Immunogenicity of Decidual Stromal Cells in an Epidermolysis Bullosa Patient and in Allogeneic Hematopoietic Stem Cell Transplantation Patients

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Allogeneic mesenchymal stromal cells (MSCs) are widely used in regenerative medicine, but little is known about their immunogenicity. In this study, we monitored the therapeutic and immunogenic effects of decidual stromal cells (DSCs) from term placentas when used as a therapy for generalized severe junctional epidermolysis bullosa (JEB) (previously termed Herlitz JEB), a lethal condition caused by the lack of functional laminin-332. An 11-month-old JEB patient was treated with five infusions of allogeneic DSCs within a 3-month period. Amniotic membranes (AMs) were applied to severe wounds. After the treatment, wounds started to heal in the middle of the blisters, but the improvements were transient. After two infusions of DSCs, the JEB patient had developed multispecific anti-HLA class-I antibodies. No antibodies to laminin-332 were detected, but the patient had high levels of anti-bovine serum albumin antibodies, which could bind to DSCs. Peripheral blood mononuclear cells (PBMCs) from the patient had a higher proliferative response to DSCs than to third-party PBMCs, which contrasts with the pattern observed in healthy donors. Human DSCs and MSCs induced similar xenoreactivity in mice. Two of 16 allogeneic stem cell-transplanted patients, treated with DSCs for graft-versus-host disease or hemorrhagic cystitis, showed a positive flow cytometric crossmatch test. One patient had anti-HLA antibodies before DSC infusion, whereas the other had no anti-HLA antibodies at any time. AM and DSC infusions may have improved the healing process in the JEB patient, but DSCs appeared to induce anti-HLA antibodies. The risk of alloimmunization by DSCs seems to be low in immunocompromised patients.

Introduction

EPIDERMOLYSIS BULLOSA (EB) is a group of inherited diseases that are characterized by skin and mucosal fragility and blister formation. The most severe form of this disease is generalized severe junctional EB (JEB), which previously was termed Herlitz JEB [1]. This autosomal recessive disorder is most often caused by homozygous null mutations in the genes, *LAMA3*, *LAMB3*, or *LAMC2*, encoding the heterotrimer laminin-332 (laminin-5). Loss of function in this skin adhesive protein prevents proper attachment of the epidermis to the basement membrane of the skin and the papillary dermis, leading to extensive mucocutaneous manifestations. Currently, there is no established cure for JEB, so treatment is mainly aimed at relieving the

symptoms and preventing complications [2]. The average life span of patients with generalized severe JEB has been reported to be around 6 months [3,4], with a mortality rate of almost 90% within the first year of life [5]. The most common causes of death are failure to thrive, respiratory failure, infections, and dehydration [3].

Recently, Tolar et al. introduced allogeneic hematopoietic stem cell transplantation (HSCT) as a putative treatment for another form of severe epidermolysis bullosa, recessive dystrophic EB (RDEB), which is caused by a deficiency in collagen type VII. HSCT partly resolved the disease in the majority of patients [6]. No beneficial effect of HSCT has so far been reported in patients with generalized severe JEB [7].

Fetal membranes have been used to treat severe burn injuries for over a century, and the use of amniotic membranes

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(AMs) as a treatment for chronic ulcers in RDEB patients has been reported [8]. Furthermore, intradermal injections of allogeneic fibroblasts [9] or bone marrow-derived mesenchymal stromal cells (BM-MSCs) [10] in RDEB patients transiently improved reepithelialization of chronic ulcers. These kinds of experimental therapies have not been assessed in JEB patients. The use of BM-MSCs as a preconditioning regimen before HSCT has also been proposed in the treatment of RDEB to prevent aggravation of the symptoms of the disease [11].

We have previously used decidual stromal cells (DSCs) from term fetal membranes to treat graft-versus-host disease (GVHD) in HSCT patients [12,13]. These cells share many characteristics with BM-MSCs, which have been more extensively studied and used as treatment for several indications in regenerative medicine. We have found that DSCs have a consistent suppressive effect *in vitro* and have high expression of integrins and coinhibitory markers [14]. In contrast to BM-MSCs, DSCs can be isolated without invasive procedures and they have greater expansion capability.

BM-MSCs have been suggested to escape immune recognition by alloreactive cells [15,16], which have supported the use of allogeneic MSCs in cell therapy. However, stromal cells express MHC class-I molecules [14] and they have been shown to stimulate proliferation of HLA-disparate human T cells *in vitro* and to induce both B cell and T cell-mediated alloresponses in animal models [17–19]. Thus, MSCs have an immunostimulatory capacity. Other possible antigens include xenobiotic serum components, such as bovine serum albumin (BSA) in fetal calf serum (FCS), which is used for expansion of MSCs. The immunogenicity of stromal cells in clinical settings has not been extensively studied [20], and the induction of alloantibodies has only been investigated in immunocompromised patients [21].

Materials and Methods

Patient characteristics

The JEB patient. An 11-month-old girl was found to be homozygous for the mutation R635X in *LAMB3*, resulting in a defect in one of the three polypeptide chains of the heterotrimer laminin-332. The patient had typical symptoms of generalized severe JEB, including blister formation in the skin, problems with upper airways, and infections requiring antibiotic treatment. Blisters were treated with AMs at the age of 11 months, and subsequently with DSC infusions intravenously at the age of 12, 12.5, 13, 13.5, and 14.5 months. A median of 2.3×10^6 (range $1.8\text{--}2.5 \times 10^6$) cells per kg body weight were infused at each time point. She had received two erythrocyte transfusions, 3 months and 1 month before her first treatment with AMs, and one at the time of the third DSC infusion. Concurrent treatment consisted of standard treatment of the disease, that is, proper treatment in the case of pain, local infection, and nutrient deficiency, including erythrocyte transfusions if needed. Informed consent was obtained from the parents.

HSCT patients. Sera from patients who had undergone HSCT and had been treated with DSCs for acute GVHD ($n=7$), chronic GVHD ($n=3$), hemorrhagic cystitis ($n=5$), or both hemorrhagic cystitis and acute GVHD ($n=1$) were analyzed for the presence of anti-HLA antibodies. Median age was 44 (range 8–65) years. These patients were condi-

tioned with myeloablative ($n=10$) or reduced-intensity conditioning ($n=6$). They were treated with a median of 2 (range 1–5) DSC infusions. Immunosuppression was either cyclosporine and methotrexate or sirolimus and tacrolimus [22]. The transplantation procedure, treatment, and supportive care have already been described in detail [23]. Ethical approval for DSC therapy was obtained from the institutional ethics review board (2010/452-31/4).

Preparation of AMs and DSCs

Term placentas were obtained from healthy mothers after cesarean section. Informed consent was obtained from the mothers, and ethical approval was obtained from the institutional ethics review board (2009/418-31/4, 2010/2061/32). The donors were seronegative for HIV, hepatitis B virus, hepatitis C virus, and syphilis. The placenta was washed in PBS (Hyclone, Thermo Scientific) to remove contaminating blood. For preparation of the amnion, the membrane was carefully peeled from the underlying chorion, starting from incisions made close to the umbilical cord. The amnion was washed in PBS to remove any remaining blood and stored in PBS supplemented with penicillin–streptomycin (Hyclone) at 4°C overnight to await confirmation of seronegativity for the above-mentioned viruses.

DSCs were isolated as previously described [12,14]. Briefly, the fetal membrane was dissected from the placental structure, trypsinized, and cut into 4-cm² pieces. The pieces were incubated in 182.5-cm² flasks in DMEM (Hyclone) containing 10% FCS (Hyclone) and penicillin–streptomycin (ie, complete DMEM). The explants were removed from the flasks when colonies of fibroblast-like cells appeared. When the DSCs were confluent, the cells were harvested with trypsin/EDTA (Hyclone), washed in complete DMEM, and seeded in new flasks at 2.9×10^3 cells per cm² in complete DMEM. The cells were cultured to passage 3 or 4 and frozen in complete DMEM containing 10% DMSO (WAK-Chemie Medical GmbH). As previously described [12,14], the DSCs were positive for CD29, CD44, CD73, CD105, and HLA class-I, but negative for CD34, CD14, CD45, and HLA class-II. The origin of the DSCs was analyzed by microsatellite polymorphism using capillary electrophoresis, which showed that the cells were of maternal origin [12].

Application of AMs to wounds

AMs taken from term placentas were washed and kept in saline until applied to wounds. The membranes were covered with dressings and these were kept in place using a nylon net.

Infusion with DSCs

The DSCs were thawed and diluted in ClinMACS PBS/EDTA buffer (AMCell Miltenyi Biotec GmbH) supplemented with 5% human AB plasma. The cells were washed, filtered through a 70-μm cell strainer (BD), suspended in an infusion solution containing NaCl (B. Braun Melsungen AG) supplemented with 10% AB plasma, and transferred to a heparinized syringe (BD) at 2×10^6 cells per mL. The solution was infused intravenously. The cell preparation was tested for bacterial contamination. The well being of the patients was monitored closely for 2 h after DSC infusion.

Fluorescence microscopy

The DSCs were grown on chamber slides (Nalge Nunc, Int.) for 48 h. They were fixed with 1% formaldehyde (Sigma). A 0.2% Triton-X solution (Sigma) was used to permeabilize the cells. They were stained with rabbit anti-human laminin-332 and a secondary anti-rabbit Alexa Fluor 488 antibody (Abcam). Negative controls were incubated with secondary antibodies only. To view the nuclei, a mounting fluid containing DAPI was added (Vector Laboratories, Inc.). The mounting fluid was removed and replaced with glycerol (Sigma) before analysis on an Olympus BX-51 fluorescence microscope with an Olympus XC30 camera using CellSens standard software (Olympus).

Flow cytometric crossmatching

Approximately 10^5 DSCs were incubated with 50 μ l of patient serum for 30 min at room temperature. Normal AB serum was used as a negative control, and a pool of sera from alloimmunized patients, containing both anti-HLA class-I and class-II antibodies, was used as a positive control. For blocking experiments, the serum samples were diluted 1:1 in PBS, PBS with 2% BSA, or PBS with 2% human serum albumin and incubated for 30 min before incubation with the DSCs. The cells were washed thrice in PBS and incubated with FITC-conjugated antibodies to human IgG (Jackson Immuno Research Laboratories) for 20 min at 4°C. After washing in PBS, 7AAD (BD Biosciences) was added to the cells to exclude dead cells. The cells were analyzed on a FACSCalibur (BD). The results from the flow cytometric crossmatching (FCXM) test were calculated by relating the mean fluorescence intensity (MFI) of the sample to those of the negative and positive controls according to the following formula:

$$\text{Sample value (\%)} = \frac{\text{Sample MFI} - \text{Neg. control MFI}}{\text{Positive control MFI} - \text{Neg. control MFI}} \times 100$$

A sample was considered positive if the sample value was 10% or higher.

Assessment of serum HLA antibodies by solid-phase immunoassay

Single-antigen, bead-based, anti-HLA IgG antibody determinations (Labscreen; One Lambda) were performed according to the manufacturer's instructions and analyzed on a Luminex (LABScan 100) platform. Normalized baseline MFI values above 1,000 were considered positive.

Proliferation assay

Peripheral blood mononuclear cells (PBMCs) from the JEB patient, collected before the second, third, fourth, and fifth infusions of DSCs, were plated in triplicate at 2×10^5 cells per well in 96-well plates (Nunc) in RPMI with 5% AB serum. The cells were stimulated with irradiated DSCs (2×10^4 cells/well, $n=4$) or an irradiated pool of PBMCs from five donors (2×10^5 cells/well). The same experiment was performed with PBMCs from a healthy donor. After 5 days, the PBMCs were pulsed with 1 μ Ci ^3H -thymidine

(Amersham Biosciences) per well for 18 h. ^3H -thymidine incorporation was measured with a MicroBeta TriLux liquid scintillation counter (Perkin-Elmer).

FCS and BSA ELISA

Polystyrene, 96-well, half-area EIA plates (Costar) were coated overnight with 1% FCS (Hyclone) or 1% BSA (Sigma-Aldrich) in PBS. The plates were washed in 0.15 M NaCl containing 0.05% Tween-20. Plasma samples were diluted at least 1/100 in PBS with 0.05% Tween. For BSA blocking experiments, the plasma samples were diluted in PBS with 0.05% Tween and 5% BSA and incubated for 30 min before they were added to the ELISA plates and incubated for 2 h. After washing, the plates were incubated for 1 h with biotinylated goat anti-human IgG antibodies (Jackson Immuno Research Laboratories) in PBS with 0.05% Tween, and thereafter with streptavidin-conjugated poly-HRP (Sanquin Reagents) for 20 min. Substrate solution containing 3,3',5,5'-tetramethylbenzidine (TMB) dihydrochloride (Sigma-Aldrich) was added and the reaction was stopped with 1 M H_2SO_4 . Except for coating, all incubations were carried out on a shaker at room temperature. The amount of converted substrate was measured as optical density (OD) at 450 nm in a spectrophotometer (Molecular Devices) and was analyzed with Softmax Pro 6.2.1 software (Molecular Devices).

Detection of antilaminin-332 antibodies

Immunoblot assay using purified human laminin-332 was performed as reported previously [24]. Hirako et al. recently established the preparation of a hemidesmosome-rich fraction from cultured DJM-1 cells, in which all hemidesmosomal components are concentrated [25]. An immunoblot assay using the hemidesmosome-rich fraction has also been developed recently [26].

Murine model for immunogenicity of human DSCs

To evaluate the immunogenicity of human DSCs and human bone marrow-derived MSCs in mice, $0.5\text{--}1 \times 10^6$ human DSCs, MSCs, PBMCs, or PBS were infused intravenously through the lateral tail vein of four healthy BALB/c mice. Ten to 14 days later, the animals were sacrificed and the spleens were removed aseptically. Single-cell suspensions from the spleens of treated and control animals were prepared. After lysis of red blood cells, 4×10^5 spleen cells from individual experimental animals were cocultured with 4×10^4 irradiated (30 Gy) human DSCs, BM-MSCs, or PBMCs (from the same donors as in the immunization step) in round-bottom 96-well plates (Nunc). After 5 days, the cultures were pulsed as described above.

Results

Clinical effects of AM and DSC therapy

The 11-month-old JEB patient had multiple wounds and blisters at different sites, all over the body. AMs were applied to six major lesions: on the back, the right axilla, bilaterally on the elbows, on the right medial malleolus, and over the left Achilles tendon. The first two applications, on the back and the right axilla, fell off immediately.

Two weeks after the application of the AM, she was given DSCs intravenously. Already after 3 days, the lesions over her groins had improved (Supplementary Fig. 1A; Supplementary Data are available online at www.liebertpub.com/scd). After that, she had improvements in the face (Supplementary Fig. S1B) and the fingertips (Supplementary Fig. S1C). After 3 weeks, the wound over her left ear had healed and those in her face had improved. She was given a second infusion 2 weeks after the first DSC treatment. The elbows, which had also been covered with AMs, were improved (Supplementary Fig. S1D). A healing process was seen in the middle of the blisters, as opposed to at the margin, which may occur spontaneously in EB. A third, fourth, and fifth infusion of DSCs was given, but the improvements appeared to be transient. There was no evident improvement in the upper airways after DSC therapy.

To summarize, improvements in the skin were seen both after treatment with AMs and after infusion with DSCs. The patient died from respiratory insufficiency 1 year after treatment with AMs and DSCs.

DSC therapy appeared to induce multispecific HLA antibodies in the JEB patient

The JEB patient had received DSCs from three different placental donors (Table 1). To determine whether the JEB patient had been immunized against any antigens on DSCs, the cells were incubated with patient serum. FCXM revealed that the serum of the patient contained IgG antibodies directed against DSCs from all four of the donors tested (Fig. 1A), three of whom had been used for treatment of this patient and one had not (FM1) been used (Table 1). We also observed binding of serum IgG antibodies to PBMCs from an unrelated donor, although the intensity of binding was lower than with the positive control serum (Fig. 1B). A histogram of a representative FCXM test on DSCs is shown in Fig. 1C.

To determine the specificity of the antibodies, LabScreen single-antigen bead assay was performed on plasma samples

taken at the time of the second, third, fourth, and fifth DSC infusions. As shown in Table 1, specificity to one HLA class-I antigen, A24 (MFI 3,613), and one HLA class-II antigen, DR4 (MFI 2,322), was found in the sample obtained before the second DSC infusion, which was the earliest sample available. At the time of the third DSC infusion, the patient had IgG antibodies to a total of 31 HLA class-I antigens with a median MFI of 4,951. Two of these, A3 and B35, were HLA antigens that were expressed on donor DSCs (from FM8 and FM3, respectively), whereas the others were not expressed on any of the donor DSCs. Before the fourth and fifth DSC treatments, the number of different anti-HLA class-I antibody specificities had increased further to 35 and 38, with a median MFI of 6,380 and 8,519, respectively. Except for DR4, which was present in the earliest available sample, there were no other antibodies directed to HLA class-II antigens in any of the samples tested. The MFI of anti-DR4 antibodies also remained at a constant level throughout the treatment period.

To conclude, these data indicate that DSCs may have induced multispecific HLA class-I antibodies, although it cannot be excluded that the AM and erythrocyte infusions (described in the Material and Methods section) could have caused the alloimmunization.

Proliferative responses to DSCs

To study the immunogenicity of DSCs in the JEB patient in more detail, we stimulated PBMCs, collected at several time points, with irradiated DSCs or with an irradiated pool of PBMCs from at least five donors. We found that the PBMCs from the JEB patient proliferated more vigorously in response to DSCs than to the pool of PBMCs (Fig. 1D). The median stimulation index for DSC stimulation was 6.3 (range 4.3–8.2) compared with 2.8 (range 2.1–5.0) in response to third-party PBMCs. This contrasted markedly with the pattern observed in PBMCs from a healthy donor, where the median stimulation

TABLE 1. TRANSPLANTATION SCHEME AND DEVELOPMENT OF ANTI-HLA ANTIBODIES IN THE JUNCTIONAL EPIDERMOLYSIS BULLOSA PATIENT

<i>Transplantation</i>	<i>Days after first treatment</i>	<i>Cell/tissue donor</i>	<i>HLA of donated cells</i>	<i>Specificity of anti-HLA antibodies at the time of transplantation</i>	<i>Median MFI of anti-HLA class-I antibodies (range)</i>
Amniotic membrane	0	FM11 (fetal)	ND	ND	ND
First DSC infusion	16	FM8 (maternal)	A2, A2, B14, B35, DR4, DR13	ND	ND
Second DSC infusion	37	FM3 (maternal)	A2, A3, B7, B15, DR4, DR15	A24, DR4	A24: 3,616, DR4: 2,322
Third DSC infusion	51	FM8 (maternal)	As above	A1, A3, A11, A23, A24, A25, A32, A36, A80, B13, B35, B38, B46, B49, B50, B51, B52, B53, B56, B57, B58, B59, B62, B63, B67, B71, B72, B75, B76, B77, Cw9, DR4	4,951, (1,199–11,015), DR4: 2,354
Fourth DSC infusion	65	FM11 (maternal)	A2, A24, B18, B40, DR15, DR16	As before the third infusion + B27, B37, B44, B47	6,380, (2,672–10,123), DR4: 3,275
Fifth DSC infusion	93	FM11 (maternal)	As above	As before the fourth infusion + A26, B45, B55	8,519, (1,509–12,278), DR4: 2,302

DSC, decidual stromal cells; MFI, mean fluorescence intensity; FM, fetal membrane; ND, not determined.

FIG. 1. The JEB patient had humoral and cellular immunity to decidual stromal cells (DSCs). Flow cytometric crossmatching (FCXM) showed that serum from the junctional epidermolysis bullosa (JEB) patient collected at the time of the fourth DSC infusion contained IgG antibodies to (A) four different DSC donors (FM1, FM3, FM8, and FM11) and (B) to third-party peripheral blood mononuclear cells (PBMCs). (C) A representative histogram of an FCXM test on DSCs (FM1). (D) PBMCs from the JEB patient, collected at different time points, and from a healthy donor were stimulated with irradiated DSCs from four different donors or with an irradiated pool of PBMCs from five donors (mixed lymphocyte reaction, MLR) and proliferation was measured from ^3H -incorporation on day 6.

index was 1.9 (range 1.7–2.8) for DSCs and 6.8 for PBMCs. The proliferative response of patient PBMCs was as high to DSCs that the patients had been treated with as to DSCs that the patient had not previously been exposed to.

The JEB patient had not developed antibodies to laminin-332

The above results suggest that the JEB patient had developed immunity to DSCs that were not only directed to HLA-antigens. We therefore tested for the presence of antibodies to other antigens.

As the JEB patient lacked expression of functional laminin-332, this protein may have induced an adaptive immune response after exposure. We investigated whether DSCs ex-

press laminin-332 and found using fluorescence microscopy that the protein is highly expressed in DSCs (Fig. 2A).

We tested whether the patient had developed antibodies to laminin-332. An immunoblot assay using purified human laminin-332 showed that no samples taken after DSC infusion contained IgG or IgA antibodies specific for any of the $\alpha 3$, $\beta 3$, or $\gamma 2$ subunits. Serum from a patient with mucous membrane pemphigoid (MMP) was used as a positive control and it showed specific reactivity with all three subunits of laminin-332 (Fig. 2B). An immunoblot assay using a hemidesmosome-rich fraction further confirmed that the plasma of the JEB patient did not contain any antibodies to laminin-332 or to any other antigens in the basement membrane zone, such as integrins $\alpha 6$ and $\beta 4$, bullous pemphigoid (BP) 180, BP230, or plectin (Fig. 2C).

The JEB patient had high levels of anti-BSA antibodies

The DSCs were cultured in a medium supplemented with FCS during the expansion. We next examined whether the JEB patient had developed antibodies to FCS or to BSA, the main protein in FCS. Using an FCS-specific ELISA, we found that plasma from the JEB patient contained high levels of IgG antibodies that bound to FCS (Fig. 3A). Preincubation of plasma with 2% BSA before the assay lowered the OD, indicating not only that at least a proportion of the antibodies were specific for BSA but that other bovine antigens may also have been involved. The presence of specific anti-BSA antibodies was confirmed by a BSA-specific ELISA, in which blocking of plasma by BSA completely inhibited the reaction (Fig. 3B). We ex-

amined plasma from another patient with generalized severe JEB, a 10-month-old patient about to undergo an allogeneic HSCT, which also turned out to contain high levels of anti-FCS and anti-BSA antibodies (Fig. 3C). When the plasma samples were diluted to the point at which the positive control turned negative (1/27 000), the OD of the plasma samples from the two JEB patients was still above the detection limit, indicating the remarkably high titers of anti-FCS antibodies in these patients.

To determine whether the DSCs express any antigens that can be detected by anti-BSA antibodies, we incubated patient serum with BSA to block anti-BSA antibodies before FCXM. As shown in Figure 3D, the binding of antibodies to DSCs decreased when anti-BSA antibodies were blocked, indicating that DSCs contain residual BSA that is accessible for antibody binding.

FIG. 3. Detection of anti-bovine IgG antibodies binding to DSCs. ELISA showed that the plasma of the JEB patient contained antibodies to (A) fetal calf serum (FCS) and (B) bovine serum albumin (BSA). Preincubation of plasma with 2% BSA before the assay (BSA blocking) lowered the optical density (OD) slightly in the FCS-specific ELISA and considerably in the BSA-specific ELISA, indicating not only that the BSA ELISA was indeed specific but that there might also have been antibodies to other FCS antigens. (C) Samples were diluted extensively (1/27,000), but plasma from two JEB patients still showed an OD over the limit of detection in both the FCS-specific and the BSA-specific ELISAs. The positive control was negative at this dilution. (D) FCXM with serum from the JEB patient showed that preincubation with 2% BSA, but not with 2% human serum albumin (HSA), before the assay reduced binding of IgG antibodies to DSCs (donor FM1 and FM3). This indicates that FCS-cultured DSCs contained residual BSA that allowed binding of anti-BSA antibodies.

Immunogenicity of human DSCs in mice

The immunogenicity of human DSCs was further confirmed in a mouse model by examining the capacity to mount a recall proliferative response to human cells. Splenocytes from mice that had been immunized with human DSCs showed a higher proliferative response to irradiated human PBMCs than splenocytes from PBS-treated naïve control mice (Fig. 4A). In fact, the response to human PBMCs was as high in splenocytes from PBMC-treated mice as in those from DSC-treated mice. Similar results were obtained in mice that had been treated with human BM-MSCs (Fig. 4B). This indicates that human DSCs and BM-MSCs have capacities similar to those of immunocompetent PBMCs to promote immunization in a xenogeneic setting.

DSCs do not induce anti-HLA antibodies in allogeneic HSCT patients

As DSCs appear to be immunogenic in immunocompetent patients, we investigated whether these cells could induce an alloresponse in allogeneic HSCT patients who had been treated with DSCs for acute GVHD ($n=8$), chronic GVHD ($n=3$), or hemorrhagic cystitis ($n=5$) (Table 2). The number of DSC doses ranged from 1 to 5 infusions derived

from 1 to 3 placental donors, and the patients were followed for between 4 weeks and 4 months after DSC treatment. We investigated the presence of IgG antibodies to DSCs in serum from the patients by FCXM. Two of the acute GVHD patients showed a positive FCXM result, of whom one patient (1,462) showed a positive test before the first DSC infusion (Fig. 5A). The other patient (1,583) was negative before the treatment, but had a positive response to DSCs 4 weeks after infusion (Fig. 5B). The Labscreen test was therefore performed, which showed that patient 1,462 had multispecific anti-HLA antibodies before ($n=43$, median MFI 7,877, range 1,555–10,997) and 4 weeks after ($n=34$, median MFI 2,535, range 1,004–10,933) DSC infusion, whereas patient 1,583 had no anti-HLA antibodies either before or after treatment. As shown in Figure 5C, there was no increment in IgG antibodies to BSA or FCS, as determined by ELISA, in this patient at 4 weeks after DSC infusion, indicating that the binding to DSCs was not caused by anti-BSA IgG antibodies.

Discussion

BM- and adipose-derived MSCs have been used to treat inflammatory immune disorders such as GVHD [27] and

FIG. 4. Immunogenicity of human DSCs and bone marrow-derived mesenchymal stromal cells (BM-MSCs) in mice. Splenocytes from mice that had been infused with human peripheral blood mononuclear cells (PBMCs) or with (A) human DSCs ($n=4$) or (B) human BM-MSCs ($n=2$) were stimulated with irradiated DSCs/BM-MSCs or PBMCs. The proliferative response to PBMCs was as high in mice that had been immunized with DSCs (A) and BM-MSCs (B) as in mice that had been immunized with PBMCs.

Crohn's disease [28]. Stromal cell therapy is also relevant in regenerative medicine, owing to the ability of these cells to secrete factors involved in angiogenesis and tissue repair [29]. Similar cells of both fetal and adult origin can be isolated from term placentas, a source that easily provides a large number of cells with great expansion capacity without requiring any invasive procedures.

In the present study, we treated a JEB patient with AM and DSC infusions. The rationale for using these tissues and cells in EB patients is primarily their ability to increase wound healing through paracrine factors. The fact that DSCs (Fig. 2A) and AMs [30] express laminin-322 may also be a positive feature, although we did not examine the presence of functional laminin- $\beta 3$ in the patient after DSC treatment. We chose to infuse the DSCs intravenously, rather than intradermally to skin lesions, since we assumed that systemic rather than local administration would increase the chances of

resolving blisters in both skin and mucosal surfaces. However, we observed no effect on upper airways. In a murine model of RDEB with mutations in the type VII collagen gene, Tolar et al. found that transplantation of nonhematopoietic cells, including MSCs and epidermal stem cells expressing mRNA for collagen VII, did not alter the course of the disease, whereas hematopoietic stem cells were able to correct the basement membrane zone defect [31].

In the current study, the JEB patient appeared to benefit from the AM and DSC treatment in terms of improvement in the healing process of blisters and wounds, particularly after the earlier infusions. The patient survived for 23.5 months compared with the average of 6 months [32]. There is however a range regarding survival among patients with JEB (up to 32.6 months) [32]. BM-MSCs have been suggested to be used to precondition RDEB patients before the HSCT to prevent transplant-related toxicity and rapid progression of

TABLE 2. ALLOGENEIC HEMATOPOIETIC STEM CELL-TRANSPLANTED PATIENTS

UPN#	Sex	Age at DSC treatment, years	Indication for DSC treatment	No. of DSC infusions	DSC treatment (weeks after HSCT)	FCXM test
1011	M	8	cGVHD	2	398, 406	Neg
1265	M	18	cGVHD	2	195, 206	Neg
1462	F	65	aGVHD	2	16, 31	Pos
1468	M	58	aGVHD	1	15	Neg
1475	F	18	cGVHD	1	45	Neg
1504	F	53	aGVHD	3	6, 15, 19	Neg
1531	M	62	aGVHD	1	4	Neg
1563	F	8	HC	1	13	Neg
1583	M	58	Toxicity, aGVHD	2	1, 7	Pos
1587	F	41	HC	1	9	Neg
1588	F	24	HC	1	8	Neg
1589	M	48	aGVHD	3	44, 46, 47	Neg
1590	F	43	HC	1	5	Neg
1602	M	44	HC	1	6	Neg
1619	F	43	aGVHD	4	21, 22, 23, 39	Neg
1620	M	53	aGVHD	5	16, 17, 18, 23, 44	Neg

UPN, unique patient number; aGVHD, acute graft-versus-host disease; cGVHD, chronic graft-versus-host disease; HC, hemorrhagic cystitis.

FIG. 5. DSC therapy in allogeneic hematopoietic stem cell transplantation (HSCT) patients. (A, B). FCXM done on sera from HSCT patients showed that two patients who had received DSCs for transplant-related disorders contained IgG antibodies that bound to DSCs, one of whom had anti-HLA antibodies before DSC therapy, which gradually decreased (A). (C) Anti-FCS and anti-BSA antibody ELISA showed that the positive FCXM test in patient UPN 1538 was not due to the presence of antibodies to bovine antigens.

the disease [11]. HSCT could have been considered in the JEB patient if the clinical improvement of DSCs had lasted longer.

Cells and tissues originating from the fetal membranes, the placenta, and the decidua have immunomodulatory properties, as shown by the work of others and our group [14,33–37]. The fetal membrane consists of three layers, of which the amnion and the chorion are of fetal origin and the

decidua parietalis is of maternal origin. The inner AM contains both stromal and epithelial cells, both of which have been studied in experimental models of tissue repair [38]. These cells express HLA class-I molecules, but AMs show low immunogenicity in experimental xenotransplantation [39]. DSCs express HLA class-I antigens, but not HLA class-II antigens, either in the absence or presence of the inflammatory cytokine, IFN- γ [14].

We found that the JEB patient had high levels of circulating anti-HLA class-I antibodies directed against more than 30 HLA antigens 7 weeks after the first treatment with DSCs. The number of specificities and the avidity of the antibodies gradually increased during the treatment, as indicated by an increased median MFI in Luminex analysis. The first sample available only contained antibodies to two HLA antigens (A24 and DR4). The patient had received blood transfusions 1 and 3 months before the AM and DSC treatment started and one transfusion after the third infusion of DSCs. It can therefore not be excluded that the erythrocyte transfusions induced the HLA alloantibodies. However, they first appeared 11–12 weeks after the last blood transfusion. In addition, the erythrocyte concentrations were depleted of leukocytes and it is relatively rare that such blood products induce immunization.

The AM could possibly also have induced the anti-HLA antibodies, but this appears less likely as human AMs are accepted not only over allogeneic barriers [40] but also in xenogeneic settings [39]. Thus, it is more likely that immunization was caused by the DSCs. However, it cannot be excluded that the erythrocyte transfusions and/or AMs could have caused the production of anti-HLA antibodies. Only three of the HLA antibody specificities matched the HLA of the donated DSCs, and the majority of the antibodies were specific for irrelevant HLA antigens. Cross-reactivity between different HLA antigens is high, but the alloantibodies detected in the patient serum also appeared to belong to many different cross-reactive groups (CREGs) [41].

The proliferative response of PBMCs from the JEB patient to DSCs and PBMCs showed a markedly different pattern to that of healthy donors. In the JEB patient, DSCs induced a higher proliferative response compared with a pool of PBMCs from six donors (Fig. 1D), whereas DSCs only promote a negligible activation of PBMCs from healthy donors [14]. This pattern was observed at all time points, including the first available sample collected 3 weeks after the first DSC infusion. This not only indicates that antigens other than HLA may be responsible for the proliferation but it also further supports the fact that DSCs can stimulate immunity and subsequently also may have caused the induction of anti-HLA antibodies. Unfortunately, we had no samples taken before the AM and DSC treatment was started, and we cannot exclude the possibility that, for unknown reasons, DSCs were more immunogenic in the JEB patient than in healthy donors in general. It is also possible that the exacerbated proliferative response is due to normal idiosyncratic immune responses of this patient rather than specifically to the disease or to pediatric patients in general. We did not have access to blood samples from any HSCT pediatric patients treated with DSCs, so the immunogenicity of DSCs in immunocompromised children is still unclear.

Laminins and other basement membrane zone proteins are potentially immunogenic, as demonstrated by the formation

of autoantibodies to these antigens in MMP and BP [42]. As DSCs express functional laminin-332, we had to consider the risk that the JEB patient might develop antibodies to the laminin- β 3 subunit. However, we did not detect any antibodies to laminin-332 or to any other protein of the basement membrane zone.

The JEB patient had high titers of anti-BSA antibodies, and we found that another patient with the same diagnosis also had elevated levels of bovine-specific antibodies. It is not known why the JEB patients had high anti-BSA IgG titers, but this was observed in both patients and thus independently of DSC treatment. Exposure to BSA in humans generally occurs through dietary routes or through vaccination. The proportion of healthy donors with anti-BSA antibodies has been reported to be about 50% [43]. Thus, the presence of anti-BSA antibodies is common [44], but their clinical relevance is unclear. They have been implicated in membranous nephropathy [45] and insulin-dependent diabetes mellitus [46], but the majority of BSA-seropositive patients do not develop any adverse events. However, infusion of FCS-cultured cells in patients with anti-BSA antibodies may cause hypersensitivity reactions, as demonstrated in HIV patients receiving FCS-cultured syngeneic T cells, which promoted arthus-like reactions [47]. Spees et al. showed that FCS proteins associate with MSCs after *in vitro* culture with FCS-supplemented medium [48]. The FCS proteins were partly internalized, which excludes the possibility of disposal of the bovine contamination by washing. They also showed that FCS-cultured rat MSCs can induce anti-FCS antibodies in a rat model.

The murine experiments in the present study demonstrate that xenogeneic DSCs and BM-MSCs have similar capacities to provoke adaptive immune responses. However, it should be noted that the immune response in mice against human stromal cells is different than that from alloresponses in humans since not only HLA molecules but also xenogeneic antigens are detected. Allogeneic MSCs induce lower lymphocyte proliferation than PBMCs and have been considered to be immune privileged [49]. Others have previously found that BM-MSCs do induce xenoreactivity [17–19]. Thus, in JEB patients, BM-MSCs are probably not more favorable than DSCs with regard to risk of immunization.

Allogeneic HSCT patients have long-lasting defects in adaptive immunity, making them highly susceptible to opportunistic infections. Consequently, these patients are less likely to develop alloantibodies than immunocompetent patients. Patients with GVHD are also treated with immunosuppressive drugs, further dampening their ability to induce an adaptive immune response. Sixteen HSCT patients were examined in this study and none of them developed anti-HLA antibodies after infusion with DSCs. One patient had multispecific anti-HLA antibodies before DSC treatment. This patient was treated with DSCs for acute GVHD, was a partial responder [12], and was alive over 3 years after DSC treatment. The FCXM test and the Luminex analysis indicated that the antibodies to antigens on DSCs successively dropped after the first DSC infusion. This might reflect the fact that recipient anti-HLA antibodies, which were still present in the circulation after the HSCT, started to be catabolized and that there was no *de novo* production of alloantibodies from the new donor-derived immune system.

Another patient showed a positive FCXM test 4 weeks after DSC treatment, but this could not be explained by the

presence of either anti-HLA or anti-BSA IgG antibodies. This patient first received DSCs for bleeding during the neutropenic phase, and thereafter for acute GVHD approximately 1 month after. The specificity of the DSC-binding antibodies present after the first DSC infusion is still to be determined. The potential immunogenicity of BM-MSCs in HSCT patients has been examined by Sundin et al. [21]. They did not detect any anti-HLA antibodies when analyzing 12 acute GVHD patients after MSC infusion, but anti-FCS antibodies were detected in long-term follow-up samples. It was unclear whether the MSCs, which had been expanded in FCS, could have caused the appearance of these antibodies.

Conclusions

We may need to reconsider the immunogenicity of allogeneic stromal cells as they appear to have provoked anti-HLA antibodies in an immunocompetent individual. Bovine proteins in cell culture medium, in which the therapeutic cells are cultured, could also be targets for already formed antibodies. This could be avoided by culturing the cells in human platelet lysate. DSCs and the AM may have induced positive effects on wound healing in JEB patients, and it is possible that continued cell therapy would have sustained the positive effects. A concomitant cyclosporine treatment could possibly be used to prevent immunization to alloantigens. With over 100 infusions of allogeneic FCS-cultured DSCs, we have not observed any hypersensitivity reactions in any of the patients. However, it remains to be determined whether the efficiency of stromal cell therapy might be hampered by the presence of antibodies that can bind to the cells and activate complement-mediated cell death.

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