Chapter 6.

Mesenchymal stromal cell therapy is associated with increased adenovirus-associated, but not cytomegalovirus-associated mortality in children with severe acute Graft-versus-Host Disease.

Stem cells Translational Medicine 2014; 3 (8): 899-910
Calkoen FGJ, Vervat C, van Halteren AGS, Welters MJP, Veltrop-Duits LA, Lankester AC, Egeler RM, Ball LM, van Tol MJD.

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 MSC (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. Therefore, 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 (PBMC) or after restimulation of virus-specific T cell lines. In patient-derived PBMC, 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 reactivations warrants critical viral monitoring during randomized controlled trials on second-line treatment including MSCs.

Introduction

Mesenchymal stromal cells (MSCs) are multipotent non-hematopoietic cells that can be easily expanded *in vitro*. In culture, MSCs remain genetically stable and their low immunogenicity makes them suitable candidates for immunotherapy.(Dominici, 2006; Bernardo, 2007c) In *in vitro* studies MSCs have been shown to suppress proliferation and activation of T lymphocytes, B lymphocytes, natural killer cells, and monocytes.(Sotiropoulou, 2006; Traggiai, 2008b; Krampera, 2003; Melief, 2013) Although cell-cell interaction and various soluble factors have been reported to mediate the *in vitro* (Gieseke, 2010; Meisel, 2004; Selmani, 2008; Mougiakakos, 2011) immunomodulatory effect of MSCs, the mechanism(s) underlying the *in vivo* (Ren, 2008; Gonzalez, 2009) suppressive capacity of MSCs remains further to be elucidated.

In clinical studies, MSCs showed beneficial effects in patients with steroid-refractory acute graft-versus-host disease (aGvHD), autoimmune disorders and auto-inflammatory diseases.(Sun, 2009; Le Blanc, 2008; Garcia-Olmo, 2005) Although dampening of alloreactive or autoimmune-driven inflammatory reactions is frequently observed after MSC infusion, overall down modulation 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).(Broers, 2000; Boeckh, 2003; Walls, 2003) 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.(Cantoni, 2010)

Published data on the effect of MSCs on virus-specific T cells are somewhat conflicting. Karlsson *et al*.,(Karlsson, 2008) 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) specific. However, in a recent study, von Bahr *et al*.(von Bahr, 2012) 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 graft recipients(Flomenberg, 1994; Howard, 1999; Baldwin, 2000) and are lethal in up to 50% of cases with disseminated infection.(Walls, 2003; Kojaoghlanian, 2003; Leen, 2005; van Tol, 2005b)

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/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 MSC in co-cultures with T lymphocytes at different stages of differentiation, with T cells naturally present among peripheral blood mononuclear cells (PBMC) 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 MSC on virus-specific T cell responses, PBMC 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 MSC for steroid-refractory aGvHD grade II-IV from 2004 until 2012 according to an ethical approved protocol (number LUMC-MEC: P05-089) were included in the current study. Patients received 1 to 3 third-party, bone marrow derived, MSC infusions consisting of 1-2 x 10^6 MSCs per kg recipient body-weight, as previously described.(Le Blanc, 2008; Calkoen, 2013b) 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 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 two 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 to 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 to 2004, n=13). Patient and transplant characteristics of the study cohort and both control groups are summarized in supplementary Table 1 (Table S1).

Patient materials

PBMC collected weekly prior to and after MSC infusion as well as PBMC stored after routine immunophenotyping post HSCT (ethical approved protocols LUMC-MEC P01-028 and P03-061) were used for this study. Whenever possible, PBMC were investigated at the following time-points: before the start of systemic steroids, before the first MSC infusion, 7-14 days after first MSC infusion, 7-14 days after subsequent MSC infusions and 180 and 365 days after the first MSC infusion. Cryopreserved PBMC of patients after HSCT were used after thawing and resting for 4 hours at 37˚C, 5% CO_2 in RPMI 1640 (Invitrogen, Paisley, UK) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin (P/S; Invitrogen), 10% human serum (HS, Sanquin, Amsterdam, the Netherlands).

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 ethical committee of the Leiden University Medical Center (LUMC-MEC P08-001), was performed in accordance with the Declaration of Helsinki.(World Medical Association, 1997) 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 medium with Glutamax (DMEM; Invitrogen) supplemented with P/S and 10% fetal bovine serum (FBS; VWR International, Bridgeport, NJ). Medium was refreshed every three to four days. Cultures were harvested at 80% confluency by treatment with trypsin (Invitrogen), replated and maintained for maximally 6 passages at 37˚C and 5% CO_2. 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 (all Becton Dickinson Biosciences (BD), San Diego, CA). CD105 was obtained from Ancell Corporation (Bayport, MN). The osteoblast and adipocyte differentiation potential was evaluated on cells at passage 4 to 6 as described previously.(Bernardo, 2007b; Calkoen, 2013a) After 3 weeks, fat vacuoles in adipocytes and calcified depositions in osteoblast were stained with Oil-Red-O (Sigma, St. Louis, MI) or Alizarin Red (MP Biomedicals, Solon, OH), respectively.

PBMC stimulation

PBMC were stimulated with methylene blue photoinactivated 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) or CMV pp65 peptides (1.0 nM, Department of Immunohematology, Leiden University Medical Center, the Netherlands).(Zandvliet, 2011) Phytohemagglutinin (PHA, PeproTech, London, UK) and interleukin-2 (IL-2 Novartis International, Basel, Switzerland) were used at the indicated concentrations. PBMC were stimulated directly with exogenously added peptides or with peptide-loaded mature dendritic cells (mDC) generated *in vitro* from purified autologous monocytes. In brief, monocytes were isolated from PBMC (3.0 x 10^6/mL) by 2 hours 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; TebuBio, Le Perray-en-Yvelines, France) and 40 ng/mL of IL-4 (PeproTech). Immature DC (iDC) were harvested and cultured (1.0 x 10^5/mL) with similar concentrations GM-CSF and IL-4 combined with 0.25 ng/mL CD40-ligand (Beckman-Coulter, Marseille, France) and 500 U/mL of interferon-γ (IFN-γ; Boehringer, Mannheim, Germany) for 2 days.

Generation of virus-specific T cells

PBMC 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 previously described.(Veltrop-Duits, 2006) Briefly, PBMC were stimulated with peptide-loaded mDC 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-Gray-irradiated peptide-loaded autologous PBMC and IL-2 and IL-7 for an additional 16 days, after which the virus-specific T cell lines were harvested and cryopreserved.

Co-culture experiments

PBMC (100,000 cells per well) were co-cultured with 20,000 or 2,500 (30-Gray irradiated) MSCs or without MSCs in 96-well plates. Virus-specific T cells (20,000 cells per well) were co-cultured with 20,000, 4,000 or 500 (irradiated) MSCs or without MSCs. PBMC directly stimulated with virus-derived peptides were cultured for 7 days. PBMC stimulated with viral peptide-loaded mDC 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 mDC or with no exogenously added viral peptides were included. Proliferation was assessed by addition of ^{3}H-thymidine (1 μCi per well; Perkin Elmer, Waltham, MA, USA) 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 instruction.

Flow cytometry

Monoclonal antibodies (mAbs) used to characterize the T cells proliferating in culture were: anti-CD3 PerCPC5.5 (Becton Dickinson (BD), Mountain View, CA), anti-CD4 APC (Beckman Coulter Immunotech, Marseille, France), anti-CD8 PE (BD), anti-CD8 APC (Beckman Coulter). Prior to stimulation, cells were labeled with carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) to discriminate between proliferating and non-proliferating cells. To determine activation and the differentiation stages of virus-specific T cell lines, the mAbs anti-HLA-DR FITC (BD), anti-CD45RA PE (Beckman Coulter) and anti-CCR7 FITC (R&D, Minneapolis, MN) 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 PBMC

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) were coated with anti-IFN-γ antibody (Mabtech, Stockholm, Sweden) overnight. PBMC (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. PBMC stimulated with PHA (10 ug/mL) plus a high dose of IL-2 (50 IU/mL) was used as positive controls.

IFN-γ was detected by anti-IFN-γ-biotin antibody (Mabtech) and visualized by streptavidin-alkaline phosphatase (Sigma, St Louis, MO) and its substrate 5-chromo-4-chloro-3-indolyl phosphate (BCIP; Sigma). IFN-γ spots were counted with a fully automated, computer-assisted, video-imaging analysis system (BioSys 5000, Karben, Germany). Virus-induced ELISpot results were compared to 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 PBMC per well was assessed by ^{3}H-thymidine incorporation performed overnight after 6 days of stimulation with CMV or HAdV peptides with a low dose of IL-2 (3 IU/mL). PHA (2 ug/mL) plus a high dose of IL-2 (50 IU/mL) stimulation for 5 days adding ^{3}H-thymidine at day 4 was used as a positive control.

For comparison of the different time-points, viability of PBMC was determined after thawing and four 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-squared tests were performed on categorical data. Graphpad 6 (Prism, La Jolla, USA) 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 responsive to steroids (90.5%), and did not reaching statistical significance. The latter group, being less severely affected regarding the grade and gut involvement of aGvHD, differed significantly from the MSC group (Table S1). Patients with MSCs as second-line therapy (n=16) showed a better one-year survival compared with patients with MSCs as third line therapy (n=6; 73.9% vs 33.3%, respectively p=0.049). 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 GvHD or occurred within a period of 90 days after the first MSC infusion (Table 1). Seven patients had a CMV infection, seven patients had an HAdV infection, and five patients had an EBV infection; multiple viral infections occurred in 5 out of 22 enrolled patients. Overall survival at 1 year after HSCT, censored for relapse, was higher (but not significantly) in patients without viral infection (Figure 1A). Survival in historic controls (n=13, six children with an infection) with steroid-refractory aGvHD receiving no MSCs showed the same trend (Figure 1B; HAdV, n=4; CMV, n=3; EBV, n=3). In contrast, the one-year survival in the control cohort with steroid-responsive patients was comparable for children with (n=7) and without (n=14) a viral infection (Figure 1C; HAdV, n=3; CMV, n=4; EBV, n=3). Although, not statistically significant (p=0.09), there was a trend to a higher percentage of viral infections and a longer duration of viremia in the MSC-treated children compared to patients responsive to steroids only (data not shown).

In contrast to CMV and EBV infections, HAdV infection was significantly associated with higher nonrelapse mortality (Figure 1D). HAdV infections which became appar-

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

UPN	MSC as 2nd line	MSC response[1]	Follow-up post HSCT	Cause of Death	Virus	Anti-viral treatment	Before steroids	Start steroids	MSC1	MSC1 + 30	MSC1 + 60	MSC1 + 90
2	No[2]	NR	Death (day + 99)	CMV + GvHD	CMV	(Val)ganciclovir	+	+	+	+	+	NA
11	Yes	CR	Alive (day + 1378)		CMV	(Val)ganciclovir	+	+	+	+	+	+
12	Yes	CR	Death (day + 371)	Relapse	CMV	(Val)ganciclovir	-	+	+	-	-	-
15	Yes	NR	Alive (day + 601)		CMV	(Val)ganciclovir	+	+	+	+	+	+
					EBV		+	+	+	-	-	-
17	Yes	PR	Death (day + 314)	Respiratory insufficiency,	CMV	(Val)ganciclovir	+	+	-	+	+	+
				Aspergillus infection, HAdV, CMV	HAdV	Cidofovir	+	-	-	+	-	-
19	Yes	CR	Alive (day + 487)		CMV	(Val)ganciclovir	+	-	+	-	+	-
					EBV	RTX	+	- (RTX)	-	-	-	-
20	Yes	PR	Death (day + 193)	interstitial pneumonitis	CMV	(Val)ganciclovir	+	+	-	+	+	+
				-> chronic GvHD	HAdV	Cidofovir	-	-	-	-	-	+
1	No[2]	CR	Death (day + 66)	Klebsiella pneumonia	HAdV	Cidofovir	-	-	-	+	NA	NA
4	No[2]	CR	Death (day + 1498)	Line infection; pneumonia	HAdV	Cidofovir	+	+	+	+	+	+
5	No[2]	PR	Death (day + 64)	MOF, HAdV	HAdV	Cidofovir	-	-	-	+	NA	NA
6	No[2]	NR	Death (day + 102)	MOF, HAdV,	HAdV	Cidofovir	-	-	-	-	+	NA
				klebsiella, EBV	EBV	RTX	-	-	+	- (RTX)	+	na
7	Yes	NR	Death (day + 53)	EBV, HAdV and	HAdV	Cidofovir	-	-	+	NA	NA	NA
				ongoing aGvHD	EBV	RTX	-	-	+ (RTX)	NA	NA	NA
3	No	CR	Alive (day+ 2708)		EBV		-	-	+	+	-	-
10	Yes	PR	Alive (day + 1497)		EBV	RTX	-	-	+	+	- (RTX)	-

[1]: response of aGvHD at 28 days after the first MSC infusion;

[2]: MSCs 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.

ent after start of MSC therapy were associated with a 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 a CMV (n=5) or HAdV (n=1) infection present before and persisting during aGvHD were alive at 1 year after HSCT (Table 1). Six patients in the MSC cohort, who all were from the first 3 years of the inclusion period,

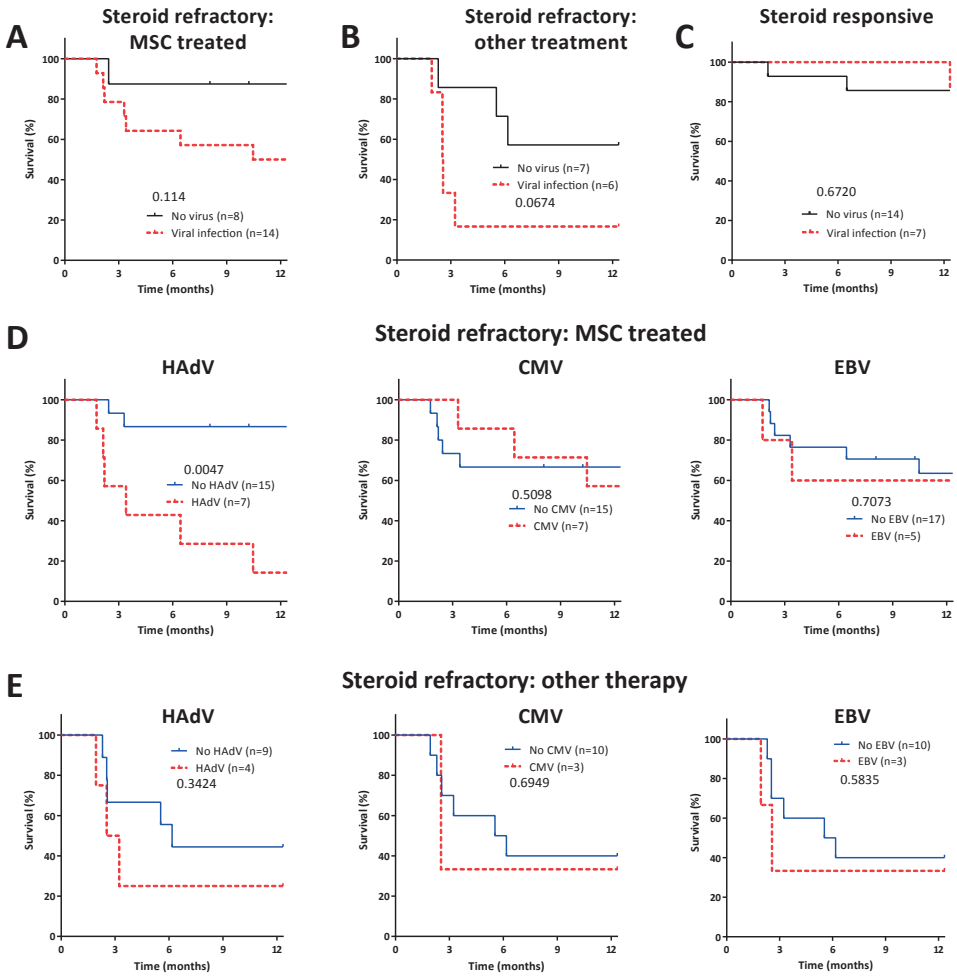

Figure 1 *HAdV infections negatively affect the survival of patients treated with MSCs for steroid refractory aGvHD.* A-C: The one-year survival of patients in relation to viral infections (HAdV, CMV and/or EBV) is depicted for steroid-refractory patients receiving MSCs (A) or other second-line therapy (B) and for steroid-responsive patients (C). D-E: HAdV, but not CMV or EBV was significantly associated with poor survival in patients treated with MSCs (D). This was not observed in steroid refractory patients receiving other therapies (E). Differences were assessed using the Log-rank Mantel-Cox test.

received other second-line treatment prior to MSC infusion. All these patients had a viral infection either prior to (n=3) or after MSC (n=3) 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 e). None of the three viruses was associated with decreased survival, as seen for HAdV in the MSC cohort (Figure 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 anti-viral immunity.

In vitro effect of MSC on virus-specific T cells

To determine the putative negative effect of MSCs on HAdV-specific T cells, PBMC 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 co-cultures (Figure 2A-B). To exclude bystander immune activation induced by TLR ligands or other non-specific virus-derived stimuli, experiments were also performed using synthetic, good manufacturing practices-grade, 15-mer viral peptides. In cultures of PBMC stimulated with HAdV hexon-derived peptides, proliferation and IFN-γ production were low, and no suppressive effect of MSCs was observed in either assay (Figure 2C-D). In contrast, after stimulation with synthetic CMV pp65-derived peptides, MSCs showed an inhibitory effect on proliferation and IFN-γ production (Figure 2C-D). 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 (Figure 2E-F).

Stimulation of PBMC with peptide-loaded autologous monocyte-derived dendritic cells

Conflicting results were obtained when investigating the interaction of MSCs and HAdV-specific T cells after employing two ways of stimulation and using two different read-out systems. Consequently, we decided to stimulate PBMC from healthy adult donors with peptide-loaded autologous mDC. MSCs suppressed PBMC proliferation after stimulation with CMV-peptides or HAdV-peptide-loaded mDC (Figure 3A). IFN-γ production was suppressed by MSCs in co-cultures after stimulation with CMV PepTivator with or without IL-2, (although not significantly, p=0.06), but not after stimulation with HAdV PepTivator (Figure 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.

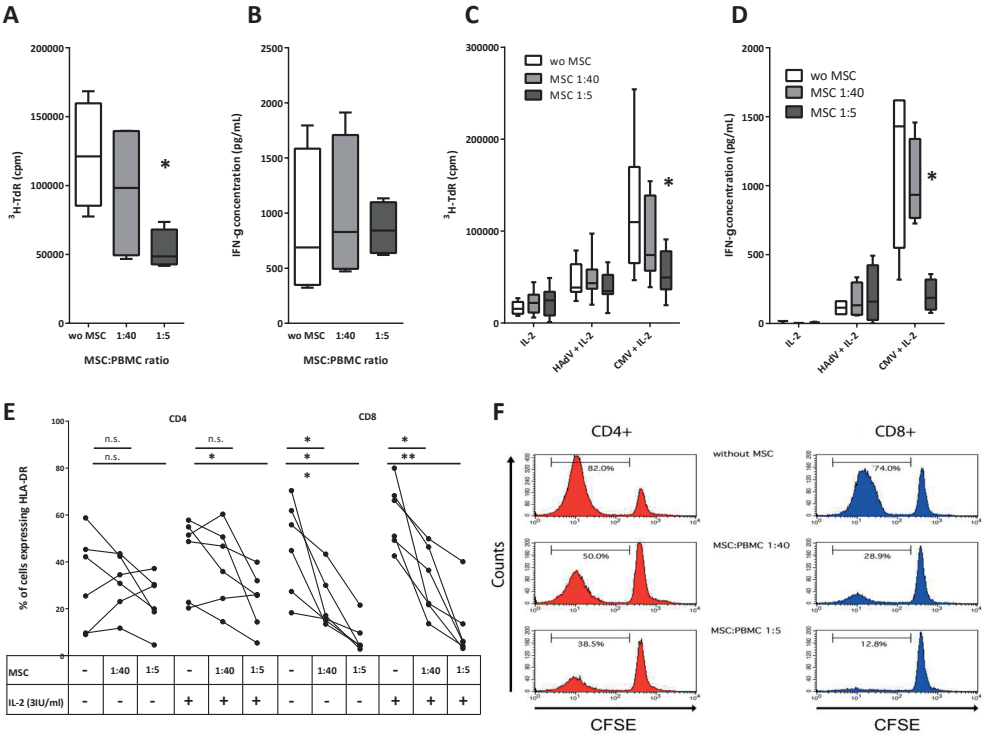

Figure 2. *MSCs suppress PBMC proliferation.* A-B: In cultures of PBMC stimulated with inactivated HAdV, proliferation at day 7 (A) was suppressed by the addition of MSCs, whereas IFN-γ concentration in supernatant obtained at day 6 (B) was not affected (n=4). C-D: After stimulation of PBMC with CMV (pp65 peptides) and IL-2 (3 IU/mL) proliferation (C) and IFN-γ concentrations (D) were lower in conditions with than without (wo) MSCs. Activation with HAdV (hexon peptides) plus IL-2 was lower compared to CMV plus IL-2 and was not affected by the addition of MSCs. E-F: In cultures of PBMC stimulated with CMV pp65 peptides, MSCs affected activation and proliferation of $CD4^+$ and $CD8^+$ T cells as demonstrated by decreased HLA-DR expression (E) and decreased CFSE dilution (F, representative picture of n=4 after stimulation with CMV pp65 peptides only), respectively. Box plots represent 4 independent experiments. Boxes represent median with standard deviation. Whiskers indicate minimum and maximum values. *: $p<0.05$; **: $p<0.01$ using paired Wilcoxon signed-rank tests.

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 cell in CMV-specific T cell lines varied (Figure S1A). 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 (Figure S1B). All cell lines were predominantly of effector memory phenotype (>50%) characterized by the lack of CCR7 and CD45RA expression (Figure S1C-D).

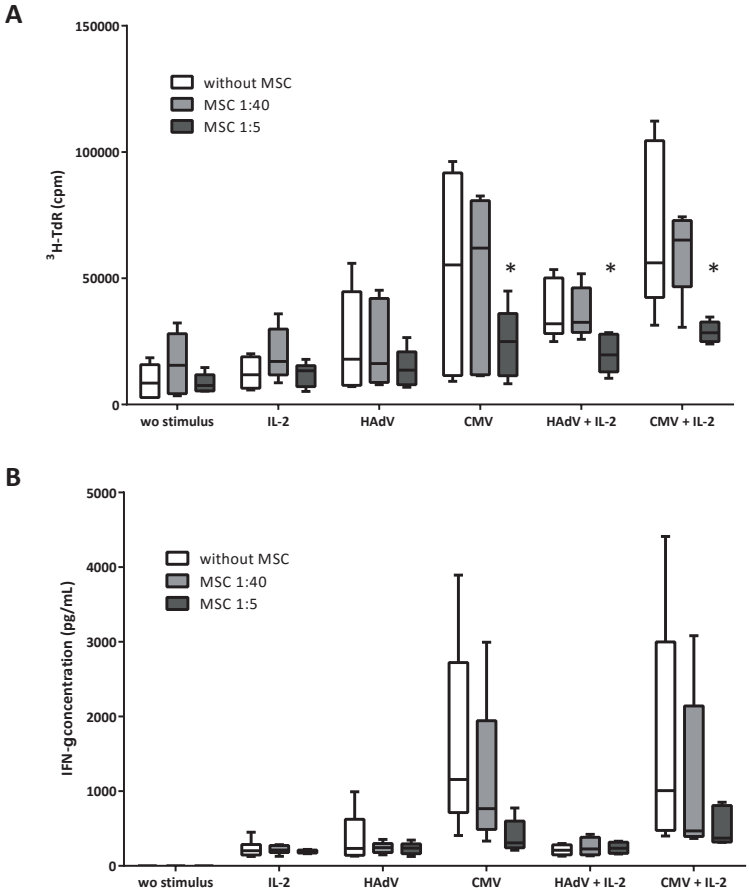

Figure 3. *Effect of MSCs on proliferation of and IFN-γ production by PBMC stimulated with peptide loaded autologous mature dendritic cells (mDC).* A: Proliferation after stimulation of PBMC with hexon peptides-loaded (HAdV) or pp65 peptides loaded (CMV) mDC was increased in co-cultures of MSC and PBMC at a ratio 1:5. In this set-up, a significant reduction in proliferation was observed. B: IFN-γ production remained relatively low for HAdV compared with CMV, and no suppression was documented after stimulation with HAdV hexon-derived peptides. Box plots represent data of six different experiments. Boxes represent median with standard deviation. Whiskers indicate minimum and maximum values. *: $p<0.05$ using paired Wilcoxon signed-rank tests. IL-2: 3 IU/mL.

Viral peptide-induced proliferation and IFN-γ production were suppressed in co-cultures of HAdV- and CMV-specific T cell lines with MSCs (Figure 4A-B). 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 (Figure 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 upon restimulation of such

T cell lines; in both cases suppression by MSCs was documented (Figure 4D-E). 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 aGvHD. At the start of MSC therapy two of the seven patients with CMV infection had an undetectable viral DNA load (UPN17 and UPN20; Table 1 and Figure 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 virema at the start of MSC therapy, the viremia resolved in this 90 days time window (UPN12 and UPN19) and persisted in the other three patients (UPN02, UPN11 and UPN15) (Table 1 and Figure 5A).

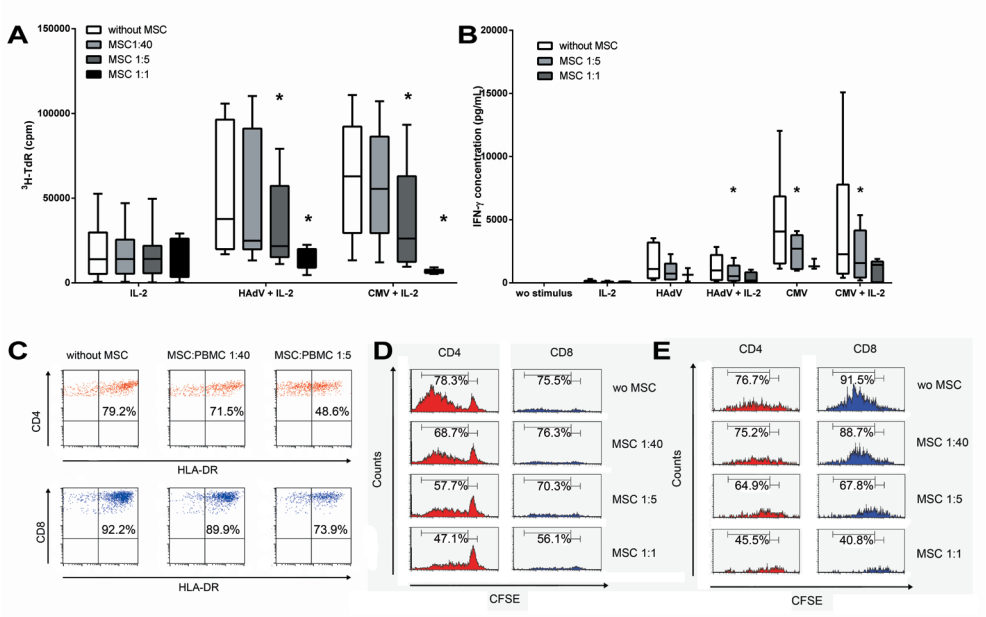

Figure 4. *Effect of MSCs on proliferation, IFN-γ production and HLA-DR expression by virus-specific T cell lines.* A-B: MSCs suppressed proliferation of and IFN-γ production by virus-specific T cell lines (n=6) stimulated with HAdV hexon peptides or CMV pp65 peptides plus IL-2 (3 IU/mL). C: HLA-DR expression was lower in HAdV-specific T cell lines stimulated with HAdV and IL-2 in co-cultures with MSCs. A representative experiment of three cell lines is shown. D-E: Dose-dependent suppression by MSCs of proliferation of both $CD4^+$ (D) and $CD8^+$ (E) T cells after stimulation with HAdV plus IL-2 measured by CFSE dilution was observed. Boxes represent median with standard deviation. Whiskers indicate minimum and maximum values. *: $p<0.05$ using paired Wilcoxon signed-rank tests.

In six of the seven patients with CMV infection, the frequency of IFN-γ producing cells was determined in the week before, 1-2 weeks after and 28 days after MSC infusion. These six patients received MSCs as second-line therapy. Both patients with

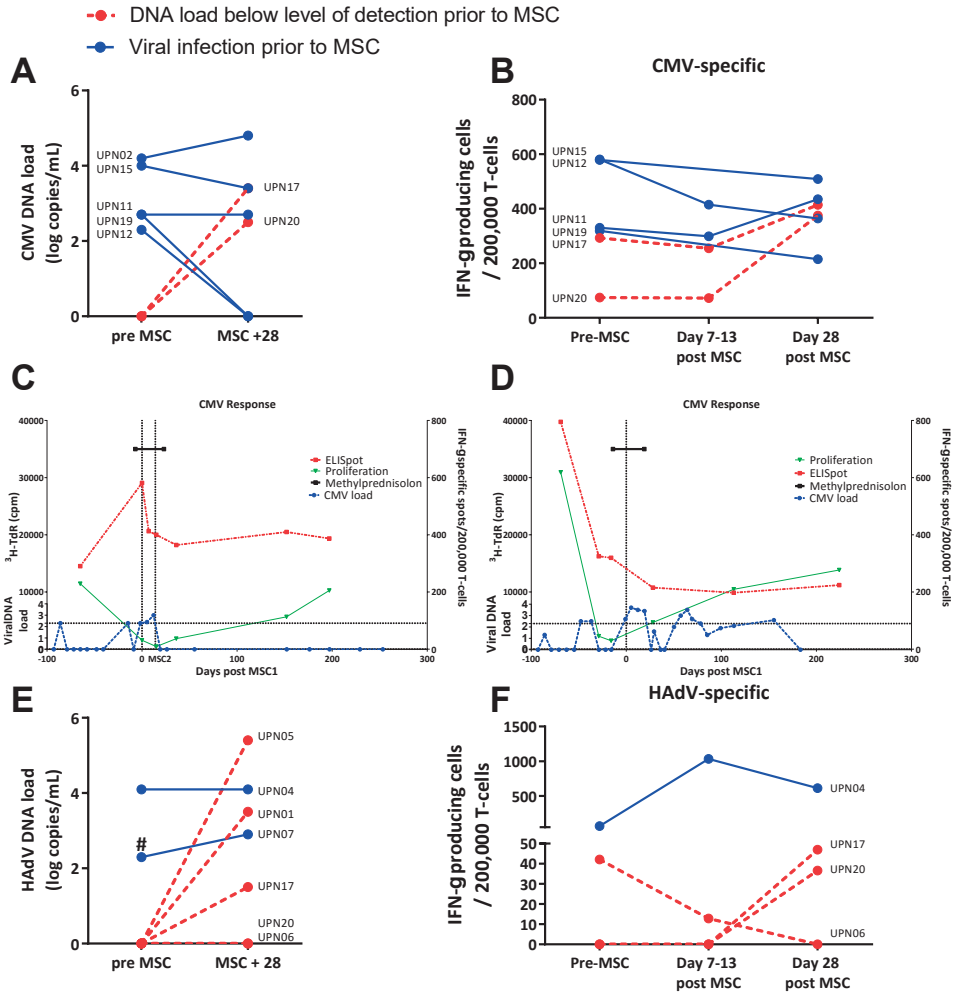

Figure 5 *Viral DNA loads increase around start of aGvHD despite the persistence of IFN-γ producing virus-specific T cells in patients treated with MSCs.* A-B: At 28 days after MSC infusion, CMV DNA loads were increased in two patients, UPN17 and UPN20, (A), however, the number of CMV-specific IFN-γ-producing cells was not affected (B). C-D: In two children UPN12 (C) and UPN19 (D), CMV-specific proliferation could be detected by ^{3}H-thymidine incorporation. Proliferation was decreased after start of steroids. E-F: In 5 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 three days prior to MSC infusion. UPN01, UPN02, UPN05, and UPN07: no PBMC were available for ELISpot assay.

Table 2. Steroid-unresponsive children receiving other second-line therapy: patient characterization and viral infections

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

Abbreviations: + or2, 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; EBV, Epstein-Barr virus; HAdV, humanadenovirus; HSCT, hematopoietic stem cell transplantation; MMF, mycofenolate mofetil; MOF, multiorgan failure; NA, not applicable; UPN, unique personal number.

recurrent viremia after MSC treatment (UPN17 and UPN020) 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 (Figure 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 a 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 (Figure 5C-D) with recurrence of CMV viremia prior to MSC infusion. Both had CR after MSC infusion which allowed tapering of steroids. CMV-induced proliferation 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 upon *ex vivo* HAdV stimulation of PBMC 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 (Figure 5F). The patient with high numbers of HAdV-specific IFN-γ-producing T cells (UPN04) experienced a persistent viremia from before the start of systemic steroids onward; however, this patient is the only survivor at one year. The other patients developed a HAdV infection after MSC infusion, and those occurred late for UPN06 (49 days after HSCT) and UPN20 (106 days after HSCT) (Table 1 and Figure 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.(von Bonin, 2009; Prasad, 2011; Le Blanc, 2004; Kebriaei, 2009; Ball, 2013) 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 data on the incidence of infections are focused on CMV.(von Bahr, 2012) The incidence of HAdV infections, which is especially relevant to children after HSCT (Chakrabarti, 2002; Flomenberg, 1994; Walls, 2003), 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 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, six out of seven children with an HAdV infection in this cohort were transplanted with a mismatched graft, thereby affecting 6 out of 10 children transplanted with such a graft, in line with previously reported data.(van Tol, 2005a) 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 MSC 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 out of 22 children (19 episodes of viral reactivation: 7 CMV, 5 EBV and 7 HAdV) included in our MSC-treated cohort.(Rao, 2009) 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 one-year survival for patients with (n=11) and without (n=11) viral dissemination in our MSC cohort is statistically different (36.4% vs 91.0%; respectively; p=0.01).

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.(Karlsson, 2008) 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.(Kang, 2005; Sundin, 2006) The latter observation is in line with our findings of a negative impact of MSCs on proliferation of both PBMC and virus-specific T cell lines after stimulation with CMV pp65 or HAdV hexon derived peptides loaded mDC or PBMC, respectively. The *in vitro* suppressive effect of MSCs on virus-induced T cell proliferation and IFN-γ production was MSC-dose dependent and most evident at high ratios of MSC 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 co-culture experiments using MSC and T cell clones specific for CMV, HAdV or control HY 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 a decreased homing of ovalbumin-pulsed dendritic cells to the lymph nodes.(Chiesa, 2011) An indirect effect of MSCs on lymphocyte activation and proliferation via monocytes has been suggested previously.(Groh, 2005) 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 infections after the initiation of MSC therapy. A negative impact of MSC 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. (Karlsson, 2008) Lucchini *et al.* came to a similar conclusion based on the analysis of IFN-γ-producing cells in two patients after MSC infusion.(Lucchini, 2012) 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 a viral reactivation that occured after initiation of MSC therapy, including three 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 extended this to HAdV-specific T cells. Of note, the level of IFN-γ-producing cells amongst peripheral blood T cells did correlate with the course of the CMV viremia or with 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 preced-

ing MSC infusion and continued thereafter. A decreased number of $CD8^+$ T cells after initiation of methylprednisolone has been reported previously,(Aubert, 2001; Cwynarski, 2001) in addition to decreased cytokine production without reduction of T cell counts.(Ozdemir, 2002) 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 PBMC prior to and after initiation of aGvHD treatment, whereas the proliferative capacity was hampered from the start of steroid infusion. This analysis could only be performed in two patients with sufficient numbers of PBMC 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 a fast reduction of methylprednisolone after MSC infusion seems to be of utmost importance in these patients following objective documentation of resolvement of the aGvHD symptoms.(Calkoen, 2013b) 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 of 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 virus 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 should also represent the ideal platform to further investigate potential side-effects of MSC, specifically with regard to viral reactivation.

Acknowledgements

We thank the medical, nursing and associated nonmedical personnel of the Pediatric Stem Cell Transplantation Unit of the Leiden University Medical Center for the excellent clinical care offered to the patients included in this study. We are grateful to Ann Vossen (Dept. of Medical Microbiology of the Leiden University Medical Center) for collecting data on viral infections in the cohort transplanted in the period 1994-2004. This study was supported by a grant from KIKA, the Dutch Children Cancer-Free Foundation (Grant 38).

Supplementary data

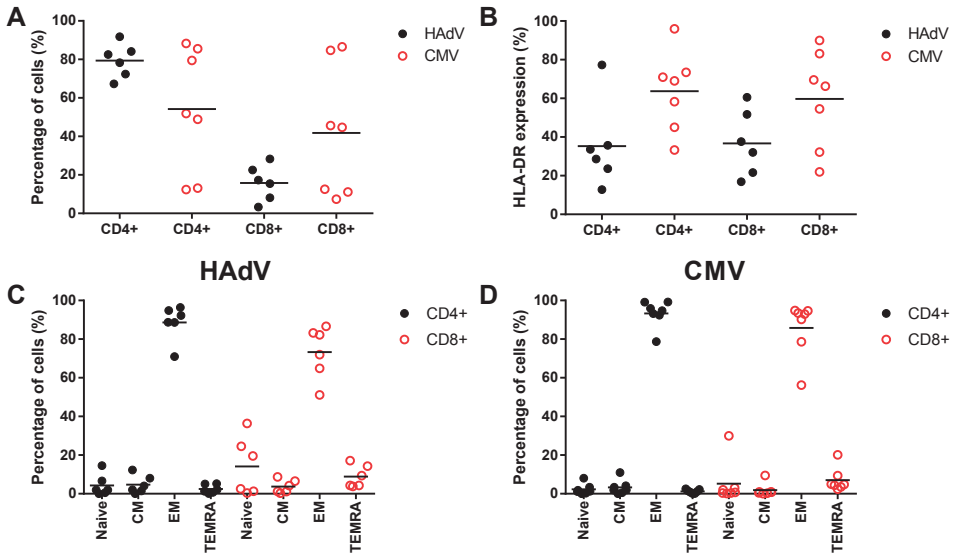

Figure S1: *Phenotype of virus-specific T-cell lines on day 28 after culture initiation.* T-cell lines raised against HAdV (hexon) or CMV (pp65) derived peptides were characterized by immunophenotyping. A: The percentage of $CD4^+$ and $CD8^+$ T-cells in the HAdV- and CMV-specific T-cell lines is depicted. B: The percentage of cells expressing HLA-DR on either $CD4^+$ or $CD8^+$ T-cells was higher in CMV-specific T-cell lines compared to HAdV-specific T-cell lines. C-D: Both HAdV-specific (C) and CMV-specific (D) T-cell lines consisted mainly of effector memory (EM) cells ($CD45RA^-CCR7^-$). Naïve, central memory (CM) and end stage effector (TEMRA) cells were defined as $CD45RA^+CCR7^+$, $CD45RA^-CCR7^+$ and $CD45RA^+CCR7^-$ cells, respectively.

Supplementary Table 1. Patient's characteristics

		Steroid refractory aGvHD; MSC	Steroid refractory aGvHD; no MSC	Steroid responsive aGvHD
N		22	13	21
Follow-up (days; median; range)		379 (53-2708)	166 (58-5463)	1123 (61-2575)*
Diagnosis	ALL/AML	9	2	13
	Other hematologic malignancies	6	6	4
	Nonmalignant disorder	7	5	4
Age at HSCT median (range)		6.3 (0.7-18.1)	5.2 (0.6-14.3)	9 (0.4-17.1)
Sex (M/F)		12/10	9/4	16/5
Donor	HLA-identical related	7	2	9
	Matched Unrelated	5	6	10
	Mismatched Unrelated	10	2	2*
	Other related	0	3	0
Stem cell source (BM/PBSC/CB)		13/4/5	9/4/0	17/3/1
Graft manipulation (none/TCD/CD34 selection)		20/1/1	10/2/1	21/0/0
Donor sex (M/F)		12/10	6/7	7/14
Conditioning	TBI based MAC	5	5	9
	Busulfan based MAC	14	7	10
	Other MAC	2	0	1
	RIC	1	1	1
Serotherapy	+ rATG/Campath	13/3	5/3	12/2#
GvHD prophylaxis	CsA	7	2	6
	CsA + MTX	10	9	13
	CsA + prednisolon (1 mg/kg)	5	0	1
	CsA + MMF	0	1	0
	none	0	1	1
Relapse		3	0	3
Median days between first MSC and start aGvHD (range)		12 (5-59)		
aGvHD organs involved	skin only	0	1	10***
	gut only	2	0	2
	gut and skin	10	8	6

Supplementary Table 1. Patient's characteristics (continued)

		Steroid refractory aGvHD; MSC	Steroid refractory aGvHD; no MSC	Steroid responsive aGvHD
	gut and liver	5	0	0
	skin and liver	0	0	3
	gut, skin and liver	5	4	0
aGvHD grade	II	0	7***	16***
	III	14	3*	5*
	IV	8	3	0**
aGvHD stage gut	0	0	1	11***
	1	0	5**	7*
	2	2	1	0
	3	16	3*	3***
	4	4	3	0
aGvHD treatment	Steroids up to 2 mg/kg	22†	13	21†
2nd line therapy	Total	6††	13***	0*
	Steroids 3-5 mg/kg	5	3	0
	MMF / Tacrolimus	1/2	6/0	0/0
	thalidomide / aCD25	0	3/1*	0

ALL acute lymphatic leukemia; AML acute myeloid leukemia; HSCT hematopoietic stem cell transplantation; HLA human leukocyte antigen; BM bone marrow; PBSC peripheral blood stem cells; CB cord blood; TCD T-cell depletion; TBI total body irradiation; MAC myeloablative conditioning; RIC reduced intensity conditioning; rATG rabbit anti-thymocyte globulin; aGvHD acute Graft-versus-Host Disease; CsA cyclosporin A; MTX methotrexate; MSC mesenchymal stromal cells; MMF mycophenolate mofetil. # one patient received Campath and rATG. † one patient received only 1 mg/kg prednisone. †† MSC were given as third-line therapy. Mismatched donor defined as: 5/6 (CB) or 9/10 HLA allele-matched (BM/PBSC). Grading and staging of aGvHD was according to the adapted Seattle criteria. (Ball LM, Egeler RM *BMT* 2008) Both control groups were compared to the MSC-group using Chi-squared and unpaired students T-tests: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.