Original Article

No alloantibodies against mesenchymal stromal cells, but presence of anti-fetal calf serum antibodies, after transplantation in allogeneic hematopoietic stem cell recipients

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ABSTRACT

Background and Objectives

Mesenchymal stromal cells (MSC) may be used in cellular therapy to treat graft-versus-host-disease and autoimmune disorders, and in regenerative medicine. Preliminary data suggest limited cellular allogeneic rejection, but less is known about humoral responses. The objective of this study was to investigate whether antibodies against MSC were present after hematopoietic stem cell transplantation (HSCT) including treatment with HLA matched or mismatched allogeneic MSC.

Design and Methods

Twelve patients were evaluated using flow cytometric cross matches (FCXM) and enzyme-linked immunosorbent assays. Expression of blood group antigens, regarded as alloantigens giving rise to humoral alloimmunity, on MSC were explored using flow cytometry and immunofluorescence.

Results

Three of 12 patients exhibited late positivity in the FCXM. In absorption studies, antibodies directed against fetal calf serum (FCS), a component of the MSC culture medium, were identified. Healthy individuals expressed varying levels of anti-FCS antibodies and the same pattern was seen in immunosuppressed HSCT patients. MSC did not express blood group antigens. The patients with positive FCXM are alive and well.

Interpretation and Conclusions

We have shown that immunosuppressed patients can exhibit anti-FCS antibodies, but no alloantibodies, which may bind to MSC. These antibodies seem clinically insignificant.

Key words: rejection, humoral, immunogenicity, cross match.

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Mesenchymal stromal cells (MSC), previously referred to as mesenchymal stem cells, are non-hematopoietic cells present in the human bone marrow. The cells have an extensive proliferative capacity *in vitro* without loss of phenotype and have the ability to differentiate into more mature lineages, e.g. bone, cartilage and fat *in vivo* and *in vitro*.[1-5] Despite their expression of HLA class I, and even when induced to express class II, MSC seem to escape allogeneic effector mechanisms and reduce alloreactivity.[6] Several mechanisms have been suggested that may act in synergy. For instance, MSC modulate dendritic cell function.[7,8] They reduce T lymphocyte proliferation and the formation and function of CD8$^+$ effectors.[9-14] The cells have also been demonstrated to inhibit lymphocyte responses to bacterial, fungal and viral agents.[15]

MSC are regarded as candidates for cellular therapy.[16] The immunomodulatory nature of MSC may be of clinical relevance in allogeneic hematopoietic stem cell transplantation (HSCT).[17] MSC reversed therapy resistant grade IV acute graft-versus-host-disease (GvHD) of skin, gut and liver, and improved survival.[18,19] Furthermore, MSC may be used in regenerative medicine,[20] treatment of autoimmune disorders[21] and inborn errors of metabolism, such as Hurler's disease.[22]

Research has mainly focused on cellular immunity, such as alloreactive T lymphocytes, as effectors of rejection in stem cell transplantation. Early studies showed that Thy1$^+$ lymphocytes were capable of inhibiting hematopoietic development after HSCT. Very few, almost undetectable, T lymphocytes that survived the conditioning regimen appeared sufficient to cause rejection defined as inhibited hematopoiesis.[23] In a murine model, antibody-dependent NK-cell mediated rejection occurred.[24] It was later shown that NK-cells alone can mediate bone marrow cell rejection in mice.[25] However, in man such conclusive results are lacking. Rejection by antibodies is less studied, but animal studies[26] and retrospective analyses of clinical data[27] identified transfusion-induced sensitization to minor histocompatibility complexes expressed on donor marrow cells as the major cause of graft rejection in patients with severe aplastic anemia (SAA). Evidence for immunological rejection by pre-formed non-HLA antibodies against donor cells has been demonstrated in HSCT patients.[28,29] Antibodies against AB0-antigens are produced and are present lifelong soon after birth in immunocompetent individuals and may play a role in the outcome of HSCT.[30]

In the present study, we aimed to investigate whether there are humoral immune responses to transplanted MSC in patients with a prior HSCT. Antibody development was compared to clinical data to evaluate possible humoral alloimmunity against transplanted MSC.

Design and Methods

Patients

Between November 2002 and May 2006, 29 patients underwent MSC transplantation at Karolinska University Hospital Huddinge, Stockholm, for the indications stated below. A total of 12 patients were included in this survey. Exclusion criteria were multiple MSC transplants and lack of serum samples.

Allogeneic HSCT and MSC transplantation procedure

The myeloablative conditioning regimen consisted of cyclophosphamide 120 mg/kg combined with busulphan 16 mg/kg (*n*=5), or fractionated (3 Gy for 4 days) total body irradiation (TBI, n=2).[31] Two patients received fludarabine 30 mg/m^2/day for 5 days, cyclophosphamide 60 mg/kg and TBI (3 Gy for 2 days). One patient with severe combined immunodeficiency received fludarabine for 5 days combined with melphalan 140 mg/m^2.[32] The reduced intensity conditioning regimen consisted of fludarabine at 30 mg/kg/day for 5 consecutive days in combination with 2 Gy TBI (*n*=1) or busulphan (*n*=1). Patients with unrelated donors were given anti-T-cell prophylaxis consisting of anti-thymocyte globulin (Thymoglobulin®; Genzyme, Cambridge, MA, USA) or alemtuzumab (CamPath®; ILEX Pharmaceuticals, San Antonio, TX, USA) as part of the conditioning regimen.[33] The source of hematopoietic stem cells was bone marrow in two cases, while the majority of patients (*n*=9) received mobilized peripheral blood stem cells. Cord blood was the source in one patient. The median CD34$^+$ cell dose was 7.7×10^6/kg (range 0.21-68). No patient was treated with granulocyte colony-stimulating factor. As GvHD prophylaxis, most patients received cyclosporine A (CsA) combined with four doses of methotrexate (*n*=8).[34] Other protocols included CsA in combination with mycophenolate mofetil (*n*=1) and prednisolone (*n*=1). Two patients were given tacrolimus and sirolimus in combination as GvHD prophylaxis. In patients with matched unrelated donors CsA was discontinued after 6-12 months in the absence of GvHD. Using HLA identical sibling donors, CsA was tapered after 2 months and discontinued at 3-4 months.[35]

MSC isolated, as described below, from healthy donors were expanded *ex vivo* in clean conditions approved by the Swedish Medical Products Agency and were given intravenously to the patients. Cells were collected for the infusion preparation in passage 1-4 and diluted in saline supplemented with 10% human AB plasma. The median MSC dose was 1.4×10^6/kg (range 0.9-2.0), and all but one patient received a single dose (*n*=11). Five patients received HLA-mismatched unrelated MSC, three were given MSC from sibling donors and four patients haploidentical MSC, i.e. from first degree relatives. Patients who had undergone prior HSCT were given MSC for the following indications: to promote engraftment in HSCT (*n*=5), to treat hemorrhagic cystitis (*n*=3) and to treat acute GvHD (*n*=4).[18,19] Clinical data were collected prospectively. Serum from the patients was sampled before transplantation and 1 to 2 weeks, 1 month, 3 months, 6 months and finally 1 year post-MSC transplantation. The study was approved by the Regional Ethics Review Board. Patients were observed for eventual adverse events and followed over time.

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Patients' immunoglobulin levels

The patients' immunoglobulin levels were measured before HSCT and at the same time points listed above. The IgA, IgG and IgM levels were determined by electrophoresis at the Laboratory of Clinical Chemistry, Karolinska University Hospital Huddinge, Stockholm.

Isolation and *ex vivo* culture of human MSC

To isolate human MSC, bone marrow aspirates of 50 mL were taken from the iliac crest of healthy donors. The harvest was approved by the Regional Ethics Review Board. MSC were isolated and cultured as previously described.[14] Briefly, heparinized bone marrow was separated by a 1073 g/mL Percoll density gradient (Amersham Biosciences, Little Chalfont, UK). Mononuclear cells were collected, washed and re-suspended in human MSC medium consisting of Dulbecco's modified Eagle's medium low glucose (Invitrogen, Paisley, UK) supplemented with 10% fetal calf serum (FCS; Swedish National Veterinary Institute, Uppsala, Sweden) and 1% antibiotic-antimycotic solution (Life Technologies, Gaithersburg, MD, USA). The cells were plated at 1.6×10^5 cells/cm^2 in culture flasks.

The serum lot was selected on the basis of optimal MSC growth with maximal retention of osteogenic, chondrogenic and adipogenic differentiation. For indicated experiments fresh bone marrow mononuclear cells were cultured in medium supplemented with 10% pooled human AB sera instead of FCS. When cultures were near confluence, the cells were detached by treatment with 0.05% trypsin and 0.35 mM EDTA (Invitrogen) and re-plated at a density of 4×10^3 cells/cm^2.[14] Characterized by flow cytometry, the MSC uniformly expressed CD73, CD90 and CD105. The cells did not express the hematopoietic markers CD14, CD31, CD34 and CD45. *Ex vivo* expanded MSC differentiated on induction into bone, cartilage and adipose tissue.[6,14]

Flow cytometric cross match for detection of antibo-dies against MSC

Donor MSC were incubated with recipient sera from before and sampled at several time points after MSC transplantation. After washing, the cells were incubated with polyclonal anti-human IgM and IgG fluoresceinated (FITC) antibodies (Jackson Immuno Research Laboratories, West Grove, PA, USA), washed in phosphate-buffered saline (PBS) and assayed in a flow cytometer (BD Biosciences, San Jose, CA, USA). Analyses were performed with Cellquest software (BD Biosciences). Fluorescence signals from 10 000 cells were counted and the percentage of FITC-positive cells was recorded. A 20 channel shift in mean fluorescence in the test sample compared to the negative control was regarded positive.[36] A pool of sera from alloimmunized patients was used as a positive control. Sera from healthy non-transfused males with blood group AB served as a negative control.

Enzyme-linked immunosorbent assay for detection of antibodies against FCS

Ninety-six well plates were coated with 1% FCS in 0.05 M carbonate buffer (pH 9.6) over night at 22° C. Plates were washed and incubated with recipient serum diluted in PBS with 0.05% Tween, washed and then incubated with alkaline phosphatase-conjugated polyclonal anti-human IgG antibodies (DakoCytomation, Carpinteria, CA, USA). Diethanolamine with addition of PNPP (Sigma-Aldrich, St Louis, MO, USA) was used as the color development substrate solution. Plates were assayed at 405 nm in a spectrophotometer. Serum from a male with known high titers of anti-bovine antibodies was used as a positive control and the negative control consisted of solely PBS-Tween. We also included 14 sera from healthy individuals as a reference group. Optical density values of more than 1.0 were regarded as positive.

Flow cytometry and immunofluorescence for determination of blood group A and B antigen expression on MSC

MSC were incubated with monoclonal mouse-anti-human blood group A (IgG; BD Biosciences) and B (IgM; Abcam, Cambridge, UK) antigen antibodies, respectively. After washing in PBS and re-suspension, the cells were incubated with fluoresceinated (FITC) polyclonal anti-mouse IgG and IgM antibodies (Jackson Immuno Research Laboratories), respectively. Peripheral blood lymphocytes (PBL) isolated from whole blood with Ficoll-hypaque gradient (1077 g/cm^3; Axis-Shield PoC AS, Oslo, Norway) were used as a control. Washed cells were analyzed by flow cytometry as above.

For immunofluorescence, MSC were cultured overnight in chambers on microscopy slides. Erythrocytes and PBL, as controls, were dried on slides and fixed in methanol-acetone solution at -20° C. Samples were incubated with polyclonal mouse-anti-human blood group A and B antigen antibodies (IgM; Biotest AG, Frankfurt am Main, Germany), washed and incubated with the fluoresceinated (FITC) anti-mouse IgM antibodies used in the flow cytometry assay. Slides with MSC and PBL were stained with DAPI for visualization of the nuclei. Counterstaining with Evans blue was applied for the MSC slides. Glycerol mounted slides were subsequently read independently by two of the authors. The analysis was performed using a fluorescence microscope (DMRXA; Leica Microsystems GmbH, Wetzlar, Germany) at 22° C with a 40x oil objective (PL Fluotar Oil; Leica Microsystems GmbH) and filters for FITC, DAPI and CY3 (Chroma Tecnology Corp, Rockingham, VT, USA). Images were captured using a camera (S/N 370 KL 0565; The Cooke Corp, Romulus, MI, USA) and SlideBook 2.1.5 software (Intelligent Imaging Innovations Inc, Denver, CO, USA). Finally, images were post-processed and mounted using Photoshop 3.0 (Adobe Systems Inc, San Jose, CA, USA).

Specificity determination of antibodies against MSC

To determine whether the late reactivity detected in the flow cytometric cross matches (FCXM) were specific for the infused MSC, the recipient sera were incubated with MSC

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Table 1. Characteristics of the 12 patients who underwent MSC transplantation following allogeneic hematopoietic stem cell transplantation.

Characteristic	
Diagnosis	
Acute leukemia	5 (42%)
Lymphoma	1 (8%)
Myeloma	1 (8%)
Aplastic anemia	1 (8%)
Severe combined immunodeficiency	2 (17%)
Myeloproliferative disorder	1 (8%)
Thalassemia major	1 (8%)
Patients' sex (male/female)	10/3 (83%/17%)
Patients' age (year), median (range)	36 (1-64)
Hematopoietic stem cell donor	
HLA-identical sibling	6 (50%)
Matched unrelated	4 (33%)
HLA subtype mismatched unrelated	1 (8%)
Major HLA mismatched unrelated	1 (8%)
CD34$^+$ cell dose ($\times 10^6$/kg), median (range)	7.7 (0.21-68)
Stem cell source	
Bone marrow	2 (17%)
Peripheral blood stem cells	9 (75%)
Cord blood	1 (8%)
Conditioning	
Bu+Cy	5 (42%)
Flu+Cy+TBI	2 (17%)
FTBI+Cy	2 (17%)
Mel+Flu	1 (8%)
RIC Flu+TBI	1 (8%)
RIC Flu +Bu	1 (8%)
Graft-versus-host disease prophylaxis	
CsA+Pred	1 (8%)
CsA+MTX	8 (64%)
CsA+MMF	1 (8%)
Tacrolimus+Sirolimus	2 (17%)
Anti-T-cell prophylaxis	9 (75%)
Granulocyte colony-stimulating factor	0

HLA: human leukocyte antigen; Bu: busulphan; Cy: cyclophosphamide; TBI: total body irradiation; FTBI; fractionated total body irradiation; Mel: melphalan; Flu: fludarabine; RIC: reduced intensity conditioning; CsA: cyclosporine A; Pred: prednisone; MTX: methotrexate; MMF: mycophenolate mofetil.

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from other donors (*n*=3). We also incubated these sera with MSC expanded and cultured in medium supplemented with human AB serum (*n*=2). Serum-exposed cells were assayed in the flow cytometer as above.

We incubated the same sera with PBL derived from healthy individuals (*n*=3) and MSC donor-derived PBL (*n*=2) to determine whether the positive FCXM resulted from a broader spectrum of alloantibodies or immunization against the donor, respectively. Serum-exposed PBL were thereafter investigated using the FCXM method.

Flow-PRA® screening kit (One Lambda, Canoga Park, CA, USA) assays were used to detect anti-HLA class I and II antibodies,[37] according to the manufacturer's instruction.

The FCXM positive sera were investigated for antibodies directed against erythrocytes, i.e. blood group antigens. For this purpose, the sera were absorbed with erythrocytes from two donors, one with blood group A, the other with blood group B. As controls, serum from the blood group A donor was absorbed with erythrocytes from the blood group B donor, and *vice versa*. Absorbed sera were thereafter re-assayed as above.

To test whether the positive findings resulted from antibodies directed to FCS, we performed absorption. Grains prepared from FCS supplemented with bovine serum albumin (Sigma-Aldrich) in 25% glutardialdehyde,[38] were used in the recipient sera and repeated five times. Thereafter the absorbed sera were re-assayed using flow cytometry. The absorption specificity was determined using an enzyme-linked immunosorbent assay (ELISA). Plates were coated with FCS and polyclonal anti-human IgG antibodies (DakoCytomation). A grain-absorbed preparation of pooled human IgG antibodies (Gammagard S/D®; Baxter Healthcare, Deerfield, IL, USA) was used as the sample. Subclass determination of anti-FCS antibodies was performed using ELISA with FCS-coated plates. Samples and controls, sera from healthy individuals and the pooled human IgG (Gammagard S/D®; Baxter Healthcare), were handled as above. After incubation with sera and washing, plates were incubated with polyclonal anti-human IgG, IgG_1, IgG_2 and IgG_3 antibodies conjugated to horseradish phosphatase (The Binding Site Ltd, Birmingham, UK), respectively. Tetramethylbenzidine (TMB; Simga-Aldrich) was used as the substrate and plates were assayed at 630 nm in the spectrophotometer.

Results

MSC transplantation

Of the 12 patients included in this study seven (58%) are alive and well, with a follow-up of 7 months to 3 years. Five patients died between 170 and 670 days post-MSC transplantation. The causes of death were mainly infectious complications, such as septicemia and fungal infection, and in one case the patient had relapse of disease, i.e. myeloma. No adverse events during or after the MSC infusion were recorded. Further characteristics and details are shown in Tables 1 and 2.

No rise in patients' immunoglobulin levels associated with MSC

The patients exhibited normal or pathological levels of immunoglobulins, which could be associated with their diseases, prior to MSC transplantation. No mono- or oligoclonal rise in immunoglobulins was detected after 1 month (*n*=5). At 3 months post-MSC transplantation, three of eight patients showed an increase. Patients 994 and 1118 showed oligoclonality as a marker of non-specific inflammation and the monoclonality of patient 1143 was concurrent with an increase in Epstein Barr virus-DNA copies. Six months after the MSC transplantation nine patients were evaluated. The majority had low immunoglobulin levels. Patient 1020 showed a monoclonal rise and was diagnosed as having herpes zoster, i.e. reactivation of varicella-zoster virus infection. In patient 1044 the monoclonality was explained by myeloma relapse. Immunoglobulins were measured at 12 months in the three available patients. One patient, 1098, showed oligoclonal bands and the remaining two had immunoglobulin levels approaching normal (*data not shown*).

Positive FCXM late after MSC transplantation

We investigated several sera with FCXM, using donor MSC, from the 12 patients who had undergone MSC transplantation. Sera pre-MSC transplantation were positive in

Table 2. Detailed characteristics of the 12 patients who underwent MSC transplantation after allogeneic hematopoietic stem cell transplantation.

Patients				Hematopoietic stem cell transplantation						Mesenchymal stromal cell transplantation						
UPN	Sex	Age[1]	Diagnosis	Match	Cell source	Cond	Anti-T-cell	GvHD prophyl	GvHD grade	Indication	Match	Passage	Dose $\times 10^6$/kg	Ninf	Day[2]	Outcome
924	M	9	ALL	MUD	PBSCT	FTBI+Cy	x	CsA+MTX	IV	GvHD	Haplo	1	2.0	2*	73/170	†d 577: Aspergillus infection & MOF
994	F	34	AML	MUD	PBSCT	Bu+Cy	x	CsA+MTX	I	Engraftm	Haplo	2	1.4	1	0	A & W: 2y 10m
995	M	59	AML	HLA-id Sib	PBSCT	Bu+Cy	x	CsA+MTX	III	GvHD	Sib	2	1.4	1	57	A & W: 3y
1020	M	38	ALL	HLA-id Sib	PBSCT	FTBI+Cy		CsA+MTX	II	Engraftm	Sib	2	1.0	1	0	A & W: 2y 6m
1044	M	60	Myeloma	HLA-id Sib	PBSCT	RIC: Flu+TBI		CsA+MMF	III	GvHD	MM	2	0.9	1	61	†d 731: relapse
1047	M	7	AA	MMUD s	BM	Flu+Cy+TBI	x	Tacro+Siro	II	Engraftm	Haplo	2	1.0	1	0	A & W: 2y
1098	M	14	Thalassemia	HLA-id Sib	BM	Bu+Cy		Tacro+Siro	–	Hem cystit	MM	2	1.6	1	24	A & W: 1y 5m
1118	M	54	AML	HLA-id Sib	PBSCT	Bu+Cy		CsA+MTX	I	Hem cystit	MM	3	0.8	1	48	A & W: 1y
1126	M	1	SCID	MMUD m	CB	Bu+Cy	x	CsA+Pred	I	Engraftm	Haplo	3	1.0	1	0	†d 255: Bronchiolitis (RSV+Aspergillus) & subdural hematoma
1129	F	64	MPD	HLA-id Sib	PBSCT	RIC: Flu+Bu	x	CsA+MTX	II	GvHD	MM	3+4	1.7	1	103	†d 223: Pneumonia & pulmonary bleeding
1143	F	1	SCID	MUD	PBSCT	Mel+Flu	x	CsA+MTX	II	Engraftm	Haplo	3	1.0	1	0	A & W: 7m
1152	M	59	Lymphoma	MUD	PBSCT	Flu+Cy+TBI	x	CsA+MTX	–	Hem cystit	MM	3	1.7	1	14	†d 170: GI-bleeding & CNS-septicemia

*UPN: unique patient number; M: male; F: female; 1: in years; ALL: acute lymphocytic leukemia; AML: acute myelogenous leukemia; AA: aplastic anemia; SCID: severe combined immunodeficiency; MPD, myeloproliferative disorder; thalassemia: thalassemia major; MUD: matched unrelated donor; HLA-id Sib: HLA-identical sibling donor; MMUD s: HLA subtype mismatched unrelated donor; MMUD m: major HLA mismatched unrelated donor; PBSCT: peripheral blood stem cell transplantation; BM: bone marrow; CB: coord blood; FTBI: fractionated total body irradiation; Cy: cyclophosphamide; Bu: busluphan; RIC: reduced intensity conditioning; Flu: fludarabine; Mel: melphalan; Anti-T-cell, anti-T-cell prophylaxis; GvHD prophyl: graft-versus-host-disease prophylaxis; CsA: cyclosporine A; MTX: methotrexate; MMF: mycofenolat mofetil; Tacro: tacrolimus; Siro: sirolimus; GvHD: graft-versus-host disease; Engraftm: engraftment; hem cystit: hemorrhagic cystitis; Haplo: haploidentical; Sib: sibling; MM, HLA mismatched; Ninf: number of infusions; 2: from HSCT; A & W, alive and well; †: deceased; d:day post-transplantation; MOF, multi organ failure; y: years; m, months; RSV: respiratory syncytial virus; GI: gastrointestinal; CNS: coagulase negative staphylococci. *Patient 924 received two infusions. The second consisted of 1×10^6/kg cells in passage 3.*

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Table 3. Frequency of antibodies against MSC detected by flow cytometric cross match / frequency of antibodies against fetal calf serum detected by enzyme-linked immunosorbent assay in 12 patients post-MSC transplantation.

UPN	Pre-MSC tx	+ 1-2 w	+ 1 m	+ 3 m	+ 6 m	+ 12 m
924	– /–	0	– /–	0	– /–	0
994	0	– /–	0	– /–	+ / –	– /–
995	– /–	0	0	– /–	– /–	– /–
1020	0	– /–	0	– /–	– /–	+ /–
1044	– /–	– /–	0	– /–	– /–	0
1047	+ /–	0	–/ –	0	– /–	– /–
1098	–/ +	– / +	– / +	– / +	– / +	+ / +
1118	+ / -	– /–	– /–	– /–	0	0
1126	– /–	– /–	– /–	– /–	0	0
1129	– / +	– / +	– /–	– /–	0	0
1143	–/ +	– / +	–/ +	– /–	– /–	0
1152	–/–	– / +	–/ +	– /–	0	0
npos:	2 / 3	0 / 4	0 / 3	0 / 1	1 / 1	2 / 1
nneg:	8 / 7	9 / 5	8 / 5	10 / 9	7 / 7	3 /4
ntot:	10	9	8	10	8	5

UPN: unique patient number; MSC tx: mesenchymal stromal cell transplantation; w: weeks; m: months; 0: sample missing; n: number; pos: positive; neg: negative; tot: total.

two cases: patient 1047, a 7-year old boy with SAA, and patient 1118, a 54-year old male with acute myelogenous leukemia (Table 3 and *Figure 1A, online supplement*). Between 1 to 2 weeks and 3 months post-transplantation none of the FCXM using MSC was positive. After 6 months patient 994, who had received MSC derived from a HLA haploidentical sibling, was positive but all others negative (*n*=7). Twelve months post-transplantation, patient 1098 treated with HLA mismatched MSC and patient 1020 treated with HLA matched MSC (*n*=2) showed positive reactions; the remaining patients (*n*=3) were negative (Table 3 and *Figure 1A, online supplement*).

Antibovine antibodies are common and found in the recipients

Five of the 14 healthy individuals in the reference group had optical density values of more than 1.0 in the FCS ELISA, similar to the positive control. A majority of the individuals in the reference group had values between 0.6 and 0.8 (*data not shown*). Two of the 11 recipient sera taken before MSC transplantation were positive for antibodies against FCS. After transplantation, the frequency of anti-FCS antibodies decreased and only one patient was positive at 12 months. This patient, 1098, was positive at all points measured (Table 3).

No expression of blood group antigens

We investigated blood group antigen expression on MSC by flow cytometry. Levels detected on PBL were comparable to those previously reported,[39] confirming the accuracy of the assay. MSC derived from three blood group A and three blood group B donors were tested. None of the examined MSC expressed blood group antigens (*Figure 2A, online supplement*). The cells were also investigated by immunoflu-

orescence. Erythrocytes expressed high levels, PBL expressed low levels, and MSC did not express blood group antigens at all (*Figure 2B, online supplement*).

The antibodies are directed against FCS

To determine the specificity of the antibodies that caused the positive FCXM in patients 994, 1020 and 1098, further FCXM were performed. First, recipient sera were incubated with MSC from donors with a different HLA type and a positive reaction was still detected. Thereafter, the sera were incubated with both MSC donor-derived PBL and PBL from other donors and FCXM became negative. Furthermore, no anti-HLA antibodies were detected in the Flow-PRA® assay *(Figure 1B, online supplement*) confirming that immunization had not occurred after transplantation with mismatched MSC.

The accuracy of the absorption of antibodies against blood group antigens was confirmed, since serum from the blood group A donor could not agglutinate erythrocytes from the blood group B donor after absorption with the same erythrocytes, and *vice versa*. The sera from the patients above were absorbed with a mixture of erythrocytes from both blood group A and B donors and then retested in the FCXM assay. The sera were still positive *(Figure 1B, online supplement*).

To test whether the antibodies were directed against the FCS, we absorbed the sera with FCS grains. Using ELISA coated with antibodies against human IgG and pooled human IgG antibodies as the sample, we confirmed that no more than a few picograms of IgG were lost during the absorption. After absorption, the reactivity in the FCS ELISA was decreased. Patients' sera were absorbed and re-assayed. The FCXM result became negative *(Figure 1B, online supplement*). To confirm the negative FCXM after absorption using FCS grains, we performed FCXM using MSC cultured in medium supplemented with human AB serum, instead of FCS, and the late positive sera were negative in this test. We used ELISA to investigate the IgG subclass of the anti-FCS antibodies. Patients and controls exhibited the same pattern: $IgG_1 > IgG_2 \approx IgG_3$. Low levels of all IgG subclasses against FCS were seen following absorption with FCS grains.

Discussion

In the present study, we aimed to investigate whether MSC transplantation in HSCT patients is complicated by humoral immunity against the cells. It has been suggested that the cells may be immunoprivileged, nevertheless this must be elucidated before entering clinical treatment protocols. Recent studies have primarily investigated T lymphocyte-mediated responses directed against MSC.[40,41] Humoral alloimmune mechanisms are of clinical importance in solid organ transplantation since both antibody formation against HLA and minor histocompatibility complexes may give rise to graft rejection.[42,43] In HSCT, graft failure has been associated with prior transfusions, mainly seen in patients with SAA, and this was explained by sensitization to non-HLA antigens such as minor histocompatibility complexes and blood group antigens.[26,27] Furthermore, antibodies from some SAA patients have been shown to react with monocytes, but not with endothelial cells, causing rejection.[29] Thus, even heavily immunosuppressed individuals can have antibodies that cause humoral immune rejection of stem cell grafts. Twelve patients undergoing HSCT were given MSC to promote engraftment or to treat hemorrhagic cystitis or GvHD. Seven patients received MSC to treat sequelae of the HSCT procedure. The survival was 58%, which may be considered a successful outcome. No immediate or delayed adverse events of MSC infusion were seen. The most common cause of death was infection.

Following HSCT, the serum immunoglobulin levels decrease before B lymphocytes are restored.[44] Still, substantial levels of immunoglobulins and plasma cells of recipient origin are present for up to 1 year post-HSCT.[45,46] Furthermore, peripheral blood HSCT is associated with increased levels of alloantibodies.[47] Thus, in spite of the induced immunodeficiency, HSCT patients may have antibodies that could interfere with MSC grafts. *In vitro* studies showed that MSC stimulate IgG production in blood and spleen cells.[48] Our patients exhibited expected levels of immunoglobulins throughout the study. No mono- or polyclonality in association with the MSC transplantation was detected. This probably indicates that no vast clonal expansion of antibody producing cells occurred as a response to the MSC, but that pre-formed antibodies may have been present. Blood group antigens are regarded as non-HLA antigens, which may give rise to alloantibodies that can cause rejection.[30] Until now it has not been known whether MSC express blood group antigens. Low levels were detected on PBL, but not on MSC. Thus, MSC cannot be rejected by an immune mechanism involving antibodies against blood group antigens if infused into an AB0-mismatched individual.

Recipient sera were investigated with FCXM, which is an easy and reliable method frequently used in solid organ transplantation to screen for antibodies to avoid hyperacute rejection.[49] Pre-transplant sera from two patients were positive in the FCXM. One of them was a multitransfused pediatric patient with SAA and must consequently be regarded as sensitized to both HLA, and non-HLA antigens. The other patient was a middle-aged male with acute myelogenous leukemia. Surprisingly, neither of these patients was positive in follow-up FCXM after transplantation. Three patients developed late positivity. Two of these patients received MSC to promote hematopoietic engraftment and one as treatment for hemorrhagic cystitis. Rapid engraftment and reversal of hemorrhagic cystitis were seen, respectively. All patients who were found to be positive in the FCXM are currently alive and well. Late positive sera were investigated further. The MSC were cultured in medium supplemented with FCS. It has been reported that MSC both internalize and have FCS components present on the cell surface. Rat MSC grown in FCS-

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supplemented medium gave rise to a humoral response after recurrent administrations in immunocompetent animals.[50] Furthermore, a pediatric patient given MSC as treatment for osteogenesis imperfecta exhibited anti-FCS antibodies post-transplantation.[51] We evaluated our patients and control subjects for anti-FCS antibodies. Surprisingly, almost all healthy individuals demonstrated varying levels of anti-FCS antibodies. Our patients exhibited a distribution of such antibodies comparable to that in the normal population. No tendency towards an overall or late presence of anti-FCS antibodies was seen in the MSC-transplanted patients. To determine the specificity of the antibodies causing the late positivity in the FCXM further investigations were undertaken (*Figure 3, online supplement*). The sera were found to be positive not only to the transplanted cells, but also to other MSC. FCXM using MSC donor-derived or other PBL were negative. This implies that the origin of the antibodies was not of a broader alloresponse and that the patients were not immunized against their donors. Furthermore, no anti-HLA antibodies could be detected in the investigated sera, indicating that MSC transplantation in HSCT recipients does not give rise to humoral alloresponses. However, late positivity against donor-derived and other MSC occurred in three patients. This was not caused by antibodies against blood group antigens, since sera were positive after absorption with erythrocytes. After absorption with FCS grains, the sera became negative, indicating that the antibodies were directed towards FCS. The absorption was specific since only small amounts of IgG were lost. Loss of non-specific antibodies would have resulted in a greater reduction in total IgG. Absorbed sera had no anti-FCS antibodies detectable by ELISA. The negative FCXM using MSC cultured in medium with human AB sera indicated that the antibodies were directed against FCS. These antibodies may be directed to different FCS antigens, since in only one instance was the FCXM positive concomitantly with the detection of anti-FCS antibodies by ELISA.

The IgG subclasses of the anti-FCS antibodies were IgG_1, which was the most abundant, and IgG_3. This pattern will give rise to opsonization, antibody-dependent cell mediated cytotoxicity (ADCC) and complement activation.[52] Our own data (*unpublished*) indicate that MSC are not sensitive to lysis by complement proteins. Hematopoietic stem cells may be rejected by NK-cell mediated ADCC,[24] but MSC are not sensitive to NK-cell mediated lysis.[12] Moreover, anti-bovine antibodies in humans are considered a natural occurrence.[53-55] Are these really functional antibodies? Almost all humans have these antibodies and immune reactions could be expected to be more frequent if they played an important role. All our patients with FCS antibodies had the intended effect from the MSC treatment with no infusion toxicity. Hence, the late positivity could be interpreted as restoration of immune function within the recipient. On the other hand, concomitantly raised titers of anti-FCS antibodies and absence of MSC engraftment was shown in one of six non-immunosuppressed children after two MSC infusions.[51] This implies that some immunocompetent individuals may have antibody formation against MSC, although our results did not confirm this in immunosuppressed HSCT patients. Studies exploring MSC engraftment may elucidate whether MSC are immune rejected or not.

In summary, we have demonstrated that HSCT patients have anti-FCS antibodies. These antibodies are also found in the normal population. Whether such antibodies are important for interactions with MSC *in vivo* remains to be proven. We could not demonstrate alloantibodies as a result of sensitization to the MSC. Furthermore, we have shown, for the first time, that MSC do not express blood group antigens.

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Authors' contributions

MS designed the study in collaboration with the other authors and collected the clinical data. Cultures and characterization of MSC were performed by BS. Flow cytometry was conducted by MS and BS. Absorption and immunofluorescence studies were performed by MS with assistance from SN. The FCS ELISA, which was originally set up by CG, and other ELISA assays, were carried out by MS assisted by SN. Results and the manuscript draft were completed by MS. KLB and OR were in charge of the study design, the analysis of results and preparation of the final manuscript. All co-authors actively participated throughout the complete process of this manuscript.

Conflict of Interest

The authors reported no potential conflicts of interest.

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