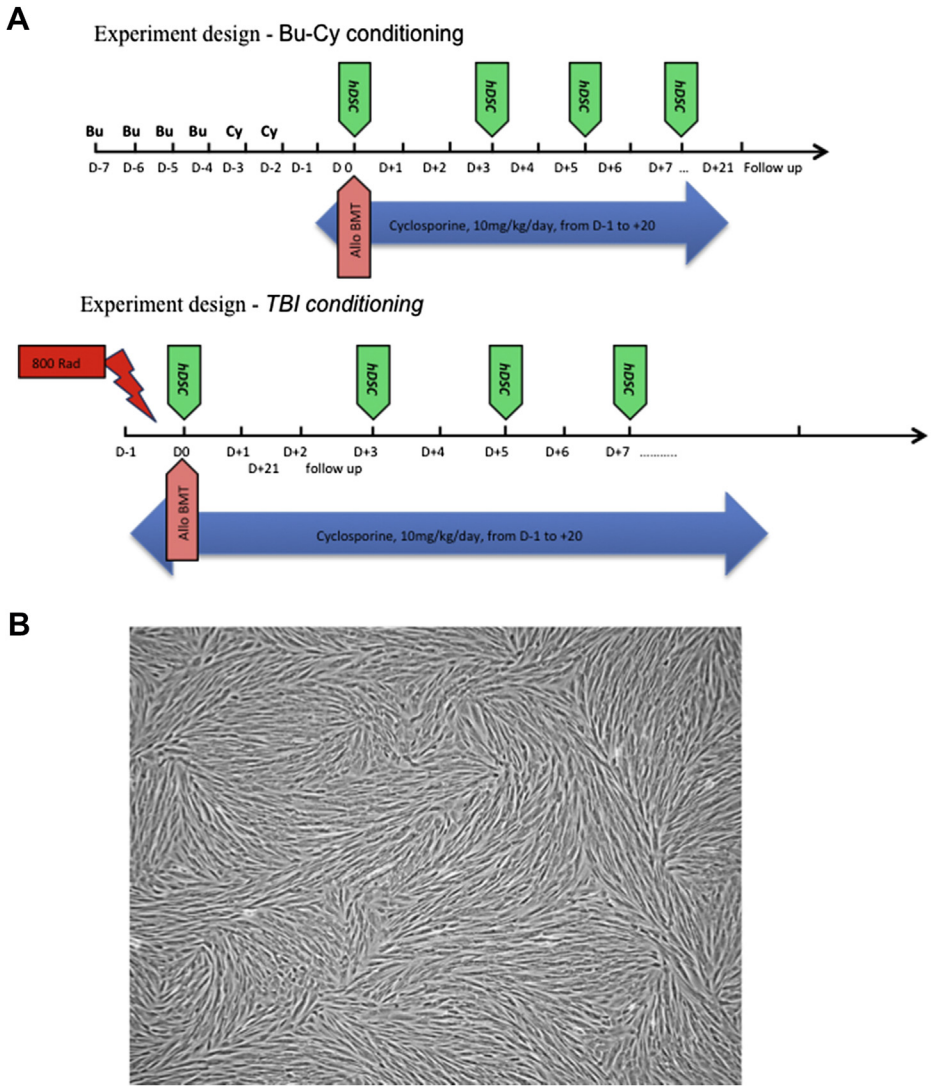

Figure 1. Experiment design and morphology of *h*DSCs. (A) *In vivo* study and experimental groups. BM transplantation and GVHD induction in mice, as well as *h*DSC infusion schedule in GVHD model. (B) Primary cultures of *h*DSCs at passage 3. Magnification ×4.

introduced a protocol for generation of large quantities of decidual stromal cells (DSCs) with significant immunosuppressive properties from fetal membrane layers of the placenta [15,16]. Compared with stromal cells isolated from BM or adipose tissue, DSCs are easily accessible without any invasive procedures. There are few or no ethical considerations because the placenta is normally discarded after delivery.

We used human DSCs (*h*DSCs) successfully for the treatment of steroid-resistant acute GVHD [17]. The preliminary results were promising, but the therapeutic protocol must be optimized because not all patients respond. The purpose of this experimental study was to investigate the underlying mechanisms of DSCs and to optimize the use of DSCs *in vitro* in MLC and after mitogenic stimulation in a well-known mouse model of acute GVHD [18].

Methods

Preparation of DSCs

Isolation and preparation of *h*DSCs has already been described [17]. Briefly, human term placentas were obtained from healthy mothers during elective cesarean section, after we had obtained informed consent. The fetal membranes were carefully dissected from the placenta, washed several times, cut into small pieces and digested with trypsin/ethylene diamine tetra-acetic acid (EDTA) (Thermo Fisher Scientific) by use of a series of incubations and washes. Trypsin-digested material (cell

Table I. Oligos used for gene cloning.

Gene	Clone information	Forward primer	Reverse primer
IDO	Thermoscientific	*EcoRI*	*NotI*
	Clone ID: 5208340	ANGCGAATTCATGGCACACGCTATGGAAAAC	ANGCGCGGCCGCTTAACCTTCCTTCAAAAGGATTTTC
IFN-γ	Thermoscientific	*EcoRI*	*BamHI*
	Clone ID: 30414644	ANGCGAATTCATGAAATATACAAGTTATATCTTGGCTTTTC	ANGC GGATCCTTACTGGGATGCTCTTCGACC
PDL-1	Thermoscientific	*EcoRI*	*NotI*
	Clone ID: 30915301	ANGCGAATTCATGAGGATATTTGCTGTCTTTATATTCATG	ANGCGCGGCCGCTTACGTCTCCTCCAAATGTGTATCAC
IL-10	Thermoscientific	*EcoRI*	*BamHI*
	Clone ID: 40035920	TNGCGAATTCATGCACAGCTCAGCACTGC	TNGCGGATCCTCAGTTTCGTATCTTCATTGTCATGTAG
EP2	Origene	*EcoRI*	*BamHI*
	SKU: SC126558	ANGCGAATTCATGGGCAATGCCTCCAA	ANGCGGATCCTCAAAGGTCAGCCTGTTTACTG

suspension or tissue) was washed and seeded in Nunc T175 flasks (Nunc A/S) with the use of complete Dulbecco's modified Eagle's medium (DMEM). When the cells from the trypsin-digested suspension and from the tissue explants were approximately 90% to 95% confluent, the cells were harvested with trypsin/EDTA, washed in complete DMEM and seeded in new T175 flasks at 2.9×10^3 cells/cm^2 in complete DMEM. The cells were cultured to passage 2 or 3 and frozen slowly in complete DMEM containing 10% dimethyl sulfoxide (DMSO) (WAK-Chemie Medical GmbH). DSCs were expanded and cultured under Good Manufacturing Practice conditions by use of a room with reverse isolation, a sterile cabinet and a separate incubator for cells from each donor.

Mice and the GVHD model

Female Balb/c (H-2d) and male B6 (H-2b) mice, 10 to 12 weeks old, were purchased from Scanbur (Sollentuna). The mice were maintained under pathogen-free conditions with controlled humidity (55% ± 5%), 12 h of alternating light and dark, controlled temperature (21 ± 2°C) and High Efficiency Particulate Air (HEPA)-filtered air. They were kept in individually ventilated cages and were fed autoclaved mouse chow and tap water *ad libitum*.

GVHD model

BM transplantation and induction of GVHD was performed according to a protocol that has already been described in detail [18]. Briefly, female BALB/c mice underwent chemotherapy (busulfan [80 mg/kg] for 4 days + cyclophosphamide [200 mg/kg] for 2 days [Bu-Cy]) or irradiation with 850 cGy total body irradiation (TBI). On day 0 (after Bu-Cy conditioning) or 6 h after TBI, recipient mice were injected through the lateral tail vein with 5 to 10×10^6 BM cells (BMCs) with or without 5 to 10×10^6 spleen cells from a B6 mouse (as donor). The South Stockholm Ethics Committee for Animal Research approved all the experiments described here (S5−12). The experimental design is shown in Figure 1A.

Assessment of GVHD

The severity of GVHD was assessed through the use of a clinical GVHD scoring system, as described previously [18,19]. Briefly, recipient mice were evaluated and scored for five clinical symptoms of GVHD: weight loss, posture, activity, texture of fur and integrity of skin. The severity of each given symptom was scored from 0 to 2. The sum of the

Table II. Oligos used for RT-qPCR.

	Gene	Forward	Reverse
1	*IDO*	GCGCTGTTGGAAATAGCTTC	AGGACGTCAAAGCACTGAAAG
2	*IFN-γ*	TGACCAGAGCATCCAAAAGAG	CTCTTCGACCTCGAAACAGC
3	*PDL-1*	TGGTGGTGCCGACTACAAG	TTGGTGGTGGTGGTCTTACC
4	*IL-10*	AGAACAGCTGCACCCACTTC	GGTCTTGGTTCTCAGCTTGG
5	*EP2*	CTTTCGCCATGACCTTCTTC	GTACTGCCCATAGTCCAGCAG

scores for all symptoms in each mouse (maximally 10) was used as an index of the severity and progression of GVHD. Liver, intestine and skin were evaluated through the use of histopathological sections to confirm GVHD.

Mouse MLC

Balb/c or C57BL/6 (B6) splenocytes (4×10^5 cells/well) were co-cultivated with 2×10^5 irradiated (30 Gy) splenocytes (from B6 or Balb/c mice, respectively) or 1×10^5 irradiated human peripheral blood leukocytes (*h*PBLs) as stimulator, with or without *h*DSCs (in different ratios; see Results section) as regulator cells, in 96-well plates (0.2 mL/well; round bottom; Costar). After 5 days, the cultures were pulsed during the final 18 h with 1 μCi/well [^{3}H] thymidine (Perkin-Elmer), and cells were harvested on a Harvester 96 (Tomtec). Beta radiation (proliferation rate) was measured with the use of a Trilux 1450 MicroBeta microplate scintillation counter (Wallac Sweden AB).

Balb/c or B6 splenocytes (2×10^5 cells/well) were cultured in 96-well plates (round bottom; Costar) with 5 μg/mL *concanavalin A* (*Con A*) or 10 μg/mL phytohemagglutinin (PHA) in the presence or absence of *h*DSCs at various ratios (see Results section). After 3 days, the cultures were pulsed with [^{3}H] thymidine, and uptake was measured as described above.

Trans-well proliferation assay

Splenocytes (1.5 or 3×10^6) from Balb/c mice were stimulated with Con A (5 μg/mL) in the lower chamber of a 24-mm-diameter trans-well plate with a 1-μm pore-size membrane (Costar). *h*DSCs or *h*BM-MSC were seeded at 3 or 6×10^5 cells per well (20% of responder) onto the trans-well membrane (insert) of the inner chamber. Control cultures that did not contain *h*DSCs/*h*BM-MSCs or *h*DSCs were added directly to the MLC. After 3 days, the maximum proliferation for Con A stimulation, the cultures were pulsed during the final 18 h with 1 μCi/200 μ [^{3}H] thymidine. Cells were transferred to 96-well plates and harvested, and β-radiation (proliferation rate) was measured as described above. To evaluate the effect of secreted mediators from *h*DSCs in MLC reactions, the supernatant of the *h*DSC culture was collected and added to the mouse proliferation assay (MLC).

DNA preparation and real-time polymerase chain reaction for detection of human DNA

Next, we tracked *h*DSCs in recipient mouse organs. Two weeks after *h*DSC infusion, mice in control and experimental groups were killed. Skin, liver and lung samples were taken and washed with phosphate-buffered saline and cut into smaller pieces with scalpel. DNA was then extracted by use of the QIAamp DNA FFPE Tissue Kit (Qiagen). DNA (5 μL) was used in a 20-μL of reaction containing 1× TaqMan Universal PCR Master Mix (Life Technologies), 300 nmol/L of each primer and 200 nmol/L of probe. Primer and probe sequences for mouse B-actin were: Forward: 5′-CAA GAA GGA AGG CTG GAA AAG A-3′, Reverse: 5′-ACG GCC AGG TCA TCA CTA TTG-3′ and Probe: 5′- (6-FAM)-CAA CGA GCG GTT CCG ATG CCC T-3′ (TAMRA). Primer and probe sequences for human B-actin were: Forward: 5′- CCA TGT ACG TGG CCA TCC A -3′, Reverse: 5′- CCC AGA GCC CAG CAT ACC T -3′ and Probe: 5′ (6-FAM)- AGT GCT ATC CCT GTA TGC TTC TGG CCG -3′ (TAMRA). The polymerase chain reaction (PCR) was performed and analyzed on the ABI 7500 Sequence Detection System with the following PCR conditions: 95°C for 10 min followed by 40 PCR amplification cycles with 95°C for 15 s and 60°C for 1 min. Relative quantification of gene expression was calculated according to the Delta Ct method. The formula used was $2^{-(\Delta Ct)}$, where $\Delta Ct = Ct$ human B-Actin − Ct mouse B-actin.

Cloning of over-expression vectors

The candidate genes were PCR-amplified from the complementary DNA (cDNA) clones (Open Biosystems/Thermo Scientific) by use of the primers indicated in Table I. The PCR-amplified candidate genes were cut with *EcoRI* and *Not1l* and sub-cloned into the lentiviral pCDH-MSCV-MCS-EF1-GFP-Puro vector (Cat. No. CD713B-1; Biocat).

Virus production and transduction of h*DSCs*

For lentivirus production, 293FT cells were co-transfected with pCDH-MSCV-MCS-EF1-GFP-Puro vectors, psPAX2 and pCMV-VSVG, by use of the calcium phosphate transfection method [8]. The virus supernatant was harvested 24 h and 48 h after transfection and concentrated by centrifugation at 6000*g* for 16 h at 4°C. The *h*DSCs were infected with the virus supernatant overnight in the presence of polybrene (8 μg/mL). Transduced cells were selected by adding Puromycin (1.5 μg/mL) for 48 h. Transduced GFP+ cells were confirmed by use of fluorescence microscopy.

Analysis of messenger RNA expression

RNA from the transduced cells was purified with the use of the RNeasy Plus Mini kit (Qiagen) and reverse-transcribed with the use of the SuperScript VILO cDNA synthesis kit (Invitrogen). Real-time quantitative PCR (RT-qPCR) was performed with the use of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and the Stepone plus Real-Time PCR system (Applied Biosystems). RNA levels were normalized to RPLPO expression. Primer sequences are listed in Table II.

Immune phenotyping of hDSCs

The phenotype of the untouched and transduced *h*DSCs was characterized by means of flow cytometry (BD Biosciences). For all flow cytometric analyses, FlowJo software version X.0.7 was used to analyze the results. The different kinds of stromal cells were stained with the most popular positive/negative markers used to characterize *h*DSCs. Phycoerythrin-positive antibodies were used for CD45, CD34, CD73 and CD29 cell-surface markers (BD Biosciences).

Histology

Tissue samples were fixed in neutral buffered formalin for 24 h, transferred to 70% ethanol, dehydrated and embedded in paraffin according to standard procedures [18]. Sections 4 μm thick were prepared and stained with hematoxylin and eosin for histological evaluation.

Statistical analysis

All data are expressed as mean ± standard error (SE) unless otherwise stated. Differences between groups were analyzed by use of the Mann-Whitney *U*-test. Values of $P < 0.05$ were considered statistically significant. Survival curves were plotted by means of Kaplan-Meier estimates. All statistical analyses were performed with the use of SPSS software version 13.

Results

Morphology and phenotypic characterization of hDSCs

Morphologically, the cells at passage 3 or 4 (which were used in this study) appeared to be stromal and spindle-shaped (Figure 1B). The phenotype and cell-surface markers of *h*DSCs have been investigated in our previous publications [15,17]. The proliferation rate of *h*DSCs was superior or similar to that observed for *h*BM-MSCs, and it was constant throughout the whole series of experiments.

In vitro immunosuppressive effects of hDSCs in allo- and xeno-stimulation of mouse splenocytes

To investigate the underlying mechanisms of *h*DSC function, it is important to know whether *h*DSCs are able to inhibit mouse spleen cell proliferation. Different ratios of *h*DSCs (0.78% to 50% relative to the responder cell number) were added to mouse spleen cells in MLC or in ConA/PHA proliferation assay. As shown in Figure 2A–C, irradiated *h*DSCs have strong xeno-inhibitory function. Clearly, increasing doses of *h*DSCs have an increasingly suppressive effect. To detect any xeno-stimulatory effects of human cells on mouse immune cells, irradiated *h*PBLs were added to the mouse MLC at the same ratio as *h*DSCs (Figure 2D,E). The results indicated that human immune cells (bearing human antigens) have a strong xeno-stimulatory effect and amplify the proliferation of allo- or polyclonal-stimulated mouse spleen cells (Figure 2D,E). Additionally, when mouse splenocytes were stimulated with *h*PBLs (xeno-stimulation), addition of *h*DSCs strongly prevented mouse cell proliferation (Figure 2F). Altogether, these findings indicate that *h*DSCs are capable of preventing mouse splenocyte proliferation in allo, xeno or Con A/PHA proliferation assays.

It is important to know whether the immunosuppressive effect of *h*DSCs in mice is strain-dependent or whether it is a universal effect. Splenocytes of C57BL/6 (responder) mice were co-cultured with irradiated Balb/c splenocytes or human PBLs (xeno-MLC), or with Con A. Addition of *h*DSCs (20% of responder) significantly reduced the proliferation rate in all sets of stimulation assays (Figure 3A).

Xeno-suppressive effect of hDSCs is based on cell-to-cell contact

Next, we wanted to determine whether or not the immuno-inhibitory effect of *h*DSCs is contact-

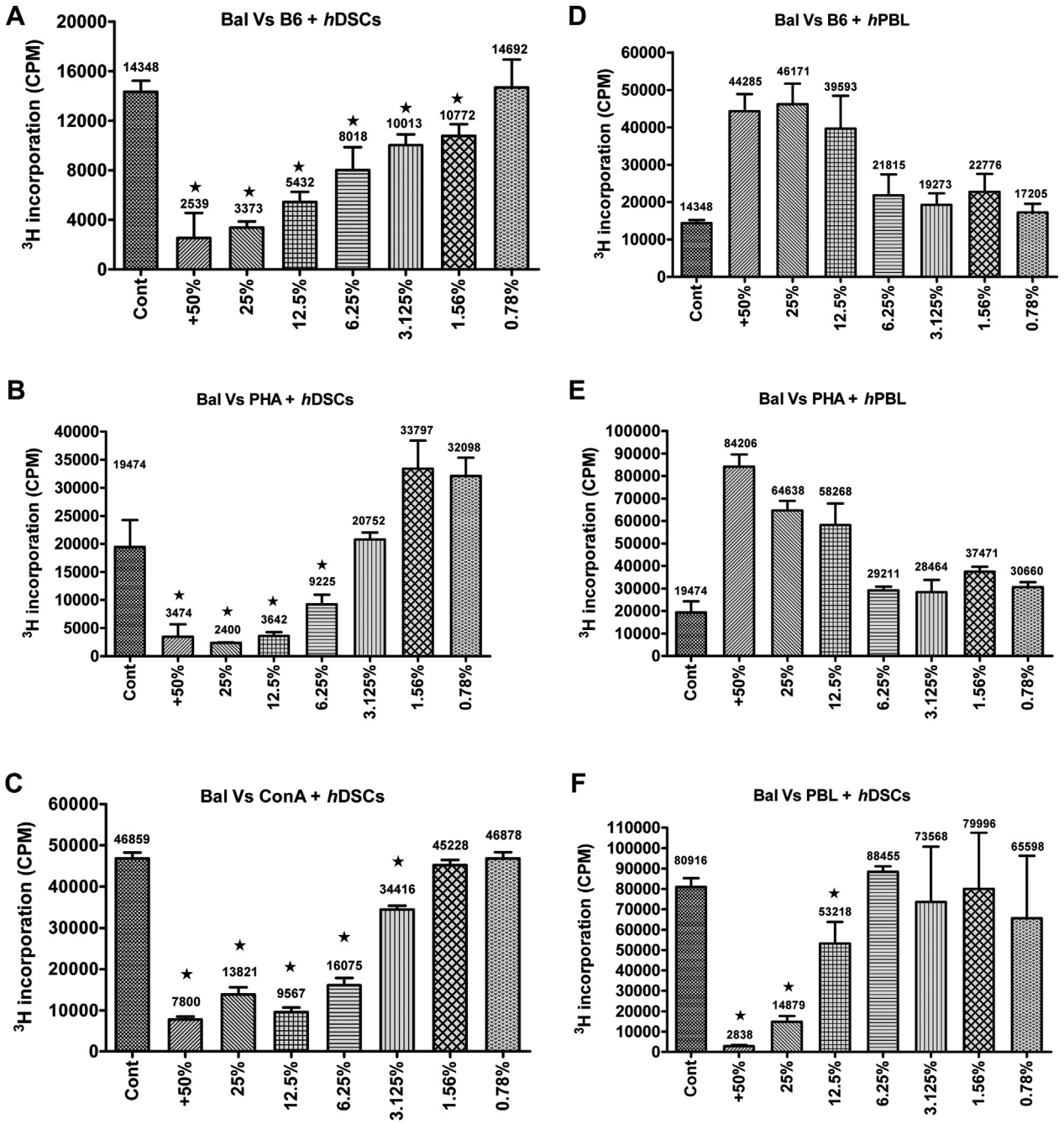

Figure 2. *h*DSCs have strong xeno-inhibitory effects. Balb/c splenocytes were stimulated with (A) irradiated spleen cells from B6 mice ($n = 2$), (B) 10 μg/mL PHA ($n = 2$) or (C) 5 μg/mL Con A ($n = 4$) and co-cultured with escalating doses of *h*DSCs. (D, E) Proliferation rate in allo-stimulation ($n = 2$) and PHA stimulation ($n = 2$) of Balb/c splenocytes when human peripheral blood lymphocytes (PBLs) were used as source of regulator cells. (F) Splenocytes from Balb/c mice were stimulated with human PBLs ($n = 2$) and *h*DSCs were added as suppressor cells. Data are presented as columns with standard errors. *$P < 0{\cdot}05$, as analyzed by non-parametric Mann-Whitney *t*-test.

dependent. Splenocytes from Balb/c mice were stimulated with 5 μg/mL Con A and *h*DSCs were added (at a ratio of 20%), either directly or in the insert of trans-well (1-μm pore size) proliferation culture. As shown in Figure 3B, the proliferation of splenocytes decreased ($P < 0.07$) when *h*DSCs were cultured in direct contact with the responder cells, whereas the proliferation was not reduced (it even increased) when the *h*DSCs were added in a trans-well setting (Figure 3B). Next, we evaluated the immune-suppressive effect of *h*DSC culture media on mouse splenocyte proliferation. When *h*DSC supernatant was added to the mouse proliferation assay (xeno-MLC), the proliferation of spleen cells did not change

(Figure 3C). It appears that *h*DSCs are effective when they are in close contact with mouse immune cells.

Viral transduction of hDSCs increases the expression

*h*DSCs were transduced with human mediator genes (prostaglandin E2 receptor [EP2], indoleamine dioxygenase [IDO], interferon [IFN]-γ, interleukin [IL-10] and programmed death ligand [PDL]-1) expressing the *GFP* marker gene (Figure 3D). To exclude viral manipulation of *h*DSCs, a group of cells was transduced with empty vector (also expressing the *GFP* gene). In the present work, we found that transduction of *h*DSCs with any of the human genes did not change the morphology, the phenotype or the proliferation pattern of cells. Transfected cells were positive for CD29 and CD73 and negative for CD45 and CD34, as we also observed in untouched *h*DSCs (Figure 3E). The transduction efficiency was confirmed by means of fluorescence microscopy (Figure 3D) and the expression levels were quantified by means of qPCR (Figure 3F). Moreover, the transduction was constitutively expressed even after several passages. As shown in Figure 3F, the expression level of the gene of interest was significantly higher in the transduced *h*DSCs than in untouched *h*DSCs. The data in Figure 3F were normalized against the housekeeping gene *RPLPO*.

Over-expression of inhibitory mediators does not affect hDSC function, either in vitro or in vivo

In an attempt to increase the immunosuppressive effect of *h*DSCs, we transduced these cells with *EP2*, *IDO*, *IL-10*, *PDL-1*, or *IFN-γ* genes of human origin (Figure 3D). We first compared the additive modulatory effect of transduced genes in an *in vitro* human proliferation assay. As shown in Figure 4A,B, untouched *h*DSCs and vector-transduced, *EP2* gene−transduced or *IDO* gene−transduced *h*DSCs decreased the proliferation rate of stimulated human PBLs in a dose-dependent manner, both in allo-MLC (Figure 4A) and in PHA stimulation assay (Figure 4B). It appears that *EP2*-expressing cells had stronger immunosuppressive function, although this was not statistically significant ($P = 0.06$). We next compared all the transduced *h*DSCs in human (Figure 4C) and mouse (Figure 4D) proliferation assays by adding these cells (10% of responder cells) in MLC. None of these transduced *h*DSCs appeared to acquire extra immunosuppressive properties compared with unmanipulated *h*DSCs. However, in mouse MLC, there was a slight tendency of better immunosuppressive properties in *EP2*-expressing cells (Figure 4D).

Effect of hDSCs on survival and clinical manifestation of GVHD in mice

To find the optimum administration time, we first infused 10^5 *h*DSCs per mouse at different time points (day 0, +3, +5 and +7). In this experiment (Figure 5A), infusion of *h*DSCs 3 days after allo-HSCT improved the survival of GVHD mice (although not significantly). Infusion at other time points did not show much improvement in mouse survival (Figure 5A). We then increased the cell dose (to 10^6 *h*DSCs per mouse) and infused *h*DSCs at the same time points (data not shown). A higher number of *h*DSCs had limited or no positive effect. Indeed, the majority of recipient mice died of embolism. It was therefore not possible to evaluate the efficacy of higher cell numbers on prevention or treatment of GVHD. We then thought of increasing the frequency of infusion occasions rather than infusing a higher cell number at once. Moreover, to make the model more comparable to the clinical setting, recipient mice received cyclosporine (intraperitoneal) at a dose of 10 mg/kg for 2 weeks (from day −1 until day +20) (Figure 1A). In this series, *h*DSCs (0.5×10^6 *h*DSCs per mouse at each time point) were infused to the recipients at days +3, +7 and +14. Despite the lower number of *h*DSCs than in the previous experiment (10^6/mouse), half of the recipient mice died after infusion at day +7. The infusion at day +7 was risky because day +7 is the nadir in the GVHD-related toxicity [18]. Thus, loading of large cells and volume had a lethal effect on the heart and lungs of the mice, especially when cyclosporine was infused on a daily basis. However, regardless of survival rate, the clinical manifestations and GVHD score (at day +14) were significantly better in animals that had received repeated doses of *h*DSCs (Figure 5B). To explore the presence of human cells in mice tissues, we measured human DNA (human actin) in the lung, liver and skin of experimental and control animals. Two weeks after cell (*h*DSC) infusion, no signals of human DNA were detectable in any of evaluated organs in experimental animals (data not shown).

Last, we evaluated the effect of transduced *h*DSCs on the clinical outcome of GVHD animals. We infused 0.5×10^6 PDL-1−over-expressing, EP2-over-expressing, IL-10−over-expressing and IDO-over-expressing *h*DSCs, respectively, to GVHD mice 3 days after allo-HSCT. Survival analysis showed that there was no improvement in survival in animals treated with transduced *h*DSCs. However, the animals that received PDL-1−transduced *h*DSCs had slightly improved clinical manifestations, especially activity and grooming.

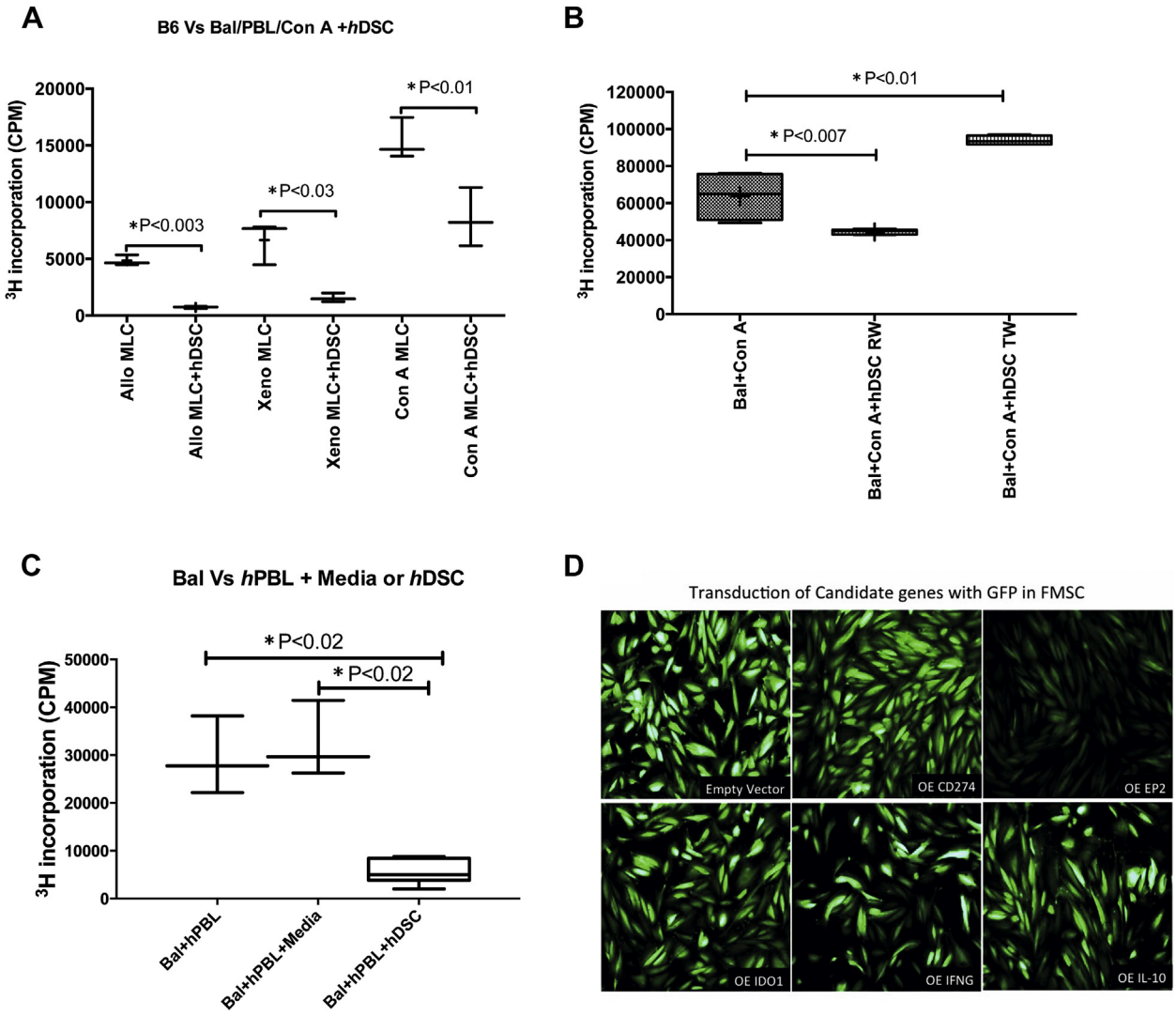

Figure 3. Function and transfection efficiency of *h*DSCs. Xeno-suppressive function of *h*DSCs is not limited to one mouse strain. (A) Splenocytes from B6 mice were stimulated ($n = 2$) with irradiated Balb/c spleen cells (allo MLC), *h*PBLs (xeno MLC) or Con A (5 μg/mL). *h*DSCs were added as suppressor cells (10% of responder cells). (B) Balb/c spleen cells were stimulated ($n = 2$) with Con A (5 μg/mL); *h*DSCs were added either directly (regular well) or indirectly to the culture (trans-well insert). (C) Splenocytes from Balb/c mice were stimulated ($n = 2$) with irradiated *h*PBLs (xeno-MLC). Supernatants of *h*DSC culture media were added as suppressor to the MLC. (D) *h*DSCs were transfected with different human genes by use of lentivirus vector. Transfection efficiency was measured with the use of immunofluorescence. Magnification ×4. (E) Phenotypic analysis of *h*DSCs by flow cytometry. Histograms represent the expression level of the different molecules (CD29, CD73, CD45 and CD34) on untouched and transduced *h*DSCs (filled gray) compared with isotype controls (red empty). (F) Level of expression of the gene of interest was measured by use of RT-PCR on transfected cells. The expression level was normalized against *RPLPO* (housekeeping gene).

Histopathological evaluation of GVHD target organs after hDSC infusion

Histopathological appearance of the liver, intestine and skin of treated and untreated animals did not show any significant improvement related to *h*DSCs infusion.

Discussion

Our recent findings show that *h*DSCs have strong immunosuppressive effects on human lymphocyte proliferation and activation. These effects, like those of BM-MSCs, are independent of the human leukocyte antigen system [20]. In the present study, we showed that xenogeneic *h*DSCs also inhibited MLC in mice. Our aim was to use the mouse model to study and optimize DSC-based treatment [15–17].

We have shown that *h*DSCs extracted from the outer layer of human fetal membrane strongly suppress mouse splenocyte activation *in vitro*. The most important finding was cross-species function of

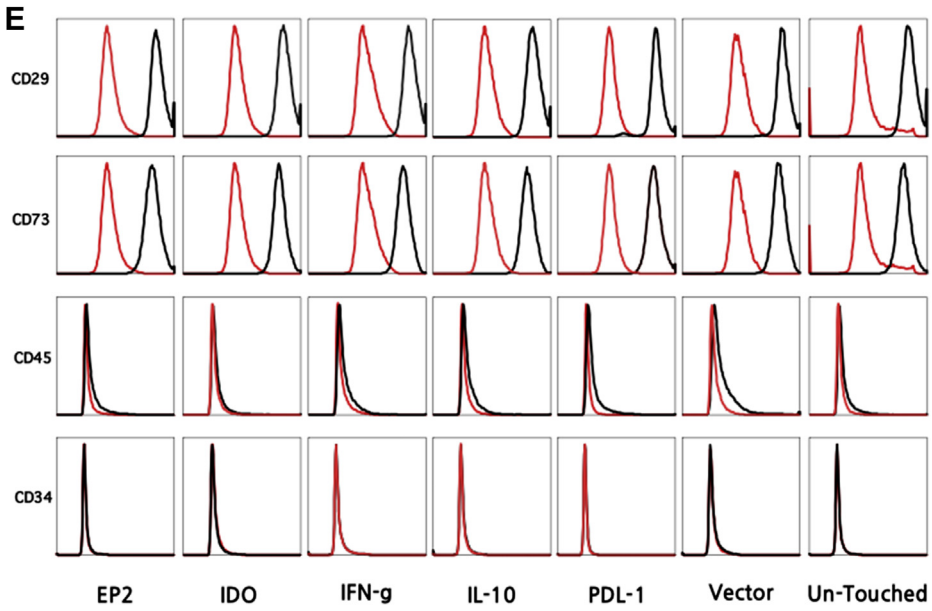

Figure 3. (*continued*).

*h*DSCs. To support the idea of similarity in function between *h*DSCs and *h*BM-MSCs, we first used varying doses of *h*DSCs in a series of *in vitro* proliferation assays. The inhibitory effect of *h*BM-MSCs *in vitro* has been shown earlier [21], and now we have found that *h*DSCs strongly induce immunosuppression even if used in a xeno situation. In addition, the inhibitory effect of *h*DSCs in mice is not restricted to one strain (BALB/c); it was also found in C57BL/6 mice. Although the immunosuppressive activity of *h*MSCs is not limited to contact-dependent activity [16,22] the *h*DSCs show their inhibitory activity through cell-to-cell contact both in human [16] and mouse experiments, as demonstrated here.

Our data indicate that *h*DSCs are able to inhibit mouse lymphocyte proliferation in a contact-dependent manner. Others have shown similar findings in a human setting [6,16]. Di Nicola *et al.* [6] reported that cell-cell contact is essential for a stronger suppressive effect of MSCs on T cells [6]. It has also been shown that expression of several integrins and/or adhesion molecules on the MSC surface increase their binding capacity to T-lymphocytes with high affinity [23]. It was also demonstrated that MSCs inhibit T-cell proliferation through an MHC-independent mechanism [20].

One of the key inhibitory molecules that express and upregulate on MSCs is CD274 (also known as PDL) [16,24]. It has been proven that engagement of PD-1 by PD-L1 results in suppression of T-cell activation [25]. Additionally, Nagamatsu *et al.* [26] have previously shown that DSCs inhibit cytokine production by CD4+ cells through the PD-L1/PD-1 pathway. In line with these results, Yan *et al.* [27] reported that MSCs are able to increase the suppressive potential of T-regulatory cells (Tregs) by upregulation of PD-1 on Tregs.

Studies have implicated that other cell-surface or locally secreted immunosuppressive factors such as prostaglandin-E2, IL-10, hemeoxygenase-1 (HO-1), PDL-1 and IL-6 have a pivotal role in the immune-suppressive ability of MSCs. Of interest, several of these mediators are common in mice and humans.

The mechanism of GVHD has been classically defined as the activation and proliferation of donor T cells in response to the allo-antigens of the recipient [1,28]. Considering the *in vitro* immune-suppressor function of *h*DSCs, we assumed that it may be possible to prevent or treat GVHD in a major MHC-mismatched mouse model, in line with what has been seen clinically in acute GVHD [4,10,11,14,21]. In contrast to the *in vitro* observations, infusion with *h*DSCs did not improve the survival of GVHD mice in all sets of experiments. In some experiments, however, especially when the *h*DSCs were infused repeatedly in lower doses (approximately $1-2 \times 10^5$), it showed benefit regarding the clinical manifestations in mice, and especially regarding the GVHD score. Even so, survival was only improved in some experiments, and this was not consistently demonstrated. Moreover, the optimum infusion time for *h*DSCs was between day +3 and day +7 after allo-BM transplantation. Our findings are in line with previous reports in mouse GVHD models that used MSCs to prevent or treat GVHD [21,29–32]. With the use of cell doses of 10^6 cells/animal (5×10^6/kg), several animals died after infusion with cells

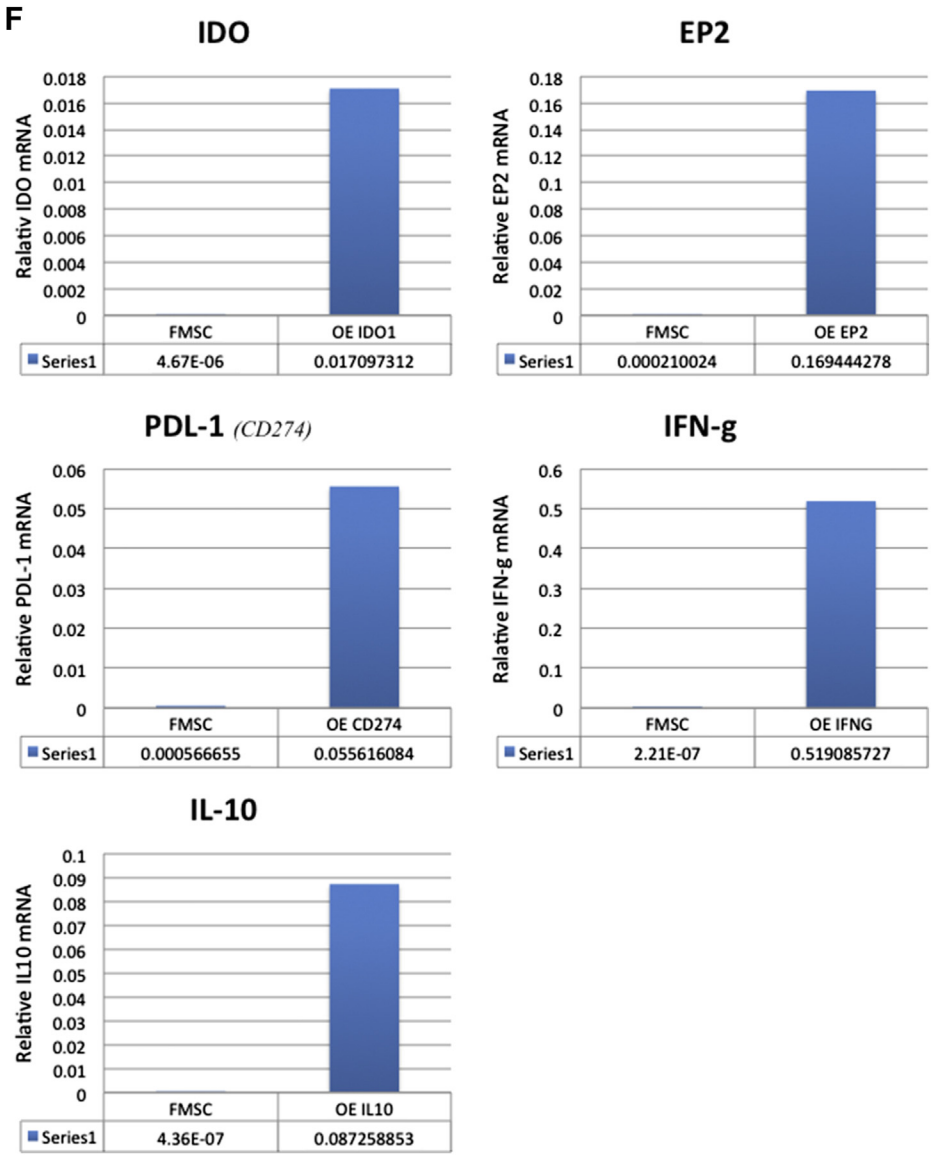

Figure 3. (*continued*).

because of massive thrombosis in the lungs. Like *h*BM-MSCs, *h*DSCs first home to the lungs after intravenous (i.v.) infusion (Erkers *et al.*, submitted and unpublished data of tracking experiments). Although there have been no reports showing that *h*DSCs may cause embolism in humans, adipose tissue–derived MCSs have been shown to result in pulmonary embolism in rare cases [32]. It is possible that *h*DSCs are too large, even though they are smaller than BM-MSCs, to be suitable for treatment of mice. With the use of higher numbers of *h*DSCs, such as 1×10^6 per mouse, the death rate associated with infusion was greater than 50%. This corresponds to 40×10^6 cells/kg. The higher cell dose that is used and tolerated in humans is 5×10^6 cells/kg [33]. Stromal cells such as MSCs also activate the coagulation system [34]. They have therefore been used to stop hemorrhaging [35]. It is possible that some of the negative effects of stromal cells in mice with GVHD are due to death from thromboembolism.

To improve the efficacy of immune-modulatory function of *h*DSCs, we introduced several inhibitory genes into the *h*DSCs. Insertion of these genes did not affect cell phenotype or proliferation rate. *In vitro* proliferation assays did not show any added value for the inhibitory effect compared with untouched *h*DSCs. Moreover, single or repeated infusion of engineered *h*DSCs had no additional protective effect on manifestation and/or outcome of GVHD in mice. Most probably, there were no effects on GVHD. Many more animals are needed

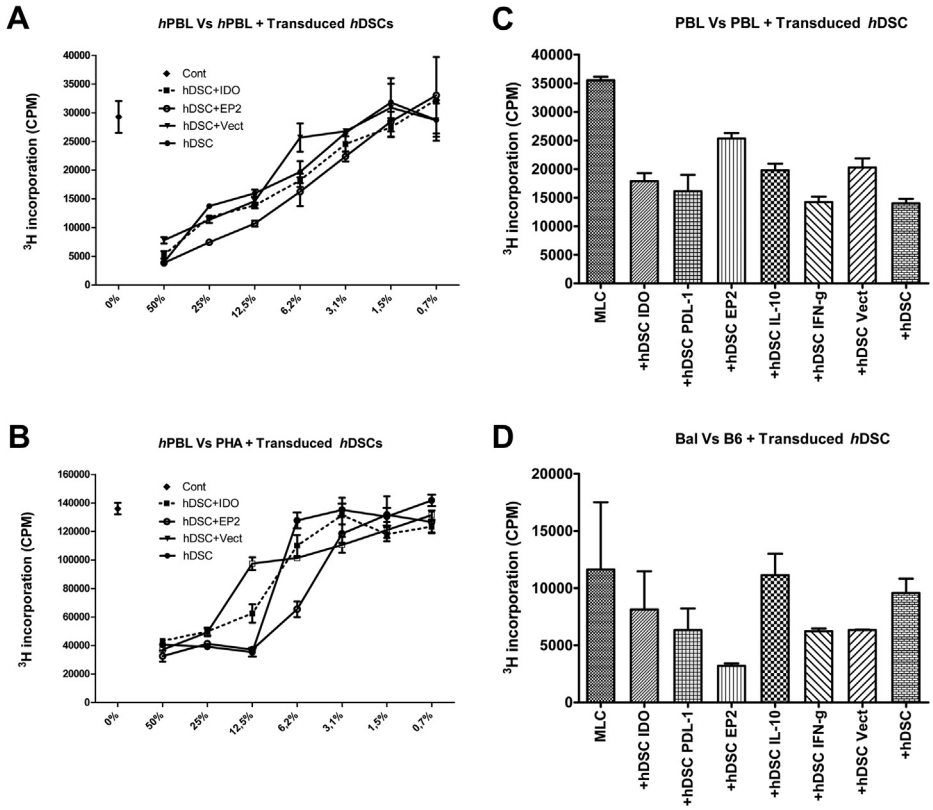

Figure 4. Transduced *h*DSCs maintain their suppressor function. Human PBLs were stimulated with (A) irradiated pooled *h*PBLs ($n = 2$) or (B) 10 μg/mL PHA ($n = 2$). Escalating doses of *IDO* gene—transfected, *EP2* gene—transfected, vector-transfected, or untouched *h*DSCs were added to the MLC as suppressor cells. (C) Human PBLs were stimulated with irradiated pooled *h*PBLs ($n = 2$). Different transduced *h*DSCs (10% of responder cells) were added to the MLC as suppressor cells. (D) Splenocytes from Balb/c mice were stimulated with irradiated spleen cells from B6 mice ($n = 2$). Different transduced *h*DSCs (10% of responder cells) were added to the MLC as suppressor cells.

in the various groups for statistical proof. In contrast to our findings, Min *et al.* [36] used IL-10—transduced mouse MSCs, which over-secrete IL-10, in a haplo-identical (parent to F1) mouse model of GVHD. Whereas the *in vitro* immunosuppressive function of genetically engineered MSCs was not much amplified, they significantly reduced GVHD-related mortality. However, the

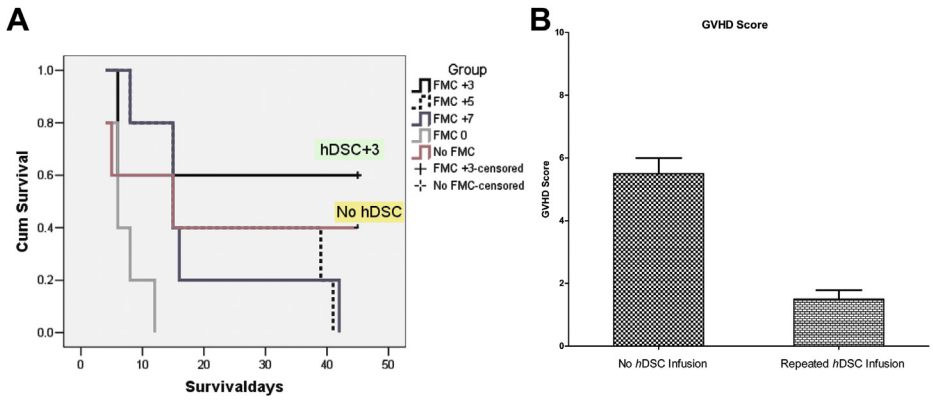

Figure 5. Effect of infusion of *h*DSCs on mouse GVHD. Balb/c mice were conditioned (either TBI or Bu-Cy) followed by allogeneic transplantation ($n = 8$) with BM and spleen cells from B6 mice (see Methods section). At different time points, single or repeated doses of *h*DSCs were infused to recipient mice. (A) *h*DSCs were infused as a single i.v. injection on day 0, +3, +5 or +7. Follow-up showed that the mice that received *h*DSCs 3 days after allo-BM transplantation had better survival. (B) *h*DSCs were infused as a repeated i.v. injection on days +3 and +5. Evaluation of clinical scores has shown that the mice that received repeated infusion of *h*DSCs had better clinical results ($n = 3$; only data from one experiment are shown).

authors did not observe any improvement in GVHD when exogenous IL-10 was infused [36]. The differences could be due to the different mouse models that were used in our study (fully mismatched) and the work of Min *et al.* (haplo-identical). Moreover, Min *et al.* used mouse BM-MSCs that were semi-allogeneic to the recipient, whereas we used xenogeneic (human source) immune-modulatory cells. As has been shown previously [8], MSCs will be licensed and activated in the presence of IFN-γ, whereas *h*DSCs are not activated [8,16]. It means that the underlying mechanism of activation and function of regulatory cells is different, and the final effect on prevention and outcome of GVHD might be the opposite. Furthermore, IL-10 is important for immunosuppression by BM-MSCs, but IL-10 may be less important for the immunomodulatory effects induced by *h*DSCs [16,37]. However, transfection with genes encoding PDL-1, EP2 and IFN-γ, which are important mediators of the immunosuppressive effect of *h*DSCs, did not enhance the immunosuppressive effects of DSCs *in vitro*.

The overall response rate of 75% in clinical steroid-resistant GVHD patients with the use of *h*DSCs seems promising, but survival was only 3 of 8. These results appear to be imitated in the mouse model, in which no clear improvement in survival is seen, despite dampening of signs of GVHD [17]. However, there are several items—including cell dose, starting time and frequency of infusions—that must be optimized. In contrast to the positive clinical findings in several clinical reports [4,10,38], the effectiveness of MSCs in prevention or treatment in experimental models of GVHD is a real challenge and is controversial [8,31]. In addition, in the xeno setting in which human MSCs are given to animals, the situation is even more complicated [30]. There have been some reports showing that MSCs cannot work across species barriers [39,40], but, on the other hand, the majority of relevant studies have indicated the functionality and/or survival of human cells in animals [41]. Overall, it is difficult to guarantee that *h*MSCs will survive and/or have long-term functionality/engraftment in animals, especially in immune-competent mice. Our finding is in agreement with a recent report indicating the antigenicity of MSCs when used in an allo or xeno setting [42].

An important issue that should be considered when using human cells in immune-competent animals is the survival and functionality of infused cells. Several reports have shown that (even in an allogeneic setting) the traceability and survival of infused cells is limited and depends on several factors including cell dose, size and so forth [43,44]. Although lack of detection of infused cells does not necessarily mean that they have been destroyed, decrease in numbers will be reflected in lower outcome and function. In line with this conclusion, we have found that *h*DSCs do not have immune privilege and will immunize healthy mice against human antigens (unpublished data).

Contradictory reports in mouse studies are more common than those observed in the clinical setting [30,32]. In this context, the induction of embolism should also be considered, as it was found in the present study. Although *h*DSCs inhibited proliferation of mouse lymphocytes after stimulation *in vitro* by mitogens and allo-antigens, this could not be translated to a successful *in vivo* effect in GVHD in mice, as has been demonstrated in the clinic. Some important considerations include donor and recipient combination (MHC disparity level), conditioning regimen (both intensity and type), graft composition (ratio of donor T cells in the graft), source, size and cell dose of MSCs, infusion time and isolation method. The use of *h*DSCs in a mouse model of GVHD may be of limited value. To obtain valuable information from a mouse model, it may be better to use mouse DSCs and a model that mimics clinical GVHD.

Conclusions

It is obvious that the success rate of MSC administration for prevention or treatment of GVHD is higher in clinical practice than in experimental studies in mice.

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References

[1] Blazar BR, Murphy WJ, Abedi M. Advances in graft-versus-host disease biology and therapy. Nat Rev Immunol 2012;12:443–58.

[2] Sullivan KM, Storb R, Witherspoon RP, Weiden PL, Anasetti C, Appelbaum FR, et al. Deletion of immunosuppressive prophylaxis after marrow transplantation increases hyperacute graft-versus-host disease but does not

influence chronic graft-versus-host disease or relapse in patients with advanced leukemia. Clinical Transplantation 1989;3:5–11.

[3] Deeg HJ. How I treat refractory acute GVHD. Blood 2007; 109:4119–26.

[4] Ringden O, Uzunel M, Rasmusson I, Remberger M, Sundberg B, Lonnies H, et al. Mesenchymal stem cells for treatment of therapy-resistant graft-versus-host disease. Transplantation 2006;81:1390–7.

[5] Bartholomew A, Sturgeon C, Siatskas M, Ferrer K, McIntosh K, Patil S, et al. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. Exp Hematol 2002;30:42–8.

[6] Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood 2002;99: 3838–43.

[7] Krampera M, Glennie S, Dyson J, Scott D, Laylor R, Simpson E, et al. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. Blood 2003;101:3722–9.

[8] Polchert D, Sobinsky J, Douglas G, Kidd M, Moadsiri A, Reina E, et al. IFN-gamma activation of mesenchymal stem cells for treatment and prevention of graft versus host disease. Eur J Immunol 2008;38:1745–55.

[9] Rasmusson I, Ringden O, Sundberg B, Le Blanc K. Mesenchymal stem cells inhibit the formation of cytotoxic T lymphocytes, but not activated cytotoxic T lymphocytes or natural killer cells. Transplantation 2003;76:1208–13.

[10] Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet 2008;371:1579–86.

[11] Parekkadan B, Milwid JM. Mesenchymal stem cells as therapeutics. Annu Rev Biomed Eng 2010;12:87–117.

[12] da Silva Meirelles L, Chagastelles PC, Nardi NB. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci 2006;119(Pt 11):2204–13.

[13] English K. Mechanisms of mesenchymal stromal cell immunomodulation. Immunology and cell biology 2013; 91(1):19–26.

[14] Le Blanc K, Ringden O. Immunomodulation by mesenchymal stem cells and clinical experience. J Intern Med 2007;262:509–25.

[15] Karlsson H, Erkers T, Nava S, Ruhm S, Westgren M, Ringden O. Stromal cells from term fetal membrane are highly suppressive in allogeneic settings in vitro. Clin Exp Immunol 2012;167:543–55.

[16] Erkers T, Nava S, Yosef J, Ringden O, Kaipe H. Decidual stromal cells promote regulatory T cells and suppress alloreactivity in a cell contact-dependent manner. Stem Cells Dev 2013;22:2596–605.

[17] Ringden O, Erkers T, Nava S, Uzunel M, Iwarsson E, Conrad R, et al. Fetal membrane cells for treatment of steroid-refractory acute graft-versus-host disease. Stem cells 2013;31:592–601.

[18] Sadeghi B, Aghdami N, Hassan Z, Forouzanfar M, Rozell B, Abedi-Valugerdi M, et al. GVHD after chemotherapy conditioning in allogeneic transplanted mice. Bone Marrow Transplant 2008;42:807–18.

[19] Cooke KR, Kobzik L, Martin TR, Brewer J, Delmonte J Jr, Crawford JM, et al. An experimental model of idiopathic pneumonia syndrome after bone marrow transplantation: I. The roles of minor H antigens and endotoxin. Blood 1996; 88:3230–9.

[20] Le Blanc K, Tammik L, Sundberg B, Haynesworth SE, Ringden O. Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. Scand J Immunol 2003;57:11–20.

[21] Sudres M, Norol F, Trenado A, Gregoire S, Charlotte F, Levacher B, et al. Bone marrow mesenchymal stem cells suppress lymphocyte proliferation in vitro but fail to prevent graft-versus-host disease in mice. J Immunol 2006;176: 7761–7.

[22] Krampera M, Cosmi L, Angeli R, Pasini A, Liotta F, Andreini A, et al. Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. Stem cells 2006;24:386–98.

[23] Majumdar MK, Keane-Moore M, Buyaner D, Hardy WB, Moorman MA, McIntosh KR, et al. Characterization and functionality of cell surface molecules on human mesenchymal stem cells. J Biomed Sci 2003; 10:228–41.

[24] Najar M, Raicevic G, Fayyad-Kazan H, De Bruyn C, Bron D, Toungouz M, et al. Immune-related antigens, surface molecules and regulatory factors in human-derived mesenchymal stromal cells: the expression and impact of inflammatory priming. Stem Cell Rev 2012;8: 1188–98.

[25] Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med 2000; 192:1027–34.

[26] Nagamatsu T, Schust DJ, Sugimoto J, Barrier BF. Human decidual stromal cells suppress cytokine secretion by allogenic CD4+ T cells via PD-1 ligand interactions. Hum Reprod 2009;24:3160–71.

[27] Yan Z, Zhuansun Y, Chen R, Li J, Ran P. Immunomodulation of mesenchymal stromal cells on regulatory T cells and its possible mechanism. Exp Cell Res 2014;324:65–74.

[28] Sadeghi B, Al-Hashmi S, Hassan Z, Rozell B, Concha H, Lundmark C, et al. Expansion and activation kinetics of immune cells during early phase of GVHD in mouse model based on chemotherapy conditioning. Clin Dev Immunol 2010;2010:142943.

[29] Badillo AT, Peranteau WH, Heaton TE, Quinn C, Flake AW. Murine bone marrow derived stromal progenitor cells fail to prevent or treat acute graft-versus-host disease. Br J Haematol 2008;141(2):224–34.

[30] Sadeghi B, Ringden O. Stem Cell-Dependent Therapies: Mesenchymal stem cells for graft-versus-host disease in experimental animal models. In: Gross G, Häupl T, editors. Stem cell-dependent therapies: mesenchymal stem cells in chronic inflammatory disorders. Berlin: De Gruyter; 2013. p. 125-41.

[31] Tisato V, Naresh K, Girdlestone J, Navarrete C, Dazzi F. Mesenchymal stem cells of cord blood origin are effective at preventing but not treating graft-versus-host disease. Leukemia 2007;21(9):1992–9.

[32] Kaipe H, Erkers T, Sadeghi B, Ringden O. Stromal cells-are they really useful for GVHD? Bone marrow transplantation 2014;49(6):737–43.

[33] Lazarus HM, Koc ON, Devine SM, Curtin P, Maziarz RT, Holland HK, et al. Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. Biology of blood and marrow transplantation: journal of the American Society for Blood and Marrow Transplantation 2005;11(5):389–98.

[34] Moll G, Rasmusson-Duprez I, von Bahr L, Connolly-Andersen AM, Elgue G, Funke L, et al. Are therapeutic human mesenchymal stromal cells compatible with human blood? Stem cells 2012;30(7):1565–74.

[35] Ringden O, Uzunel M, Sundberg B, Lonnies L, Nava S, Gustafsson J, et al. Tissue repair using allogeneic mesenchymal stem cells for hemorrhagic cystitis, pneumomediastinum and perforated colon. Leukemia 2007;21(11):2271–6.

[36] Min CK, Kim BG, Park G, Cho B, Oh IH. IL-10-transduced bone marrow mesenchymal stem cells can attenuate the severity of acute graft-versus-host disease after experimental allogeneic stem cell transplantation. Bone marrow transplantation 2007;39(10):637–45.

[37] Rasmusson I, Ringden O, Sundberg B, Le Blanc K. Mesenchymal stem cells inhibit lymphocyte proliferation by mitogens and alloantigens by different mechanisms. Exp Cell Res 2005;305:33–41.

[38] Kebriaei P, Isola L, Bahceci E, Holland K, Rowley S, McGuirk J, et al. Adult human mesenchymal stem cells added to corticosteroid therapy for the treatment of acute graft-versus-host disease. Biol Blood Marrow Transplant 2009;15:804–11.

[39] Lyngbaek S, Ripa RS, Haack-Sorensen M, Cortsen A, Kragh L, Andersen CB, et al. Serial in vivo imaging of the porcine heart after percutaneous, intramyocardially injected 111In-labeled human mesenchymal stromal cells. Int J Cardiovasc Imaging 2010;26:273–84.

[40] Jeon MS, Lim HJ, Yi TG, Im MW, Yoo HS, Choi JH, et al. Xenoreactivity of human clonal mesenchymal stem cells in a major histocompatibility complex-matched allogeneic graft-versus-host disease mouse model. Cell Immunol 2010;261:57–63.

[41] Li J, Ezzelarab MB, Cooper DK. Do mesenchymal stem cells function across species barriers? Relevance for xenotransplantation. Xenotransplantation 2012;19:273–85.

[42] Griffin MD, Ryan AE, Alagesan S, Lohan P, Treacy O, Ritter T. Anti-donor immune responses elicited by allogeneic mesenchymal stem cells: what have we learned so far? Immunol Cell Biol 2013;91:40–51.

[43] G. Chen, F. Tian, Y. Zhang, Y. Zhang, C. Li, Wang Q. Tracking of Transplanted Human Mesenchymal Stem Cells in Living Mice using Near-Infrared Ag2S Quantum Dots. Advanced Functional materials 2014;24:2481–8.

[44] Sohni A, Verfaillie CM. Mesenchymal stem cells migration homing and tracking. Stem Cells Int 2013;2013:130763.