Mesenchymal Stromal Cell Therapy Is Associated With Increased Adenovirus-Associated Mortality in Children With Severe Acute Graft-Versus-Host Disease

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ABSTRACT

Beneficial effects of mesenchymal stromal cells (MSCs) in patients with severe steroid-refractory acute graft-versus-host disease (aGvHD) have been reported. However, controversy exists about the effect of MSCs on virus-specific T cells. We evaluated 56 patients with grade II–IV aGvHD who responded to steroids (n = 21) or were steroid refractory receiving either MSCs (n = 22) or other second-line therapy (n = 13). Although the overall incidence of cytomegalovirus (CMV), Epstein-Barr virus, and human adenovirus (HAdV) infections was not significantly increased, HAdV infection was associated with decreased survival in children treated with MSCs. Thus, we investigated in vitro the effects of MSCs on virus-specific T cells. Both CMV-specific and, to a lesser extent, HAdV-specific T-cell activation and proliferation were negatively affected by MSCs either after induction of a response in peripheral blood mononuclear cells (PBMCs) or after restimulation of virus-specific T-cell lines. In patient-derived PBMCs, CMV-specific proliferative responses were greatly decreased on first-line treatment of aGvHD with systemic steroids and slowly recovered after MSC administration and tapering of steroids. HAdV-specific T-cell proliferation could not be detected. In contrast, the proportion of CMV- and HAdV-specific effector T cells, measured as interferon-γ-secreting cells, remained stable or increased after treatment with MSCs. In conclusion, although in vitro experimental conditions indicated a negative impact of MSCs on CMV- and HAdV-specific T-cell responses, no solid evidence was obtained to support such an effect of MSCs on T-cell responses in vivo. Still, the susceptibility of steroid-refractory severe aGvHD patients to viral reactivation warrants critical viral monitoring during randomized controlled trials on second-line treatment including MSCs.

INTRODUCTION

Mesenchymal stromal cells (MSCs) are multipotent nonhematopoietic cells that can be easily expanded in vitro. In culture, MSCs remain genetically stable and their low immunogenicity makes them suitable candidates for immunotherapy [1, 2]. In vitro studies, MSCs have been shown to suppress proliferation and activation of T lymphocytes, B lymphocytes, natural killer cells, and monocytes [3–6]. Although cell-cell interaction and various soluble factors have been reported to mediate the in vitro [7–10] immunomodulatory effect of MSCs [7–10], the mechanisms underlying the in vivo suppressive capacity of MSCs remains to be elucidated [11, 12].

In clinical studies, MSCs showed beneficial effects in patients with steroid-refractory acute graft-versus-host disease (aGvHD), autoimmune disorders, and autoinflammatory diseases [13–15]. Although dampening of alloreactive or autoimmune-driven inflammatory reactions is frequently observed after MSC infusion, overall downmodulation of immune responses might increase the risk of viral infections. Infectious complications are a major cause of morbidity and mortality after allogeneic hematopoietic stem cell transplantation (HSCT) [16–18]. In part, this is related to the delayed immune reconstitution following HSCT; however, infections can also be exacerbated by additional immunosuppression, such as systemic steroids given for the treatment of aGvHD [19].

Published data on the effect of MSCs on virus-specific T cells are somewhat conflicting. Karlsson et al. [20] reported no effect of MSCs on the two major viral pathogens in HSCT related to T-cell expansion and cytotoxicity specific to
cytomegalovirus (CMV) and Epstein-Barr virus (EBV). However, in a recent study, von Bahr et al. [21] commented on high peak levels of CMV DNA load shortly after MSC infusion, suggesting a suppressive effect of MSCs on CMV-specific immunity. No data are available on the effect of MSCs on human adenovirus (HAdV) infections, which occur at a high frequency (up to 40%) in pediatric stem cell transplant recipients [22–24] and are lethal in up to 50% of cases with disseminated infection [18, 25–27].

At the Leiden University Medical Center, a large cohort of children with steroid-refractory aGvHD has been monitored closely after receiving MSC infusions. In the present study, the prevalence, course, and outcome of viral infections after HSCT in this cohort were documented. These data were compared with a cohort consisting of children developing aGvHD responding to steroids and with a group of historic controls with steroid-refractory aGvHD who did not receive MSCs as second- or third-line treatment.

Because both viral infections and aGvHD often occur coincidentally, it is important to further investigate the impact of MSCs on virus-specific T-cell responses. Thus, we studied the influence of MSCs in cocultures with T lymphocytes at different stages of differentiation, with T cells naturally present among peripheral blood mononuclear cells (PBMCs) and with in vitro expanded virus-specific T-cell lines. We focused on CMV and HAdV because of the potentially severe clinical impact of these viruses in pediatric HSCT. To analyze the in vivo effect of MSCs on virus-specific T-cell responses, PBMCs of patients treated with MSCs after HSCT were longitudinally investigated for their reactivity against CMV and HAdV.

MATERIAL AND METHODS

Patients and Definitions

All patients (n = 22) treated with MSCs for steroid-refractory aGvHD grade II–IV from 2004 until 2012 according to an ethical, approved protocol (LUMC-MEC P05-089) were included in the current study. Patients received one to three third-party, bone marrow-derived MSC infusions consisting of 1 or 2 × 10^6 MSCs per kilogram of recipient body weight, as described previously [14, 28]. Full resolution of symptoms at 28 days after the first MSC infusion was defined as complete response (CR). Partial response (PR) was defined as at least one grade of improvement, and no response (NR) was defined as stable disease or worsening of symptoms. Viral status of CMV, EBV, and HAdV was routinely monitored by polymerase chain reaction on plasma samples. For the purpose of the study but in contrast to the cutoff of log 3.0 copies per milliliter commonly used to define a disseminated infection, viral infection, or reactivation (referred to in this paper as “infection”) was defined as the presence of at least log 2.3 copies per milliliter in two samples taken with a time interval of at least 3 days. This allowed the inclusion of all patients with viral infections. Monitoring frequency in the first 2 months after HSCT varied between weekly and every 2 weeks thereafter until immune recovery (defined as ≥300 CD3+ T cells per milliliter of blood) was observed. Pre-emptive treatment with ganciclovir (CMV), rituximab (EBV) or cidofovir (HAdV) was initiated on detection of log ≥3 viral DNA copies per milliliter at two or more consecutive time points. Viral infections resolving before onset of severe aGvHD (defined as start of systemic steroid therapy) or occurring more than 90 days after the first MSC infusion were not taken into account.

Control cohorts consisted of patients with grade II–IV aGvHD who either responded to steroids only (HSCT in the period 2004–2012, n = 21) or were steroid refractory but received second- or third-line treatment other than MSCs (historic controls: HSCT performed in the period 1994–2004, n = 13). Patient and transplant characteristics of the study cohort and both control groups are summarized in supplemental online Table 1.

Patient Materials

PBMCs collected weekly prior to and after MSC infusion as well as PBMCs stored after routine immunophenotyping after HSCT (ethical, approved protocols LUMC-MEC P01-028 and P03-061) were used for this study. Whenever possible, PBMCs were investigated at the following time points: before the start of systemic steroids, before the first MSC infusion, 7–14 days after the first MSC infusion, 7–14 days after subsequent MSC infusions, and 180 and 365 days after the first MSC infusion. Cryopreserved PBMCs of patients after HSCT were used after thawing and resting for 4 hours at 37°C, 5% CO2 in RPMI 1640 (Invitrogen, Paisley, U.K., http://www.invitrogen.com) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (P/S; Invitrogen) and 10% human serum (HS; Sanquin, Amsterdam, The Netherlands, http://www.sanquin.nl/en/).

MSC Isolation and Culture for In Vitro Experiments

Fresh bone marrow samples of 10 healthy pediatric stem cell donors were used for MSC expansion. Parental donor and age-appropriate pediatric donor informed consent forms were signed in all cases. The study, approved by the ethics committee of the Leiden University Medical Center (LUMC-MEC P08-001), was performed in accordance with the Declaration of Helsinki [29]. After Ficoll separation, bone marrow mononuclear cells were plated in polystyrene culture flasks at a density of 0.16 × 10^6 cells/cm^2.

Cells were cultured in Dulbecco’s modified Eagle’s medium with Glutamax (Invitrogen) supplemented with P/S and 10% fetal bovine serum (VWR International, Bridgeport, NJ, https://us.vwr.com). Medium was refreshed every 3–4 days. Cultures were harvested at 80% confluence by treatment with trypsin (Invitrogen); replated; and maintained for, maximally, six passages at 37°C and 5% CO2. All MSC cell lines were phenotypically characterized at their second or third passage using antibodies against CD3, CD45, CD86, human leukocyte antigen-DR (HLA-DR), CD31, CD34, CD73, and CD90 (BD Biosciences, San Diego, CA, http://wwwbdbiosciences.com). CD105 was obtained from Ancell Corporation (Bayport, MN, http://www.ancell.com). The osteoblast and adipocyte differentiation potential was evaluated on cells at passages 4–6 as described previously [30, 31]. After 3 weeks, fat vacuoles in adipocytes and calcified depositions in osteoblasts were stained with Oil Red O (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com) or alizarin red (MP Biomedicals, Solon, OH, http://www.mpbio.com), respectively.

PBMC Stimulation

PBMCs were stimulated with methylene blue photoactivated HAdV (multiplicity of infection: 100) or 11 amino acids overlapping 15-mer peptide pools with HAdV hexon PepTivator (0.6 nM; Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com) or CMV pp65 peptides (1.0 nM; Department of Immunohematology, Leiden University Medical Center, Leiden, Netherlands) [32]. Phytohemagglutinin (PHA; PeproTech,
London, U.K., http://www.peprotech.com) and interleukin-2 (IL-2; Novartis International, Basel, Switzerland, http://www.novartis.com) were used at the indicated concentrations. PBMCs were stimulated directly with exogenously added peptides or with peptide-loaded mature dendritic cells (mDCs) generated in vitro from purified autologous monocytes. In brief, monocytes were isolated from PBMCs (3.0 × 10^6 per milliliter) by 2 hours of plastic adherence and cultured for 6 days in RPMI 10% fetal calf serum containing 800 U/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF; tebu-bio, Le Perray-en-Yvelines, France, http://www.tebu-bio.com) and 40 ng/ml of IL-4 (Peprotech, Rocky Hill, NJ, http://www.peprotech.com). Immature DCs were harvested and cultured (1.0 × 10^5 per milliliter) with similar concentrations of GM-CSF and IL-4 combined with 0.25 ng/ml CD40 ligand (Beckman-Coulter, Marseille, France, http://www.beckmancoulter.com) and 500 U/ml of interferon-γ (IFN-γ; Boehringer Mannheim, Mannheim, Germany, http://www.boehringer.com) for 2 days.

**Generation of Virus-Specific T Cells**

PBMCs from CMV-seropositive healthy adult Sanquin blood bank donors and from donors previously screened for a measurable proliferative response to HAdV peptides were selected for the generation of CMV- and HAdV-specific T-cell lines, respectively, as described previously [33]. Briefly, PBMCs were stimulated with peptide-loaded mDCs for 12 days in the presence of IL-2 (10 IU/ml) and IL-7 (5 ng/ml; PeproTech). T-cell lines were harvested on day 12 and restimulated with 30-Gy-irradiated peptide-loaded autologous PBMCs and IL-2 and IL-7 for an additional 16 days, after which the virus-specific T-cell lines were harvested and cryopreserved.

**Coculture Experiments**

PBMCs (100,000 cells per well) were cocultured with 20,000 or 2,500 MSCs (30-Gy irradiated) or without MSCs in 96-well plates. Virus-specific T cells (20,000 cells per well) were cocultured with 20,000, 4,000, or 500 MSCs (irradiated) or without MSCs. PBMCs directly stimulated with virus-derived peptides were cultured for 7 days. PBMCs stimulated with viral peptide-loaded mDCs or virus-specific T-cell lines were cultured for 5 days after defining the optimal culture duration in preliminary experiments. Negative controls with cells cultured with either unpulsed mDCs or with no exogenously added viral peptides were included. Proliferation was assessed by addition of 3H-thymidine (1 μCi per well; PerkinElmer Life and Analytical Sciences, Waltham, MA, http://www.perkinelmer.com) for the last 16 hours of culture. IFN-γ concentration in culture supernatants was measured by enzyme-linked immunosorbent assay (Sanquin) performed according to the manufacturer’s instructions.

**Flow Cytometry**

Monoclonal antibodies (mAbs) used to characterize the T cells proliferating in culture were: anti-CD3 PerCPC5.5 (BD Biosciences), anti-CD4 APC (Beckman Coulter), anti-CD8 PE (BD Biosciences), and anti-CD8 APC (Beckman Coulter). Prior to stimulation, cells were labeled with carboxyfluorescein succinimidy l ester (CFSE; Invitrogen) to discriminate between proliferating and nonproliferating cells. To determine activation and the differentiation stages of virus-specific T-cell lines, the mAbs anti-HLA-DR FITC (BD Biosciences), anti-CD45RA PE (Beckman Coulter), and anti-CCR7FITC (R&D Systems Inc., Minneapolis, MN, http://www.rndsystems.com) were applied. T-cell differentiation stages were defined as “naïve,” CD45RA+CCR7+; “central memory,” CD45RA−CCR7+; “effector memory,” CD45RA−CCR7−; and “end-stage effector cells,” CD45RA+CCR7−.

**Virus-Specific T-Cell Stimulation of Patient-Derived PBMCs**

Disseminated EBV reactivations in three HSCT patients were successfully treated with rituximab; therefore, functional studies focused on the detection of CMV- and HAdV-specific T cells. To detect IFN-γ-secreting cells, ELISPot plates (Millipore, Billerica, MA, http://www.millipore.com) were coated with anti-IFN-γ antibody (Mabtech, Stockholm, Sweden, https://www.mabtech.com) overnight. PBMCs (0.2 × 10^6 cells per well) were stimulated with CMV or HAdV peptides with or without a low dose of IL-2 (3 IU/ml) for 20 hours. PBMC cultured without stimuli or with low-dose IL-2 only were used as negative controls. PBMCs stimulated with PHA (10 μg/ml) plus a high dose of IL-2 (50 IU/ml) were used as positive controls.

IFN-γ was detected by anti-IFN-γ-biotin antibody (Mabtech) and visualized by streptavidin-alkaline phosphatase (Sigma-Aldrich) and its substrate 5-Bromo-4-chloro-3-indolyl phosphate (BCIP; Sigma-Aldrich). IFN-γ spots were counted with a fully automated, computer-assisted, video-imaging analysis system (BioSys 5000; BioSys, Karben, Germany, http://www.biosys.de). Virus-induced ELISPot results were compared with background levels observed in cultures without viral peptides. Values of more than 10 spots per 200,000 cells and at least two times higher than background were considered positive. All conditions were performed in duplicate.

Proliferation of 1 × 10^5 PBMCs per well was assessed by 3H-thymidine incorporation performed overnight after 6 days of stimulation with CMV or HAdV peptides with a low dose of IL-2 (3 IU/ml). Stimulation with PHA (2 μg/ml) plus a high dose of IL-2 (50 IU/ml) for 5 days and adding 3H-thymidine at day 4 was used as a positive control.

For comparison of the different time points, viability of PBMCs was determined after thawing and 4 hours of resting using trypan blue. In addition, PHA-induced proliferation and IFN-γ production were considered measures of general functionality of the thawed cells. In cases of negative PHA proliferation, time points were excluded from the analysis.

**Statistical Analysis**

Survival analysis was modeled with log-rank Mantel-Cox tests. Paired Wilcoxon signed-rank tests were used to compare subsets prior to and after MSC infusion and for evaluation of the in vitro effect of MSCs. Chi-square tests were performed on categorical data. GraphPad 6 (GraphPad Software, Inc., San Diego, CA, http://www.graphpad.com) was used for data analysis, and p values <0.05 were considered statistically significant.

**RESULTS**

**Clinical Results**

One-year survival after HSCT in the patients treated with MSCs was higher (62.9%) than in historic controls (33.3%) but lower than in children who were responsive to steroids (90.5%) and did not reach statistical significance. The latter group, being less
severely affected regarding the grade and gut involvement of aGVHD, differed significantly from the MSC group (supplemental online Table 1). Patients with MSCs as second-line therapy (n = 16) showed better 1-year survival compared with patients with MSCs as third-line therapy (n = 6; 73.9% vs. 33.3%, respectively; p = .049). One-year survival in the latter subgroup was comparable to historic controls with steroid-refractory aGVHD not treated with MSCs (n = 13; 38.5%).

In 14 of the 22 consecutive children treated with MSCs for steroid-refractory aGVHD, grade III–IV viral infections were present either at the onset of severe aGVHD or occurred within a period of 90 days after the first MSC infusion (Table 1). Seven patients had a CMV infection, 7 had an HAdV infection, and 5 had an EBV infection; multiple viral infections occurred in 5 of 22 enrolled patients. Overall survival at 1 year after HSCT, censored for relapse, was higher (but not significantly) in patients without viral infection (Fig. 1A). Survival in historic controls (n = 13, 6 children with an infection) with steroid-refractory aGVHD receiving no MSCs showed the same trend (Fig. 1B; HAdV, n = 4; CMV, n = 3; EBV, n = 3). In contrast, 1-year survival in the control cohort with steroid-responsive patients was comparable for children with (n = 7) and without (n = 14) a viral infection (Fig. 1C; HAdV, n = 3; CMV, n = 4; EBV, n = 3). Although not statistically significant (p = .09), there was a trend toward a higher percentage of viral infections and a longer duration of viremia in the MSC-treated children compared with patients responsive to steroids only (data not shown).

In contrast to CMV and EBV infections, HAdV infection was significantly associated with higher nonrelapse mortality (Fig. 1D). HAdV infections that became apparent after start of MSC therapy were associated with poor outcome; none of these patients (n = 6), including two patients with recurrence of CMV, were alive at 1 year after HSCT, whereas five of six patients with CMV (n = 5) or HAdV (n = 1) infection present before and persisting

### Table 1. Steroid-unresponsive children receiving MSC: patient characteristics and viral infections

| UPN | MSC as second line | MSC responsea | Follow-up after HSCT | Cause of death | Virus | Antiviral treatment before steroids | Start steroids | MSC1 | MSC1 +30 | MSC1 +60 | MSC1 +90 |
|-----|-------------------|--------------|---------------------|----------------|-------|-----------------------------------|--------------|------|---------|----------|---------|
| 2   | Nob               | NR           | Death (day +99)     | CMV + GvHD    | CMV   | Ganciclovir                       | +            | +    | +       | +        | +       |
| 11  | Yes               | CR           | Alive (day +1,378) |                | CMV   | Ganciclovir                       | +            | +    | +       | +        | +       |
| 12  | Yes               | CR           | Death (day +371)   | Relapse       | CMV   | Ganciclovir                       | –            | +    | +       | –        | –       |
| 15  | Yes               | NR           | Alive (day +601)   |                | CMV   | Ganciclovir                       | +            | +    | +       | +        | +       |
| 17  | Yes               | PR           | Death (day +314)   | Respiratory insufficiency, Aspergillus pneumonia, HAdV, CMV | CMV   | Ganciclovir                       | +            | +    | –       | +        | +       |
| 19  | Yes               | CR           | Alive (day +487)   |                | CMV   | Ganciclovir                       | +            | –    | –       | +        | –       |
| 20  | Yes               | PR           | Death (day +193)   | Interstitial pneumonitis Caused by chronic GvHD | CMV   | Ganciclovir                       | +            | +    | –       | +        | +       |
| 1   | No                | CR           | Death (day +66)    | Klebsiella pneumonia | HAdV  | Cidofovir                         | –            | –    | –       | +        | NA      |
| 4   | No                | CR           | Death (day +1,498) | Line infection; pneumonia | HAdV  | Cidofovir                         | +            | +    | +       | +        | +       |
| 5   | No                | PR           | Death (day +64)    | MOF, HAdV     | HAdV  | Cidofovir                         | –            | –    | –       | +        | NA      |
| 6   | No                | NR           | Death (day +102)   | MOF, HAdV, Klebsiella, EBV | HAdV  | Cidofovir                         | –            | –    | –       | +        | NA      |
| 7   | Yes               | NR           | Death (day +53)    | EBV, HAdV + ongoing aGVHD | HAdV  | Cidofovir                         | –            | –    | –       | +        | NA      |
| 3   | No                | CR           | Alive (day +2,708) | EBV           | –      | –                                  | +            | +    | +       | –        | –       |
| 10  | Yes               | PR           | Alive (day +1,497) | EBV           | RTX   | +                                  | (RTX)        | NA   | NA      | NA       | NA      |

aResponse of aGVHD at 28 days after the first MSC infusion.
bMSCs were applied as third line treatment.

Abbreviations: + or ++, indicative of a viral DNA load greater than or equal to log 2.3 copies per milliliter; aGVHD, acute graft-versus-host disease; CMV, cytomegalovirus; CR, complete response; EBV, Epstein-Barr virus; HAdV, human adenovirus; HSCT, hematopoietic stem cell transplantation; MMF, mycofenolate mofetil; MOF, multiorgan failure; MSC, mesenchymal stromal cell; NA, not applicable; NR, no response; PR, partial response; RTX, rituximab; UPN, unique personal number.
Table 2. Steroid-unresponsive children receiving other second line therapy: patient characteristics and viral infections

| UPN | Second line treatment | aGvHD resolution | Follow-up after HSCT | Cause of death | Virus | Antiviral treatment | Before steroids | Start steroids | Steroids +14 | Steroids +30 | Steroids +60 | Steroids +90 |
|-----|-----------------------|------------------|----------------------|---------------|-------|--------------------|----------------|--------------|-------------|-------------|-------------|-------------|-------------|
| 31  | Thalidomide           | Yes              | Alive (day +5,463)   |               | CMV   | +                  | −              | −            | +           | −           | −           | −           | −           |
| 32  | Prednisolone          | No               | Death (day +97)      | aGvHD, HAdV   | HAdV  | −                  | −              | −            | −           | +           | NA          | −           | −           |
| 33  | MMF                   | No               | Death (day +77)      | HAdV, line infection | EBV   | −                  | −              | −            | +           | NA          | −           | −           | NA          |
| 34  | MMF                   | No               | Death (day +76)      | Liver failure, HAdV | CMV   | +                  | +              | +            | +           | +           | NA          | −           | NA          |
| 35  | anti-CD25             | Yes              | Death (day +58)      | aGvHD, liver failure, HAdV | HAdV  | −                  | −              | −            | +           | NA          | NA          | −           | −           |
| 36  | MMF                   | No               | Death (day +76)      | Respiratory failure, MOF | CMV (Val) ganciclovir | +          | +              | +           | +           | NA          | NA          | −           | −           |

Abbreviations: + or −, indicative of a viral DNA load greater than or equal to 2.3 copies per milliliter; aGvHD, acute graft-versus-host disease; CMV, cytomegalovirus; EBV, Epstein-Barr virus; HAdV, human adenovirus; HSCT, hematopoietic stem cell transplantation; MMF, mycofenolate mofetil; MOF, multiorgan failure; NA, not applicable; UPN, unique personal number.

during aGvHD were alive at 1 year after HSCT (Table 1). Six patients in the MSC cohort, all from the first 3 years of the inclusion period, received other second-line treatment prior to MSC infusion. All of these patients had a viral infection either prior to (n = 3) or after (n = 3) MSC infusion (Table 1).

In the steroid-refractory patients receiving other second-line therapy and no MSCs, CMV was present prior to initiation of steroid therapy, whereas HAdV reactivated after start of aGvHD (Table 2). None of the three viruses was associated with decreased survival, as seen for HAdV in the MSC cohort (Fig. 1E).

The observed association between infections appearing after MSC infusion and decreased survival in these patients warranted further research into the possible effect of MSC infusion on antiviral immunity.

In Vitro Effect of MSCs on Virus-Specific T Cells

To determine the putative negative effect of MSCs on HAdV-specific T cells, PBMCs obtained from healthy adult donors with demonstrable precursor frequencies of viral-specific T cells (data not shown) were stimulated with inactivated HAdV in the presence or absence of MSCs. MSCs suppressed the proliferative response but not the IFN-γ production in these cocultures (Fig. 2A, 2B). To exclude bystander immune activation induced by TLR ligands or other nonspecific virus-derived stimuli, experiments were also performed using synthetic, good manufacturing practices-grade, 15-mer viral peptides. In cultures of PBMCs stimulated with HAdV hexon-derived peptides, proliferation and IFN-γ production were low, and no suppressive effect of MSCs was observed in either assay (Fig. 2C, 2D). In contrast, after stimulation with synthetic CMV pp65-derived peptides, MSCs showed an inhibitory effect on proliferation and IFN-γ production (Fig. 2C, 2D). Phenotypic analysis of T cells in CMV-stimulated cultures demonstrated a suppressive effect of MSCs on both CD4+ and CD8+ T cells. This was assessed in proliferation assays using CFSE staining and in assays measuring the percentage of cells expressing HLA-DR after antigen-specific activation (Fig. 2E, 2F).

Stimulation of PBMCs With Peptide-Loaded Autologous Monocyte-Derived Dendritic Cells

Conflicting results were obtained when investigating the interaction of MSCs and HAdV-specific T cells after using two types of stimulation and two different read-out systems. Consequently, we decided to stimulate PBMCs from healthy adult donors with peptide-loaded autologous mDCs. MSCs suppressed PBMC proliferation after stimulation with CMV peptides or HAdV-peptide-loaded mDCs (Fig. 3A). IFN-γ production was suppressed by MSCs in cocultures after stimulation with CMV PepTivator with or without IL-2 (although not significantly, p = .06) but not after stimulation with HAdV PepTivator (Fig. 3B). Altogether, these data suggest that the height of the response generated in vitro is of importance for the detection of a suppressive effect of MSCs.

Inhibitory Effect of MSCs on Virus-Specific T-Cell Lines

To combat viral infection, activation and clonal expansion of memory T cells is of importance. Consequently, the effect of MSCs on virus-specific T-cell lines was investigated. T-cell lines raised against HAdV-derived peptides consisted mainly of CD4+ T cells. The ratio of CD4+ to CD8+ T cells in CMV-specific T-cell lines varied (supplemental online Fig. 1A). The percentage of T cells expressing HLA-DR was significantly higher in CMV T-cell lines than in HAdV T-cell lines, regardless of the ratio of CD4+ to CD8+ T cells (supplemental online Fig. 1B). All cell lines were predominantly of effector memory phenotype (>50%), characterized by the lack of CCR7 and CD45RA expression (supplemental online Fig. 1C, 1D).

Viral peptide-induced proliferation and IFN-γ production were suppressed in cocultures of HAdV- and CMV-specific T-cell lines with MSCs (Fig. 4A, 4B). HLA-DR expression on T-cell lines stimulated with HAdV and IL-2 was decreased when T cells were activated in the presence of MSCs (Fig. 4C). In contrast, the high percentage of HLA-DR-expressing cells on CMV-specific T cells was not altered (data not shown). Although T-cell lines raised against HAdV consisted mainly of CD4+ T cells, both CD4+ and CD8+ T-cell-dominated expansions were observed on
restimulation of such T-cell lines; in both cases, suppression by MSCs was documented (Fig. 4D, 4E). In conclusion, in vitro MSCs have a suppressive effect on proliferation and activation of effector memory-type T cells.

**Virus-Specific Responses in HSCT Patients**

Sufficient frozen material collected at multiple time points was available from 8 of the 14 children with viral infections in the cohort treated with MSCs for steroid-refractory acute graft-versus-host disease. At the start of MSC therapy, two of the seven patients with CMV infection had an undetectable viral DNA load (unique personal number 17 [UPN17] and UPN20) (Table 1; Fig. 5A). In the period of 90 days after start of MSC therapy, these patients showed persistent recurrence of viremia. In two of the five patients with CMV viremia at the start of MSC therapy, the viremia resolved in this 90-day time window (UPN12 and UPN19) and persisted in the other three patients (UPN02, UPN11, and UPN15) (Table 1; Fig. 5A).

In six of the seven patients with CMV infection, the frequency of IFN-γ-producing cells was determined in the week before MSC infusion and at 1–2 weeks and 28 days after MSC infusion. Both patients with recurrent viremia after MSC treatment (UPN17 and UPN20) had low numbers of IFN-γ-producing cells detected by ELISpot in the 2 weeks after receiving MSCs and showed an increase in IFN-γ-producing cells at 28 days after MSC infusion (Fig. 5B). In the four evaluable patients with detectable CMV DNA loads before receiving MSCs, the number of IFN-γ producing cells did not
change significantly after MSC infusion and was not associated with the course of the CMV DNA load, which remained stable in two cases and diminished in the other two cases. Two children had late development of aGvHD (at 83 days and 92 days, respectively, after HSCT), and CMV-specific proliferation was observed prior to the start of systemic steroids. Both showed a decrease of proliferation after start of steroids (Fig. 5C, 5D) with recurrence of CMV viremia prior to MSC infusion. Both had CR after MSC infusion, and that allowed for tapering of steroids. CMV-induced proliferation was restored slowly in these patients. Another child with CR showed CMV-specific proliferation from 53 days after MSC infusion onward (data not shown). In the other three children (PR, PR, and NR to MSC, respectively), virus-specific proliferation was not detectable despite the continued presence of IFN-γ-producing cells (data not shown).

In four of the seven patients with HAdV infection, the presence of IFN-γ-producing cells on ex vivo HAdV stimulation of PBMCs could be analyzed longitudinally. In all four patients tested, IFN-γ-producing T cells were detected: in two children before and after MSC infusion, installed as third-line therapy, and in the other two only after initiation of MSC therapy as second-line treatment (Fig. 5F). The patient with high numbers of HAdV-specific IFN-γ producing T cells (UPN04) experienced persistent viremia from before the start of systemic steroids onward; however, this patient is the only survivor at 1 year. The other patients developed HAdV infections after MSC infusion, and those occurred late for UPN06 (49 days after HSCT) and UPN20 (106 days after HSCT) (Table 1; Fig. 5E). HAdV-specific proliferation was not detectable in any of the patients.

**DISCUSSION**

Numerous studies have demonstrated the feasibility of MSC therapy for steroid-refractory aGvHD after allogeneic HSCT and have indicated its potentially beneficial effects [34–38]. Response rates in these nonrandomized studies varied but were higher than in reported historic controls, in which various highly immunosuppressive medications were applied as second- or third-line...
treatment. Patient numbers included so far were too small to draw firm conclusions not only with respect to efficacy but also about the potential occurrence of adverse events such as leukemia relapse and viral infections. Currently available clinical data on the incidence of infections are focused on CMV [21]. The incidence of HAdV infection, which is especially relevant to children after HSCT [18, 22, 39], has not been addressed. In addition, the in vitro effect of MSCs on HAdV-specific T cells has not been reported.

In a pediatric cohort treated with MSCs for steroid-refractory severe aGvHD in the Leiden University Medical Center, the occurrence of HAdV infection after MSC treatment was associated with decreased survival. This was not seen either in children with aGvHD grade II–IV who were responsive to steroids or in children receiving second-line therapy other than MSCs. A likely explanation is the relatively shorter duration of immune suppression in the patients responding to steroids. The difference between steroid-refractory children receiving MSCs versus other second-line treatment might point to an effect of MSCs; however, the effect might be influenced by the comparatively high number of children (10 of 22) in the MSC cohort receiving a mismatched graft. Of note, 6 of 7 children with an HAdV infection in this cohort were transplanted with a mismatched graft, thereby affecting 6 of 10 children transplanted with such a graft, in line with previously reported data [40]. Graft modulation, either T-cell depletion or CD34+ enrichment, did not differ between the two groups.

One-year survival in patients with steroid-refractory aGvHD receiving second- or third-line therapy other than MSCs (historic controls) was significantly lower than in patients receiving MSCs as second-line therapy (38.5% vs. 73.9%) and was comparable to that of children receiving MSCs as third-line therapy (33.3%), which can be attributed to ongoing severe aGvHD. In a study on the use of monoclonal antibodies for the treatment of steroid-refractory aGvHD in children, 9 of 22 patients had viremia...
(13 episodes of viral reactivation: 5 CMV, 4 EBV, and 4 HAdV) compared with viremia in 14 of 22 children (19 episodes of viral reactivation: 7 CMV, 5 EBV, and 7 HAdV) included in our MSC-treated cohort [41]. We deliberately choose log 2.3 copies per milliliter as a cutoff for infection and reactivation, regardless of the virus involved, and chose to include patients with controlled viral infections. This might explain the somewhat higher viremia rate in our study cohort. In addition, when applying the presence of a concentration of log 3.0 copies per milliliter in at least two consecutive plasma samples—the widely accepted criterion for a disseminated infection—the rate of 1-year survival for patients with and without viral dissemination in our MSC cohort is statistically different (36.4% vs. 91.0%, respectively; \( p = .01 \)).

This is the first study describing the interaction of MSCs with HAdV-specific T cells. Karlsson et al. have published experimental data indicating the absence of an in vitro effect of MSCs on CMV- and EBV-specific T cells [20]. These data are in contrast to results from other studies demonstrating a downmodulating effect of MSCs on the autologous EBV-B-lymphoblastoid cell line-induced proliferation of EBV-specific T cells [42, 43]. The latter observation is in line with our findings of a negative impact of MSCs on proliferation of both PBMCs and virus-specific T-cell lines after stimulation with CMV pp65 peptides or HAdV hexon-derived peptide-loaded mDCs or PBMCs, respectively. The in vitro suppressive effect of MSCs on virus-induced T-cell proliferation and IFN-\( \gamma \) production was MSC-dose dependent and most evident at high ratios of MSCs to target cells. This and the fact that the relevant in vivo effect of MSCs most probably occurs at the sites of infection and tissue damage might explain that no increase in CMV-related disease is observed. Of note, suppression of proliferation was not observed in coculture experiments using MSC and T-cell clones specific for CMV, HAdV, or control H-Y minor histocompatibility antigen (data not shown).

In a recent study in mice, the suppression of the induction of an ovalbumin-specific T-cell response in vivo after infusion of MSCs was explained by decreased homing of ovalbumin-pulsed dendritic cells to the lymph nodes [44]. An indirect effect of MSCs on lymphocyte activation and proliferation via monocytes has been suggested previously [45]. In our MSC cohort, survival of patients with infections already present at the time of MSC infusion was significantly higher than that of patients with an onset of infection after the initiation of MSC therapy. A negative impact of MSCs on antigen-presenting cells involved in the induction of a response in vivo might be an explanation for the differences in lethality of HAdV and CMV infections occurring prior to and after MSC infusion, respectively. However, based on our data, we cannot exclude a differential effect of immunosuppressive drugs on the initiation of a response versus an ongoing response.

Using CMV pentamers, Karlsson et al. were the first to show that the percentage of virus-specific T cells was stable prior to and after MSC infusion in two patients [20]. Lucchini et al. came to a similar conclusion based on the analysis of IFN-\( \gamma \)—producing
cells in two patients after MSC infusion [46]. However, one of these patients showed a decrease in the number of IFN-γ-secreting cells and a recurrence of CMV viremia after multiple MSC infusions. In addition, 11 of 24 patients had viral reactivation occur after initiation of MSC therapy, including 3 patients with HAdV infection. Interestingly, no viral-infection-related death occurred in this patient group. Our data confirm the previously reported observation that MSC infusion did not affect the number of circulating CMV-specific T cells and extend this to HAdV-specific T cells. Of note, the level of IFN-γ-producing cells among peripheral blood T cells did not correlate with the course of either the CMV viremia or the HAdV viremia, suggesting that more in-depth analysis of T-cell subpopulations combined with other functional aspects might be of interest.

A major difficulty in interpreting data of ex vivo analysis of virus-specific T cells in MSC-treated patients is the immune suppression by methylprednisolone preceding MSC infusion and continued thereafter. Fewer CD8+ T cells after initiation of methylprednisolone have been reported previously [47, 48], in addition to decreased cytokine production without reduction of

Figure 5. Viral DNA loads increase around start of acute graft-versus-host disease despite the persistence of IFN-γ-producing virus-specific T cells in patients treated with MSCs. (A, B): At 28 days after MSC infusion, CMV DNA loads were increased in two patients, UPN17 and UPN20 (A); however, the number of CMV-specific IFN-γ-producing cells was not affected (B). (C, D): In two children, UPN12 (C) and UPN19 (D), CMV-specific proliferation could be detected by ³H-thymidine incorporation. Proliferation was decreased after start of steroids. (E, F): In five patients, HAdV DNA loads were detected at 28 days after MSC infusion (E). Plasma HAdV DNA load became positive in UPN06 and UPN20 at 49 days and 106 days, respectively, after MSC infusion. Despite the presence of IFN-γ-producing cells (UPN04) (F), the load did not decrease. #, Viral DNA load detected 3 days prior to MSC infusion. UPN01, UPN02, UPN05, and UPN07: no peripheral blood mononuclear cells were available for ELISpot assay. Abbreviations: CFSE, carboxyfluorescein succinimidyl ester; CMV, cytomegalovirus; HAdV, human adenovirus; HLA-DR, human leukocyte antigen-DR; ³H-TdR (cpm), tritiated thymidine incorporation in counts per minute; IFN-γ, interferon-γ; IL-2, interleukin 2; MSC, mesenchymal stromal cell; UPN, unique personal number.
T-cell counts [49]. Previous studies did not comment on the influence of methylprednisolone tapering on recovery of immunity. We demonstrated a similar number of IFN-γ-producing cells in PBMCs prior to and after initiation of aGvHD treatment, whereas the proliferative capacity was hampered from the start of steroid infusion. This analysis could be performed only in two patients with sufficient numbers of PBMCs prior to MSC infusion. No CMV-specific proliferation was detected up to 3 months after MSC infusion in three patients in whom steroids could not be tapered due to a lack of response to MSC infusion.

Although our in vitro data demonstrate a suppressive effect of MSCs on T-cell proliferation, the effect of methylprednisolone in vivo is likely to be stronger. Consequently, aiming for fast reduction of methylprednisolone after MSC infusion seems to be of utmost importance in these patients following objective documentation of resolution of the aGvHD symptoms [28]. Interestingly, infections already present at the start of aGvHD did not negatively affect the outcome. Thus, patients with active viral infections should not be excluded from future randomized controlled trials on MSCs as second-line therapy for steroid-refractory aGvHD; however, critical viral monitoring is advised.

CONCLUSION

In this study, we show an association between HAdV infections occurring after MSC infusion and decreased survival in patients treated for severe steroid-refractory aGvHD. In addition, our in vitro data demonstrate a suppressive effect of MSCs on proliferation and activation of, among others, HAdV-specific T cells, whereas no solid evidence was obtained to support a negative impact of MSCs on antiviral T-cell driven immune responses in vivo. However, the beneficial effect of MSC therapy on steroid-refractory aGvHD is strongly supported by the observation that CR or PR was established at 28 days after start of MSC therapy in 10 of the 14 children with a viral infection. In conclusion, the results of the present study indicate that the benefits of MSCs outweigh the potentially increased risk of infections. Randomized controlled trials, currently under development, will not only further address the efficacy of MSC treatment but also should represent the ideal platform from which to further investigate potential side effects of MSCs, specifically with regard to viral reactivation.

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AUTHOR CONTRIBUTIONS

F.G.J.C.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; C.V.: collection and assembly of data, data analysis and interpretation; A.G.S.v.H.: conception and design, final approval of the manuscript; M.J.P.W.: collection and assembly of data, final approval of the manuscript; L.A.V.-D.: conception and design, final approval of the manuscript; A.C.L.: conception and design, provision of study material and patients, final approval of manuscript; M.J.B.: conception and design, provision of study material and patients, manuscript writing; M.J.D.V.T.: conception and design, data analysis and interpretation, manuscript writing.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

A.G.S.v.H. has compensated employment as R&D project manager at Immunobank N.V. Amsterdam.

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