Platelet-lysate-Expanded Mesenchymal Stromal Cells as a Salvage Therapy for Severe Resistant Graft-versus-Host Disease in a Pediatric Population

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Despite advances in graft-versus-host-disease (GVHD) treatment, it is estimated that overall survival (OS) at 2 years for hematopoietic cell transplantation (HCT) recipients who experience steroid-resistant GVHD is 10%. Among recent therapeutic approaches for GVHD treatment, mesenchymal stromal cells (MSCs) hold a key position. We describe a multicenter experience of 11 pediatric patients diagnosed with acute or chronic GVHD (aGVHD, cGVHD) treated for compassionate use with GMP-grade unrelated HLA-disparate donors' bone marrow-derived MSCs, expanded in platelet-lysate (PL)-containing medium. Eleven patients (aged 4-15 years) received intravenous (i.v.) MSCs for aGVHD or cGVHD, which was resistant to multiple lines of immunosuppression. The median dose was 1.2×10^6/kg (range: 0.7-3.7×10^6/kg). No acute side effects were observed, and no late side effects were reported at a median follow-up of 8 months (range: 4-18 months). Overall response was obtained in 71.4% of patients, with complete response in 23.8% of cases. None of our patients presented GVHD progression upon MSC administration, but 4 patients presented GVHD recurrence 2 to 5 months after infusion. Two patients developed chronic limited GVHD. This study underlines the safety of PL-expanded MSC use in children. MSC efficacy seems to be greater in aGVHD than in cGVHD, even after failure of multiple lines of immunosuppression.

INTRODUCTION

Graft-versus-host disease (GVHD) is one of the most severe complications in the setting of allogeneic hematopoietic stem cell transplantation (HSCT). Despite the improvement in HLA matching technique, about 50% of HSCT recipients experience acute GVHD (aGVHD), and only 30% to 50% of them benefit from first-line steroid treatment [1]. Second-line treatment for steroid-resistant GVHD is not univocally established, and varies according to the recipient conditions, involved organs, and GVHD stage. Any immunosuppressive strategy is burdened by increased infection rate and toxicity, which in turn, augments treatment-related morbidity and treatment-related mortality (TRM). Today it is estimated that overall survival (OS) at 2 years for HSCT recipients who experience steroid-resistant GVHD is 10% [2].

Among the most recent therapeutic approaches for GVHD, mesenchymal stromal cells (MSCs) hold a key position. These are multipotent progenitor cells that present extensive immunomodulatory properties. They inhibit T and B cell response by arresting them in the G0/G1 phase of the cell cycle, prevent monocytes from their antigen-presenting function, and increase regulatory T cell expansion. Of importance,

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MSCs are known to escape immune rejection, thus allowing their use in an HLA-mismatched setting [3,4].

The first documentation of MSC clinical efficacy for GVHD treatment was published in 2004 [5]. Since then, various trials reported nonhomogeneous results. Differences in GVHD features and treatment schedules caused complete MSC response to vary from 15% to 55% of treated patients [6-9]. The largest cohort of patients reported so far was described in 2008 by Le Blanc et al. [10]. Fifty-five patients with grade II to IV aGVHD were treated with MSC infusions of either related or unrelated origin. Thirty patients had a complete response, and 9 showed variable grades of GVHD improvement. Complete responders had a lower 1-year TRM and a higher 2-year OS than patients with partial or no response to MSCs.

Standard conditions for MSC expansion include the presence of serum, in most instances fetal bovine serum (FBS). Regulatory guidelines, recommending avoiding the use of animal additives, have reinforced a search for possible alternatives. For this reason, platelet-lysate (PL) is currently used in many centers for MSC expansion for clinical use. A recent article from von Bonin and colleagues [11] reported 15% of complete response and 45% of partial response for GVHD treatment with the use of bone marrow (BM)-derived MSCs expanded in PL-containing medium from unrelated HLA-disparate donors [11].

Recent controversial observations come from the company Osiris (http://www.nature.com/news/2009/090909/full/news.2009.894.html), declaring the failure of the Prochymal product (http://investor.osiris.com/releasedetail.cfm?ReleaseID=407404), which performed no more effectively than a placebo in GVHD treatment in late-stage clinical trials. The same product was, on the other hand, more efficacious in treating de novo aGVHD in adult patients with an initial response rate of 94% [12]. Moreover, recent data point out that multiple infusions of prochymal (biweekly infusions of 2×10^6 cells/kg for 4 weeks, with an additional 4 weekly infusions after day 28 in patients with a partial response) were able to produce a 64% response rate in patients with otherwise refractory severe GVHD [13].

Quite contentious opinions are therefore present today in the literature concerning MSC efficacy. In the present study, we describe a multicenter experience of 11 pediatric patients diagnosed with resistant aGVHD or chronic GVHD (cGVHD) treated with PL-expanded MSCs for compassionate use on top of multiple lines of immunosuppressant. All MSC were expanded in 2 authorized good manufacturing practice (GMP) facilities (Laboratory of Cell therapy "S. Verri," "Ospedale San Gerardo," Monza, Italy, and Laboratory "G. Lanzani," "Ospedali Riuniti di Bergamo," Bergamo, Italy), exclusively by means of GMP-grade qualified reagents and according to GMP indications. Our observations in a small set of

pediatric patients seem to confirm a positive effect of PL-cultured MSCs on various patterns of GVHD, particularly those with acute skin involvement.

METHODS

Patients

Between May 2008, and June 2009, 11 patients aged 4 to 15 years received MSC intravenous infusions for aGVHD grade I to IV or cGVHD, which had proved resistant to multiple lines of immunosuppressants. All patients had developed GVHD after HSCT from unrelated or related donor, which had been performed for malignant disease in 8 cases and nonmalignant diseases in 3 cases. Conditioning regimens and GVHD prophylaxis are described in details in Table 1.

GVHD was diagnosed upon clinical evidence in all patients and confirmed by histology in 1 case (patient number 8). aGVHD and cGVHD were graded according to internationally accepted criteria by physicians at individual centers [14].

All patients received steroid as first-line GVHD treatment. Further lines of treatment depended on patients and GVHD characteristics (Table 2a and Table 2b). All patients had failed at least 2 lines of treatment before receiving MSCs. All patients continued to receive ongoing immunosuppressant while receiving MSCs. Patients 3, 4, and 7 presented skin aGVHD stage 2 (grade I), which had proven resistant to 3 lines of treatment; they were therefore considered eligible to MSC treatment.

At the time the first MSC infusion was performed, 4 patients had aGVHD involving skin only (overall grade I or II), 2 had gut aGVHD (overall grade IV). Two patients suffered aGVHD grade II to IV involving skin and gut. Three other patients had cGVHD involving skin and mucosa and/or liver, overall grade I-III (Table 2a and 2b).

Seven patients were treated at "Ospedale San Gerardo," Monza (Italy), 1 patient was treated at "Istituto Giannina Gaslini," Genova (Italy), 1 at "Centro Trapianti Cellule Staminali Eritropoietiche Ospedale Microcitemico," Cagliari (Italy), 1 at "Divisione di Ematologia, Università Cattolica Sacro Cuore," Roma (Italy), and 1 at "Istituto Scientifico San Raffaele," Milano (Italy). Patients were treated for compassionate use. MSC infusion was approved by the local Ethical Committee, and the competent National Authorities were notified. Donors and patients, or their legal guardians, signed written informed consent.

Definitions

Patients were evaluated for response at 7 and 28 days after MSC infusion. Because some patients

Table 1. Patients' Characteristics

Pts	Age (Years)	Sex	Dx	Type of Transplantation Source HLA Matching	Conditioning Regimen	GVHD Prophylaxis
1	5	Female	ALL 2nd CR	Related Marrow 8/10	TBI + Cy + VP16	CsA + MTX
2	7	Male	ALL 2nd CR	Unrelated Marrow 9/10	TBI+ Cy +VP16	CsA + ATG + MTX
3	11	Male	ALL 2nd CR	Unrelated Marrow 10/10	TBI + VP16	CsA + ATG + MTX
4	9	Male	ALL 1st CR	Unrelated Marrow 8/10	TBI+VP16	CsA + ATG + MTX
5	15	Female	ALL 2nd CR	Unrelated Peripheral 9/10	TBI + VP16	CsA + ATG + MTX
6	12	Male	ALL 2nd CR	Unrelated Marrow 8/10	TBI + VP16	CsA + ATG + MTX
7	8	Male	ALL 2nd CR	Unrelated Marrow 9/10	TBI + VP16	CsA + ATG + MTX
8	10	Male	Fanconi anemia	Unrelated Marrow 9/10	Flu + Cy	CsA + ATG + MTX
9	14	Male	AML	Unrelated Cord Blood 4/6	Flu + Bu + TT	CsA + MMF + ATG
10	4	Male	Thalassemia	Unrelated Marrow 9/10	Bu + Cy	CsA + PDN + MMF
11	11	Female	Thalassemia	Related Marrow 10/10	Bu + Cy	CsA + PDN + MTX

ALL, acute limphoblastic leukemia; CR, complete remission; AML, acute myelogenous leukemia; Bu, busulphan; Cy, cyclophosphamide; TT, thiotepa; Flu, fludarabin; TBI, total body irradiation; VP16, vepeside; CsA, cyclosporine A; PDN, prednison; MTX, methotrexate; MMF, mofetil mycophenolate; ATG, antytomocyte-globulin; GVHD, graft-versus-host disease.

presented multiple GVHD flares, response rate was calculated separately considering each single GVHD episode. We defined a response to be complete as disappearance of all signs and symptoms of GVHD; partial response as GVHD improvement of at least 1 stage in single organ scoring, or 1 grade in overall GVHD scoring, if more than 1 organ was involved. Stable disease was defined as no change in GVHD staging and grading. Progressive disease was defined as worsening of GVHD, intended as either involvement of new organs or worsening of the previously involved organs. Patients were judged to have responded if they had either complete or partial response. Responding patients were defined as temporary responders if they showed any signs of GVHD reduction at day +7 after MSC infusion but reflared earlier than 28 days after MSC therapy. Definitive responders were patients with stable response 28 days after MSC infusion.

MSC Preparation for Clinical Use

MSCs were isolated from unrelated HLA-mismatched BM donors, after having obtained written informed consent from the donors. Clinical-grade mesenchymal stromal cells were generated under GMP conditions, as we have recently extensively described [15]. Briefly, total nucleated cells were isolated from the washouts of sealed bone marrow collection bags and filters, and cells were plated, without further separation, at 800×10^3 cells/cm^2 in complete medium. Complete medium consists of α-modified-Eagle medium (α-MEM, Invitrogen, Milano, Italy) supplemented with 5% freshly thawed PL and 2 mM L-glutamine (LiStarFish, Milano, Italy). MSC cultures were detached with triple Select (Invitrogen) when the subconfluence was reached and cells were replated at 100 to 200 cells/cm^2 up to the final detachment and freezing. The final cell products have been subject to all quality controls required for clinical use. Release criteria included: lack of detectable microbial contamination (aerobic or anaerobic bacteria, fungi, and mycoplasma) according to European pharmacopoeia, cell viability ≥90%, endotoxin levels in the final product ≤5 EU/kg, cell characterization with high expression (≥70%) of CD73, CD90, and CD105 and lack (≤10%) of CD14, CD34, CD45, normal karyotype

Table 2a. Patients and Graft-versus-Host Disease (GVHD) Features Prior and After Mesenchymal Stromal Cell (MSC) Infusion

Pat	GVHD Grading at MSC Infusion	GVHD Treatment at MSC Infusion	No. of MSC Infusion/Day from HCT	Infused MSC (Dose/kg)	Response After MSC Infusion	Tapering of Immunosuppression	GVHD Reflaring	Follow-up
1A	Chronic overlap syndrome (skin and mucosae grade III)	Steroid+ MMF+ECP+CsA+ azatioprine	1/ day+ 148	1 x 10^6/kg	Partial response temporary Skin and mucosae grade I	Yes, steroid 2 days and CsA 16 days after MSC infusion	Yes, gut GVHD grade II 70 days after MSC infusion	Alive with chronic skin and mucosae GVHD grade II
2A	Chronic overlap syndrome (skin and mucosae grade II)	Steroid+ MMF+ECP+ etanercept+ CsA	1/ day +210	1 x 10^6/kg	Complete response definitive No GVHD	Yes, stop steroid 30 days, MMF tapering 50 days and Etanercept 65 days after MSC infusion	Re-flair of gut, skin, and liver GVHD grade II 95 days after MSC infusion	Alive with skin chronic GVHD grade I
2B	Chronic mucosae and liver grade III	Steroid+ FK506+ budesonide+ etanercept	1/ day +300	1.4 x 10^6/kg	Partial response temporary Chronic mucosae and liver grade II	Yes, stop budesonide and steroid 40 days and etanercept 21 days after MSC infusion	None	
3A	Acute skin grade I	Steroid+ MMF+CsA+ etanercept	1/ day + 62	1 x 10^6/kg	Complete response definitive No GVHD	Yes, steroid stopped 17 days after MSC infusion	None	Alive, no GVHD
4A	Acute skin grade I	Steroid+MMF+CsA+ etanercept	1/ day +34	0.7 x 10^6/kg	No response Skin GVHD grade I	Stopped Etanercept, but for concomitant infection 18 days after MSC infusion	GVHD persistance	Alive, no GVHD
4B	Chronic skin and liver grade III	Steroid+ CsA+MMF+ etanercept	4/ day +150 day +157 day +164 day +169	0.7 x 10^6/kg 0.7 x 10^6/kg 0.7 x 10^6/kg 0.7 x 10^6/kg	Partial response temporary Skin and liver grade II	Steroid slow and partial tapering	GVHD smooth recovery after sirolimus introduction	
5A	Acute skin grade II	Steroid+ etanercept+ CsA	1/ day +43	1.2 x 10^6/kg	Complete response definitive No GVHD	CsA stopped 18 days after MSC infusion, steroid tapering	New GVHD reflair 46 days after MSC infusion, in chronic form	Alive, no GVHD
5B	Chronic skin GVHD grade I	Steroid	1/ day + 94	1.2 x 10^6/kg	No response Skin GVHD grade I	None	Reintroduction of steroid, MMF and etanercept. Slow complete response	

GVHD indicates graft-versus-host disease; CsA, cyclosporine A; MMF, mycophenolate mofetil; ECP, extracorporeal photopheresis.

Table 2b. Patients and Graft-versus-Host Disease (GVHD) Features Prior and After Mesenchymal Stromal Cells (MSC) Infusion

	GVHD	Treatment	MSC infusion / day	Dose	Response	Steroid tapering	Persistant GvHD till ECP introduction	Outcome
6A	Chronic overlap (Skin and mucosae grade II)	Steroid + CSA + MMF + Etanercept	I/ day +68	1.2 × 10⁶/kg	No response Skin and mucosae grade II	None	Persistant GvHD till ECP introduction	Alive, no GVHD
7A	Acute skin grade I	Steroid + CsA + etanercept	2/ day +90 day +95	1.2 × 10⁶/kg 1.2 × 10⁶/kg	Complete response definitive No GvHD	Steroid tapering	None	Alive, no GVHD
8A	Acute (skin II gut grade IV)	Steroid + FK506 + etanercept + ECP	2/ day +98 day +119	1.6 × 10⁶/kg 1.6 × 10⁶/kg	Partial response, temporary Skin and gut grade II	Slow steroid tapering for 4 months	GVHD reflair 5 months after MSC infusion, refractory to all attempts (MMF, ECP, Glivec).	Death on day +329 from HCT for progressive gut GVHD and pulmonary Aspergillosis
9A	Acute (skin and gut grade IV)	Steroid + ATG + ECP + infliximab	2/ day+77 day +111	1 × 10⁶/kg 1 × 10⁶/kg	No response Skin and gut grade IV	Steroid tapering for infectious complications	Persistent GVHD	Death on day +126 from HCT because of sepsis
10A	Acute gut grade IV	Steroid + CsA + MMF + ECP + etanercept	2/ day +66 day +77	1.2 × 10⁶/kg 1.2 × 10⁶/kg	Partial response temporary Grade III after first infusion	Steroid tapering, interruption of ECP and Etanercept	Yes, 20 days after second MSC infusion. Slow, complete response	Alive, no GVHD
11A	Acute gut grade IV	Steroid + MMF + FK506 + etanercept + ATG	I/ day +43	3.7 × 10⁶/kg	No response	None	None	Death on day +56 from HCT because of gut GVHD (recurrent intestinal subocclusions despite ileostomy, gut bleeding and interstitial-alveolar pneumonia

CsA indicates cyclosporine A; MMF, mofetil mycophenolate; ATG, antimocyte globulin; ECP, extracorporeal-photopheresis; GVHD, graft-versus-host disease.
Patients were labeled as A in case they received single or multiple MSC infusions for an unique GVHD flair or B in case they received single or multiple MSC infusions for a further flaring of GVHD.

and inability to grow without anchorage in a semisolid fluid. Cell lots were cryopreserved and thawed right before patient intravenous infusion.

Immune Monitoring: Sample Collection and Cytokine Plasma Level Detection

After having obtained an informed consent, blood samples were collected from 4 GVHD patients infused with MSCs. Plasma samples were obtained by centrifugation within 24 hours from blood collection. Tumor necrosis factor (TNF)-α and inteferon (IFN)-γ plasma levels were assessed using commercial ELISA kits (eBioscience, San Diego, CA, USA, detection limit = 4 pg/mL).

RESULTS

Three hundred million mononucleated cells were isolated from the washouts of the BM collection bags and filters of a single donor. Mononucleated cells, plated at 800×10^3 cells/cm² in complete medium, generated in 2 weeks a subconfluent layer (80% confluence) of 15×10^6 cells with a fibroblastic-like shape. Cells were frozen in multiple vials and, after all the quality controls were adequately performed and checked, cells were thawed and plated in complete medium at 100 to 200 cell/cm² for a further 2-week expansion in 5 different productions. Finally, overall, we obtained 1780×10^9 cells frozen in 70 vials of around 25×10^6 cells each vial, ready to be infused. For more details on cell expansion methods and quality control methods, see Capelli et al. [15].

Twenty-one MSC infusions were given to 11 patients; 4 patients received a single infusion, whereas 7 had 2 to 5 infusions. The median dose was 1.2×10^6/kg (range: 0.7-3.7×10^6/kg). No patient had acute side effects either during or after infusion, and none had late side effects, so far, at a median follow-up of 8 months (range: 4-18 months). Median time from HSCT to MSC treatment was 95 days (range: 34-300 days).

Fifteen of 21 MSC infusions induced either a complete or a partial response in 8 of 11 patients. Moreover, all responding patients were able to eventually taper ongoing immunosuppressive treatment after MSC infusion. Details on patients' treatment and response to MSCs are resumed in Table 2a and 2b. None of our patient presented GVHD progression upon MSC administration, but patients 1, 2, 5, and 8 presented a GVHD recurrence 2 to 5 months after MSC infusion. Overall response in our patients was 71.4%, with complete response in 23.8% and partial response in 47.6%. Eight of 11 treated patients are alive and in complete remission (CR) from their hematologic disease with a median follow-up of 10 months from HSCT (range: 6 to 24 months) and of 8 months
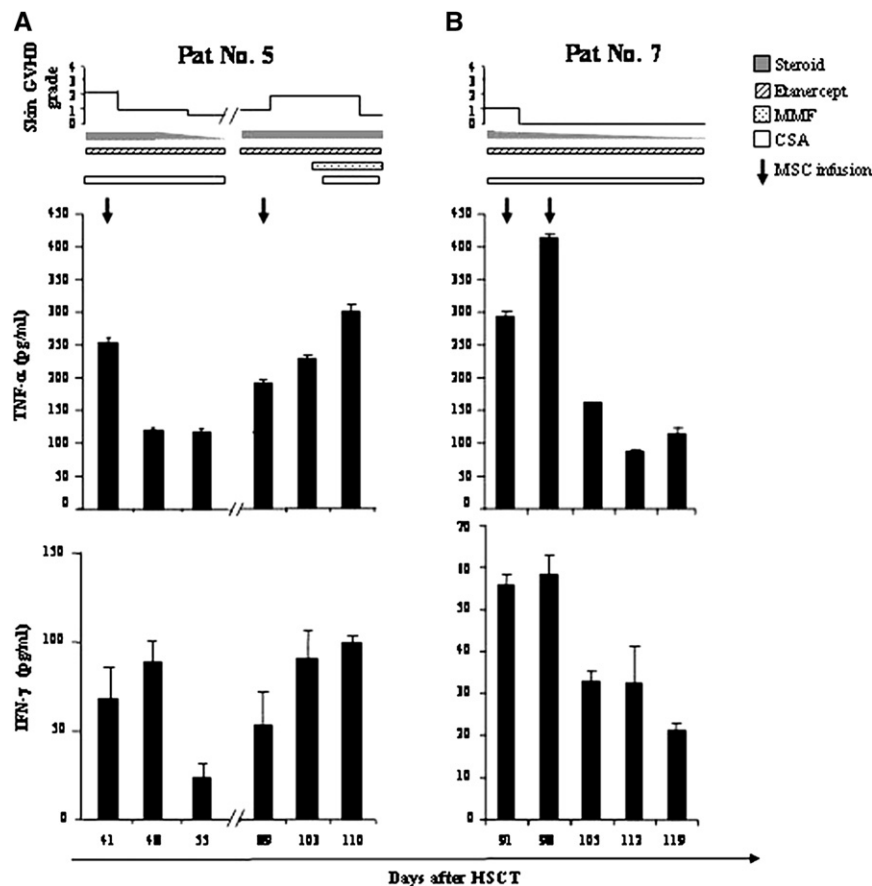
Figure 1. Time-based monitoring of TNF-α and IFN-γ levels in patients treated with mesenchymal stromal cells (MSCs) (A, B): plasma levels of TNF-α and IFN-γ in patients with graft-versus-host-disease (GVHD) were monitored at different time points after MSC infusion. Monitoring of 2 patients is shown. The GVHD of those patients was limited to the skin and GVHD score is expressed as overall grading.

from MSC infusion (range: 4-18 months). Patient number 8 died from GVHD progression and lung aspergillosis on day +329 after HSCT, patient number 9 died of sepsis on day +126 after HSCT, and patient number 11 died of GVHD progression and lung infection on day +56 after HSCT. Currently, 5 patients are free from GVHD (2 of them with skin cGVHD did not benefit from MSC, but later responded to further treatment), whereas patient number 1 has developed a skin and mucosal vGVHD (grade II overall), which still needs treatment with oral steroids and MMF. Patient 2 has developed a cutaneous cGVHD (grade I overall), which still requires FK506 oral and topic treatment. Patients 3, 4, 5, 6, 7, and 10 are alive and well, without any ongoing immunosuppressive treatment.

As far as concerns immune monitoring of MSC treatment, patient 5, who was suffering from a grade II skin aGVHD, had a complete definitive response to the first MSC infusion, as assessed by clinical observations (Figure 1A). TNF-α levels, which resulted elevated before MSC infusion (mean concentration = 253 pg/mL), persistently decreased (mean decrease = 2.3-fold) starting 24 hours after treatment. IFN-γ levels diminished 2.9-fold if compared to the preinfu-

sion values. Interestingly, upon GVHD reflair in chronic form on day +94 after HSCT, the patient received a further MSC infusion. cGVHD did not respond to MSC treatment and TNF-α and IFN-γ plasma levels remained stable or even increased after the infusion, although 4 lines of treatment were added. After a transient decrease, TNF-α levels showed a progressive increase when compared to levels at GVHD resolution on day 55. Accordingly, IFN-γ levels showed a stable increase (4.4 times) compared to levels observed at disease resolution. Patient 7 (Figure 1B) was treated in the presence of acute skin grade I GVHD, that was persistent after introduction of i.v. steroid. He presented a complete definitive response after receiving 2 MSC infusions. After MSC administration, TNF-α and IFN-γ plasma levels remained stable and finally decreased, allowing a simultaneous steroid tapering.

DISCUSSION

The present study underlines the feasibility of MSC administration on top of multiple lines of immunosuppressive therapy in pediatric patients with

refractory grade I to IV GVHD. This study addresses the particular issue of GVHD treatment in children. Although GVHD is less common in children than adults, long-term side effects of prolonged immunosuppressive treatment is a major issue in the pediatric setting. It is therefore of the utmost importance to propose a treatment strategy that may be able to reduce the burden of conventional immunosuppression. In our study, MSC administration proved its efficacy in more than half of the treated patients and allowed the tapering of other treatment lines.

MSC infusion caused a complete GVHD remission in 23.8% of our patients and a partial response in 47.6% of cases. Other groups reported a higher rate of CR (Le Blanc et al. [5] 55% complete response, 16% partial response). Isolated skin was the mostly involved organ and mainly responded (at various degree) in the aGVHD phase (3 of 4 patients). Similar results in the treatment of skin GVHD were reported by von Bonin and colleagues [11] when using PL-expanded MSCs. This is relevant because other studies, including a phase III randomized one [16], declared the inefficacy of MSC for skin GVHD treatment. Although our and von Bonin et al. studies number of treated patients is limited, it might be the case that PL-expanded MSCs harbor different trafficking properties, which allow a better chemotaxis toward the skin flogistic environment. This hypothesis needs further investigations to acquire scientific robustness. On the other hand, the same prochymal product, which was declared ineffective for skin GVHD treatment in a phase III randomized study [16], obtained a 47% rate of response in skin GVHD in a pediatric cohort of patients [13]. Younger patients may be, therefore, more prone to respond to this kind of treatment, either for peculiarities in their immunologic setting or for higher infused MSC dosages.

In our hands MSC did not prove their efficacy in isolated skin cGVHD with hyperchromic and lichenoid characteristics (partial response in 2 and no response in other 2 patients). In our limited cohort of patients, which was treated with delay and after other lines of immunosuppression, GVHD characteristics (aGVHD versus cGVHD) may have represented the first issue in influencing the patient response. For instance, patient number 5 who received 2 treatments with MSCs for 2 different GVHD flairs (the first with an aGVHD pattern, the second with a cGVHD pattern) responded to the first, but not to the second infusion, thus supporting this hypothesis.

These data are in line with ongoing studies underlining the different mechanisms of action of MSC in the acute and chronic phases of GVHD. It is probable that MSCs need to be licensed by an inflammatory environment, which is much more common in the aGVHD setting than in the cGVHD setting. aGVHD is characterized by inflammatory responses, during which the production of several pro-inflammatory cytokines (eg, IFN-γ, IL-1, TNF-α) enhances the ability of donor T lymphocytes to attack host tissues and to produce more pro-inflammatory cytokines. On the contrary, cGVHD is associated with a polarized TH-2 state, which is consistent with the clinical manifestations of cGVHD in both human and animal models (elevated IL-5 with eosinophilia, IL-4 with scleroderma), and which may weaken the efficacy of MSC infusion. Indeed, several articles have reported that the inflammatory milieu is crucial for inducing the immunosuppressive activity of MSC [17]. In particular, Polchert and colleagues [18] demonstrated that IFN-γ is required to trigger the initial MSC effect. Recipients of IFN-γ–/– T cells did not respond to MSC treatment and succumbed to GVHD. IFN-γ pretreated MSCs became immediately active and could suppress GVHD more efficiently than nontreated ones. Activation of MSC by IFN-γ induced the production of large amount of immunosuppressive cytokines, adhesion molecules, and chemokines, which attract and inhibit T lymphocytes. This phenomenon was amplified in the presence of TNF-α and IL-1 [19,20]. This could explain why MSCs are more active in aGVHD than in cGVHD.

Our data suggest a reduced efficacy of MSCs when applied in severe gut GVHD (2 partial responses and 2 nonresponses with further GvHD progression in 3 of 4 cases). Our data do not allow to draw any relevant consideration on MSC efficacy for liver GVHD, because of the limited number of cases (2 cases). The present article underlines that the same patients may respond differently to MSC infusion at different time points of their clinical history.

Several studies have demonstrated that plasma proteins involved in multiple processes such as T cell alloreactivity, inflammation, and tissue damage and repair, are altered in patients with GVHD. We analyzed plasma levels of TNF-α and IFN-γ, 2 cytokines crucially involved in GVHD pathogenesis [21-23], in 4 patients before and after MSC treatment. Not all patients' samples were analyzed because of the complex immunosuppressive schedule of treatment that most of them were receiving. We chose the 4 patients with the most linear pattern of immunosuppression to theoretically obtain more reliable and analyzable data. The levels of TNF-α and IFN-γ were elevated before MSC infusion, transiently decreased in nonresponder patients, and persistently decreased in responder patients, correlating and supporting the clinical data. In the other 2 tested patients, no relevant cytokine modification was observed, but it is noteworthy that they had a quite complex scheme of multidrug therapy that may have influenced the biologic data independently from MSC infusion, thus impairing the robustness of the analysis.

The benefit of MSCs for GVHD is very controversial, because recent results of late-stage clinical trials sponsored by Osiris go in a different direction compared with what previously published by Le Blanc and colleagues [5], particularly for skin GVHD. In the Osiris report, even though a significant improvement was observed for liver and gut steroid-refractory GVHD (*P* = .02 and .018, respectively), no relevant amelioration was registered for skin GVHD. Many issues should be considered to justify such differences with previously published data. As reported by *Nature* (http://www.nature.com/news/2009/090909/full/news.2009.894.html), the prochymal drug may work in certain subsets of patients and it probably depends on GVHD localization and rapidity of MSC use (earlier stage). Other major concerns have to be considered. First, a pediatric population (such as the one considered in the present study) may be more prone to respond. Patients enrolled in the Osiris trial were mostly adults, but in the pediatric cohort of 28 patients, prochymal showed a strong trend of improvement in response rates. Certainly, Osiris trials were much larger than our and Le Blanc's studies, as they included placebo groups and were double blinded. This gives statistical robustness to their results. The first study included 192 patients and the second 260. In neither of them did any patient live longer when compared with the placebo group. These observations are, in our opinion, a confirmation that MSCs may not be considered a curative option for GVHD of any stage and organ. At any rate, their activity has been demonstrated at various levels, and the preliminary results of the phase III prochymal study are encouraging for steroid-refractory liver and gut GVHD [16].

We emphasize here that, even if GVHD in some of our patients was not graded higher than I overall, at the time of MSC administration, all patients were already receiving 3 to 5 lines of treatment, without any sign of GVHD improvement before MSC infusion. Because our study describes patients treated with MSCs under compassionate use, the treatment schedule was tailored for each patient based upon clinical needs, and multiple doses of MSC were infused in a single patient, either to prevent GVHD reflaring after immunosuppressive therapy tapering, or to retreat new flairs of previously responsive GVHD. For the same reason, we did not treat with multiple MSC infusions the first 2 reported patients. Despite their partial response, we considered that they were far from transplantation, already receiving multiple lines of immunosuppression and exhibiting a pattern of cGVHD, thus being likely less prone to receive any benefit from MSC treatment. To our knowledge, no evidence has been produced about the ideal timing and doses of MSC infusions in the setting of GVHD treatment. Nevertheless, multiple infusions are commonly reported in the literature

[5]. Our patients responded to a median dose of 1.2 $\times$ 10^6/kg MSC, which can be considered a low cell dose in comparison to other published data [12]. This may suggest that, in the pediatric setting, repeated administrations might be more effective than single high-dose MSC administration, although such a theory deserves further studies. Also, recently published observations [13] seem to corroborate this hypothesis, particularly in the pediatric setting.

The present study also underlines the safety of MSC use in children. None of the treated patients, who already had been exposed to multiple lines of treatment and intensive chemotherapy regimens, presented acute reactions to MSC infusion. Following MSC administration we did not register any infectious complication, including bacterial, fungal, and viral diseases. This was also reported by other studies and the different activity of MSC on alloantigens and virus-specific T cell response has recently been investigated by Karlsson et al. [24]. Moreover, no disease recurrence or second malignancies has been observed up to now in our cohort.

Last, but not least, our present data underline the importance to produce MSCs as cellular "drugs," under strict GMP conditions in an authorized laboratory, using PL as a growth-promoting factor instead of FBS. PL-medium, besides avoiding the use of animal-derived material, accelerates MSC expansion reducing the duration of ex vivo manipulation and therefore the possibility of microbial contamination. As we previously described in detail [25,26], MSC growth in 5% PL is superior to 10% FBS in terms of cell proliferative capacity. PL lots have been standardized in their platelet concentration, which has been previously demonstrated to be the most relevant element for the release of growth factors. In Salvadè et al. [26], we analyzed differences in the release of growth factors possibly responsible for the accelerated growth rate. ELISA tests were performed to determine the amounts of epidermal growth factor (EGF), tumor growth factor (TGF)-β1, platelet-derived growth factor (PDGF)-αα, PDGF-ββ, vascular endothelial growth factor (VEGF), and basic figroblast growth factor (FGF), well known in the literature to induce MSC proliferation. We observed that all PL lots contain a great quantity of the above-mentioned growth factors, particularly if compared to human plasma, whose GF levels were undetectable. Certainly, such great amount of growth factors detected in the PL-medium is somehow responsible for accelerated MSC proliferation, but further studies are necessary to determine which ones are truly relevant in promoting MSC growth .

The GMP-grade production of an MSC bank presents the double advantage of having a certified product that respects already established well-defined release criteria (which ensure the high quality of the

cell product), and also of establishing a third-party cell bank, which allows the immediate use of defined numbers of identical vials in case of clinical need, starting from the same donor. This latest observation should ensure in the future a wider (and more rapid) use of MSCs and an easier evaluation of their clinical effects in larger studies.

The results of the present study induced us to open a trial in pediatric patients in which 2 doses of PL-cultured MSCs (1×10^6/kg dose at a 5-7-day interval) will be administered as an early treatment of steroid-resistant GVHD (EudraCT number 2008-007869-23). This strategy will allow us to have a more homogeneous population and to possibly observe real benefits of a more prompt use of MSCs as a second-line treatment for GVHD.

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