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Clinical and laboratory features of mesenchymal stromal cells in pediatric stem cell transplantation

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Part 4

Viral Infections

“Where so many hours have been spent in convincing myself that I am right, is there not some reason to fear I may be wrong”?

- Jane Austen 1775-1817

Chapter 9

Mesenchymal stem cells exert differential effects on alloantigen and virus-specific T-cell responses

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Summary

Mesenchymal stem cells (MSC) suppress alloantigen-induced T-cell functions in vitro and infusion of third-party MSC appears a promising therapy for graft-versus-host-disease (GvHD). Little is known about the specificity of immunosuppression by MSC, in particular the effect on immunity to pathogens. We have studied how MSC affect Epstein-Barr virus (EBV) and cytomegalovirus (CMV)-specific T-cell responses. We found that EBV and CMV-induced proliferation and interferon- γ (IFN- γ) production from peripheral blood mononuclear cells (PBMC) was less affected by third-party MSC than the response to alloantigen and that MSC had no effect on expansion of EBV and CMV pentamer-specific T-cells. Established EBV-specific cytotoxic T-cells (CTL) or CMV-CTL cultured with MSC retained the ability to proliferate and produce IFN- γ in response to their cognate antigen and to kill virally infected targets. Finally, PBMC from 2 patients who received MSC for acute GvHD showed persistence of CMV-specific T-cells and retained IFN- γ response to CMV post MSC infusion. In summary, MSC have little effect on T-cell responses to EBV and CMV, which contrasts to their strong immunosuppressive effects on alloreactive T-cells. These data have major implications for immunotherapy of GVHD with MSC and suggest that the effector functions of virus-specific T-cells may be retained after MSC infusion.

Introduction

Human mesenchymal stem cells (MSC) can differentiate into a variety of tissues including bone, cartilage and muscle¹. MSC are found in low frequency in the bone marrow, but can be isolated and expanded in vitro. One important feature of MSC is their immunoregulatory functions. MSC suppress alloantigen and mitogen-induced proliferation²⁻⁴, interferon- γ (IFN- γ) production⁵ and cytolytic killing^{6,7} in vitro in a non-MHC restricted manner³, but the mechanisms of suppression by MSC are still largely unclear. MSC also seem to escape recognition of alloreactive cells^{3,8-10}. The immunomodulatory effects of MSC in the allogeneic setting have provided a rationale for the clinical use in graft-versus-host disease (GvHD). Severe acute GvHD after allogeneic stem-cell transplantation (SCT) is associated with high mortality, but infusion of third-party MSC appears a promising therapy for GvHD refractory to conventional immunosuppressive treatments^{11,12}.

Very little is known about the specificity of immunosuppression by MSC and, in particular, the effect on cell-mediated immunity to infectious pathogens. This is an important issue as infections are a major cause of morbidity and mortality after allogeneic SCT, particularly in the setting of intensive immunosuppression required for the treatment of GvHD. Two major viral pathogens in this setting are cytomegalovirus (CMV) and Epstein-Barr virus (EBV). Between 40-70% of SCT recipients who are CMV seropositive or have a seropositive donor develop CMV reactivation¹³⁻¹⁵, and this is frequently seen when patients are further immunosuppressed for GvHD. EBV reactivation may result in post-transplant lymphoproliferative disease (PTLD) and occurs in 11-26% of SCT patients where selective T-cell depletion has been used for prevention of GvHD¹⁶⁻¹⁸. Anti-viral T-cell effector functions are essential for preventing viral reactivation and progression to virus-associated disease. Thus, if MSC have regulatory effects on anti-viral cell-mediated immunity, administration of MSC to immunocompromised patients could exacerbate their susceptibility to infectious pathogens. Indeed, at least one patient treated with MSC for GvHD developed EBV-associated PTLD and there has been several cases of CMV-reactivation post MSC infusion¹². However, there is insufficient clinical experience as yet to determine whether administration of MSC affects the

development of virus-associated disease. We have therefore systematically studied how MSC affect anti-viral T-cell effector functions in vitro. Additionally, we have monitored cellular immune responses to CMV in 2 patients who received MSC for treatment of GvHD refractory to conventional therapies.

Methods

Donors and isolation of cells

Peripheral blood was taken from healthy EBV- and CMV-seropositive volunteers with their informed consent. Ethical approval for the study was obtained through the non-clinical institutional review board at University College London. PBMCs were isolated by density gradient centrifugation over Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden).

MSC were isolated and expanded from bone marrow taken from the iliac crest of adult volunteers, following the approval by the ethics Committee at Huddinge University Hospital, Sweden, and were cultured as previously reported³. Briefly, mononuclear cells were isolated from Percoll-separated bone marrow, resuspended in human MSC medium consisting of DMEM (Life Technologies, Gaithersburg, MD, USA), supplemented with 10% FCS (Sigma, St Louis, MO, USA) and antibiotic–antimycotic solution (Life Technologies). Cultures were maintained at 37°C for 2-4 passages and harvested by treatment with trypsin (Gibco BRL, Grand Island, NY, USA). The cells were classified as MSCs based on their ability to differentiate into bone, fat, and cartilage³ and by flow cytometric analysis (positive for CD44, CD29, CD73, CD166, and CD105 but negative for CD14, CD34 and CD45).

Patients

Patient 1 was a 3-year old boy with Kostmann's syndrome who was transplanted with PBSC from a single antigen mismatched unrelated donor in February 2005. He developed Grade IV steroid-refractory acute GVHD of the gut, which evolved into chronic GVHD refractory to immunosuppression. He received 1.7×10^6 third-party MSC/kg generated by Leiden University Medical Centre, the Netherlands, in March 2006. Prior to MSC infusion this patient had recurrent CMV viraemia associated with retinitis. He had a good transient clinical and histological response to MSC from 14

days to 6 weeks after infusion. Patient 2 was an 8-year old boy with relapsed juvenile myelomonocytic leukemia who developed Grade IV steroid-refractory acute GVHD of the skin and gut, evolving into extensive chronic GVHD after a second matched unrelated donor PB SCT in August 2006. He received 2.5×10^6 third-party MSC/kg generated by Imperial College School of Medicine, London in June 2007 and had a good partial clinical response which has been durable to date. He is currently reducing immunosuppression 3 months post-MS infusion. The prospective phase I/II study was approved by Leiden University Medical Centre scientific and medical ethical review board.

Viral antigens and vectors

EBV-transformed B-lymphoblastoid cells lines (LCLs) were generated by culturing PBMC with concentrated EBV-containing supernatant of cultured B95-8 cells using standard protocols¹⁹.

The pp65 pepmix, which consists of 138 different pp65 peptides restricted by both MHC class I and class II, were purchased from JPT Peptide Technologies GmbH (Berlin, Germany).

We used 2 adenoviral vectors carrying either the eGFP gene alone (Ad5f35-GFP) or a transgene coding for a fusion protein consisting of the immunodominant CMV antigen pp65 and eGFP (Ad5f35pp65-GFP) (described in detail elsewhere²⁰). These vectors were purchased from Baylor College of Medicine (Houston, TX).

Generation of virus-specific CTL

EBV-specific CTL lines were generated by stimulating PBMC with autologous LCL for a total of 4 rounds of stimulation. For the first stimulation, 2×10^6 PBMC were cultured with 5×10^4 irradiated (40 Gy) autologous LCL per well (40:1) on 24 well plates in CTL-medium (45% Click's medium, Irvine Scientific, Santa Ana, CA, 45% RPMI 1640, Hyclone, Logan, UT and 10% FCS, Hyclone) for 9–10 days. The CTL (10^6 /well) were then stimulated weekly with LCL at a 4:1 ratio for 7 days. On day 14, the cultures were supplemented with 40 U/ml IL-2 and subsequently feed twice weekly. Generation of CMV-specific CTL has been described in detail elsewhere²⁰. Briefly, Ad5pp65GFP-

transduced autologous monocytes were used for the first round of stimulation followed by 3 weekly stimulations with Ad5pp65GFP-transduced autologous LCL.

Proliferation assay

PBMC were plated in triplicates at 1.5×10^5 per well in 96-well plates in CTL-medium and were stimulated with irradiated autologous LCL (3.75×10^3 per well), Ad5f35-vector (20 infectious units/cell), pp65 pepmix (1 $\mu\text{g/ml}$) or irradiated allogeneic PBMC (1.5×10^5 per well) in the absence or presence of irradiated MSC (MSC/effector cell ratio 1:10). EBV-CTL (10^5 per well) were cultured with irradiated autologous LCL (5×10^3 per well) in the presence or absence of MSC (MSC/effector ratio 1:10). After 4 days, PBMC or EBV-CTL were pulsed with 1 μCi ^3H -thymidine (Amersham Biosciences, Little Chalfont, UK) per well for 16 h. To study the proliferative response of CD45RA^+ and CD45RO^+ T-cells, CD3^+ T-cells were first separated by Pan T-cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany) from normal donor PBMC followed by CD45RO -bead positive selection (Miltenyi) according to manufacturers' instruction. $\text{CD3}^+\text{CD45RA}^+$ or $\text{CD3}^+\text{CD45RO}^+$ cells were stimulated with allogeneic dendritic cells (DC, 5:1 ratio) or with PHA (10 $\mu\text{g/ml}$) in the absence or presence of MSC for 4 days. ^3H -thymidine incorporation was measured with a MicroBeta TriLux (Perkin-Elmer Weiterstadt, Germany). The data are presented with the response from unstimulated responding cells, stimulating cells alone and irradiated MSC alone subtracted from the response of test cultures.

Enzyme-linked immunospot assay (Elispot) assay

Elispot assay was used to determine the frequency of virus- or allo-specific $\text{IFN-}\gamma$ producing cells using anti- $\text{IFN-}\gamma$ monoclonal antibodies purchased from Mabtech, Stockholm, Sweden (described in detail elsewhere²⁰). The following stimulators were used to monitor antiviral or allo-responses of PBMC or CTLs: autologous LCLs (irradiated at 40 Gy), PBMC pulsed with pp65 pepmix (1 $\mu\text{g/ml}$) or allogeneic PBMC (irradiated at 30 Gy). PBMC and CTL that had been stimulated in the presence or absence of MSC for 5 days were serially diluted from 5×10^4 to 6.25×10^3 and 2×10^4 to 2.5×10^3 cells per well, respectively, and plated with 10^5 irradiated stimulators per well in 200 μL AIM-V serum-free medium (Invitrogen, San Diego, CA) in triplicates and incubated for 18 h. The virus- or allospecific cell frequency was expressed as the mean

specific spot-forming cells (SFC) after subtracting the background, i.e. the frequency of unstimulated responding cells and of stimulators alone. Linear regression analysis was used to determine the number of specific SFC per 10^5 cells.

ELISA

IFN- γ and IL-10 levels in supernatants from PBMC stimulated with pp65 peptides or irradiated allogeneic PBMC for 5 days were analyzed by ELISA according to manufacturers' instructions (R&D Systems, Minneapolis, MN).

Flow cytometry

For all flow-cytometric analysis, a CyAn flow cytometer (Dako, Fort Collins, CO) was used to acquire data and Summit v4.1 software (Dako) to analyze data.

Phenotype and pentamer staining: All anti-human monoclonal antibodies were purchased from Becton-Dickinson (BD, Erembodegum, Belgium), except for Foxp3 (Clone 236A/EF, eBioscience). The following PE-labelled pentamers were used to detect viral-specific CD8⁺ T-cells (ProImmune, Oxford, United Kingdom): CMV- pp65-specific HLA-A*0201-NLVPMVATV (A2-NLV), HLA-B*0702-RPHERNGFTVL (B7-RPH) and HLA-B*0702-TPRYTGGGAM (B7-TPR); EBV- BMLF-1 specific HLA-A*0201-GLCTLVAML (A2-GLC), EBNA-3 specific HLA-B*0702-RPPIFIRRL (B7-RPP) and BZLF-1 specific HLA-B*0801-RAKFKQLL (B8-RAK). CTL were stained with pentamers and for expression of CD8 and CD3. PBMC from normal donors negative for the restricting HLA-type were used as additional negative controls. The percentage of pentamer-positive cells in the CD3⁺/CD8⁺ lymphocyte gate was expressed as a proportion of the CD8⁺ cells with the unstained control subtracted.

Intracellular cytokine staining: PBMC or CTL stimulated with autologous LCL or pp65-pulsed PBMC in the presence or absence of MSC (MSC/effector ratio 1:10) for 5 days were restimulated over night with corresponding stimuli with or without MSC. The staining was performed according to manufacturers' instructions with minor modifications (BD). Briefly, the cells were stained with surface antibodies, fixed with Cytofix (BD) and permeabilized with 0.25% saponin buffer before incubation with anti-IFN- γ antibody or corresponding isotype control antibody (BD). For co-staining

with pentamers and intracellular IFN- γ , the pentamer staining was performed before the overnight stimulation according to manufacturers' instructions (Prolimmune).

CFSE staining

EBV-CTL were labeled with 0.6 μ M CFSE (Invitrogen, Paisley, UK) in AIM-V medium for 15 minutes at 37° C and stimulated with irradiated autologous LCL (4:1 ratio) in the presence or absence of MSC for 5 days and thereafter stained for CD3.

Cytotoxicity assay

A standard 5h ^{51}Cr release assay was performed to determine the virus-specific cytolytic activity of the CTLs (described in detail elsewhere²⁰). LCLs were incubated with 2 μ g/ml pp65 pepmix the day before the assay. HSB-2 cells were used as a control for MHC-unrestricted killing (kind gift from Dr C. Rooney, Houston). Target cells were labeled with 100 μ Ci ^{51}Cr (Amersham Pharmacia Biotech, Piscataway, NJ) and were plated at 5×10^3 cells per well and cultured with CTL at different concentrations (effector to target ratios: 30:1, 5:1 and 1:1) in 96-well V-bottom plates. CTL cultured in the presence of MSC for 5 days were plated with MSC (MSC/effector ratio 1:10) during the assay. To control for lysis of MSC by virus-specific CTL, ^{51}Cr labeled MSC were pulsed with pp65 or cultured with cold LCL and plated with effector cells as described above.

Real-time quantitative PCR

MSC were cultured with PBMC stimulated with irradiated allogeneic PBMC or pp65 peptides in transwell system plates for 3 days. MSC were harvested and RT-PCR was performed as previously described²¹.

Statistical analysis

Wilcoxon signed rank test (GraphPad Prism) was used to compare the viral or alloantigen-induced proliferation of PBMC or IFN- γ SFC in the absence or presence of MSC (figure 1a and 2a). Friedman test followed by Dunn's multiple comparison test was used to analyze the percentage of CD25^{high}Foxp3⁺ cells after different stimulations in the presence or absence of MSC (figure 6b).

Results

MSC potently suppress alloantigen-induced proliferation of PBMC, but have little effect on viral-induced proliferation.

We first examined if third-party MSC have a suppressive effect on proliferation of PBMC to viral antigens. PBMC from 6 normal donors were stimulated with autologous LCL, pp65 peptides, Ad5f35-vector or allogeneic PBMC in the absence or presence of MSC to study the proliferative response to EBV, CMV, Ad, and alloantigens, respectively. As previously shown, we found that MSC strongly inhibited alloantigen-induced proliferation at an MSC to PBMC ratio of 1:10 (suppression $61.5 \pm 24.0\%$, mean $\pm$ SD) compared to control cultures without MSC (figure 1a). MSC had less, but still statistically significant, effect on the proliferative response to EBV at an MSC to PBMC ratio of 1:10 (suppression $42.3 \pm 11.5\%$), but had no suppressive effect on the response to CMV or Ad (figure 1a).

To further study how MSC affect the expansion of virus-specific T-cells when stimulated with their cognate antigens, we next cultured PBMC with autologous LCL or pp65 peptides in the presence or absence of MSC for 7 days and stained the cells with virus-specific pentamers. Stimulation with LCL or pp65 peptides resulted in a significant expansion of pentamer positive T-cells and MSC had no effect on the expansion of EBV or CMV-specific CD8⁺ T-cells (n=4). A representative FACS plot is shown in figure 1b.

MSC do not inhibit viral-induced IFN-gamma production in PBMC.

Previous reports have shown that MSC inhibit alloantigen-induced IFN- γ production⁵. We next studied whether MSC have a similar suppressive effect on IFN- γ production from PBMC stimulated with viral antigens. We cultured PBMC from 6 donors with autologous LCL, pp65 peptides or allogeneic PBMC for 5 days in the presence or

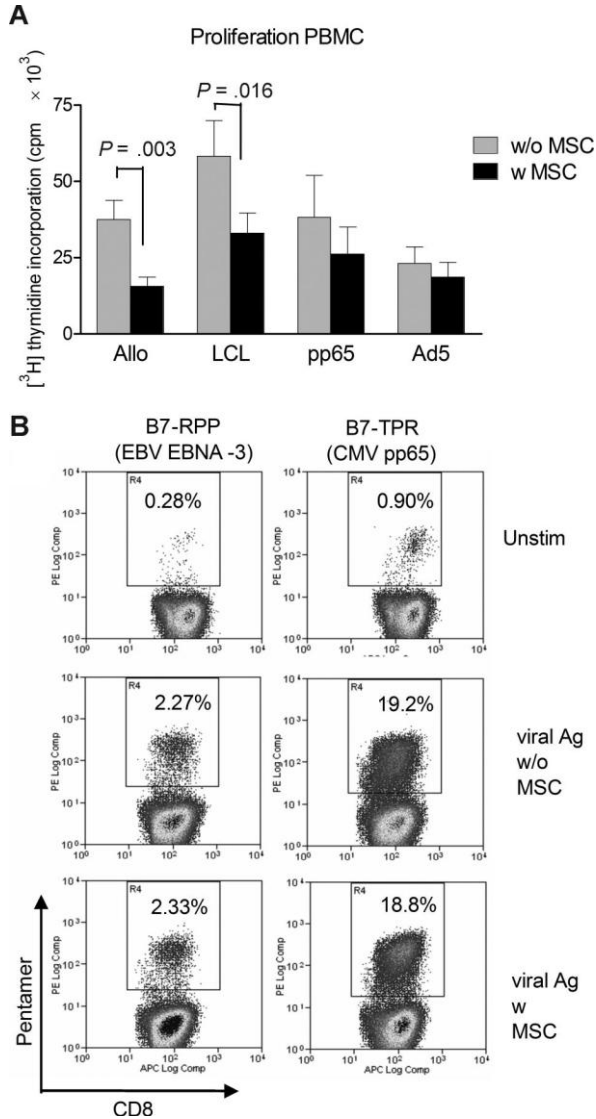


Figure 1. Effect of MSC on proliferation of PBMC in response to allo- or viral antigens.

(A) PBMC (1.5×10^5 per well) from normal donors were stimulated with irradiated (irr) allogeneic (allo) PBMC, irr autologous (autol) LCL, pp65-peptides or recombinant Ad5 viral vector in the presence or absence of third-party MSC (1.5×10^5 per well) for 5 days in (n=6). P-values refer to difference in proliferation in the presence or absence of MSC (Wilcoxon signed rank test). (B) Representative pentamer stainings of PBMC gated on CD3⁺CD8⁺ T-cells cultured with irr autol LCL or pp65-peptides in the presence or absence of MSC for 7 days.

absence of third-party MSC. Primed cells were harvested and restimulated with the same antigen over night in an IFN- γ Elispot assay. We found that MSC had no effect on the proportion of IFN- γ spot forming cells (SFC) in response to CMV in any of the donors and only a marginal (mean 31%), although significant ($P=0.03$), effect on the response to EBV (figure 2a). In contrast, the number of cells producing IFN- γ in response to allogeneic stimulation was considerably reduced in the presence of MSC in all 6 donors. Overall, there was a mean 76% suppression in cells producing IFN- γ in response to alloantigen ($P=0.02$). MSC were not added to the Elispot assay because they are adherent cells which block effector cells from interacting with the anti-IFN- γ antibody coated filter. To confirm that virus-specific T-cells had not recovered their capacity to produce IFN- γ in the absence of MSC during the Elispot incubation, we stained virus-stimulated T-cells from 2 donors that were cultured with MSC for 6 days for intracellular production of IFN- γ . When gated on CD3⁺ T-cells, we found no difference in the percentage of T-cells producing IFN- γ in response to LCL or pp65-peptides if they were cultured in the continual presence or in the absence of MSC (representative FACS plots in figure 2c). LCL and pp65-peptides mainly induced IFN- γ production from the CD8⁺ fraction of T-cells (data not shown) and there was no suppression of IFN- γ production in the CD8⁺ T-cell.

MSC do not suppress proliferation of established EBV- or CMV-specific CTL lines.

Having established that MSC have differential effects on virus- and alloantigen-induced responses from PBMC, which contain T-cells, NK cells, B cells and monocytes, we next examined how MSC influence anti-viral responses in established EBV and CMV-specific T-cell lines. The CTL cultures mainly consisted of CD3⁺ T-cells (>98%), of which the majority were CD8⁺ (mean 66.1 % for EBV-CTL and 95.5% for CMV-CTL), but there was also a proportion of CD4⁺ T-cells (mean 32.2 % for EBV-CTL and 4.5% for CMV-CTL). Established EBV-CTL lines from 5 donors were stimulated with autologous LCL and CMV-CTL from 2 donors were cultured with pp65-pulsed LCL in the presence or absence of third-party MSC for 5 days. The cells were then harvested

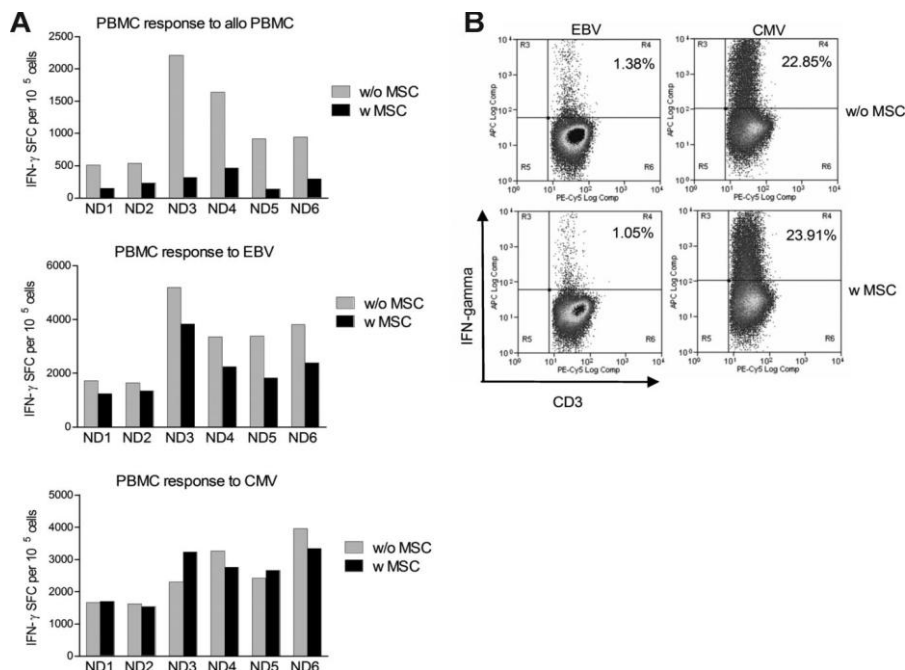


Figure 2. Effect of MSC on viral or allo-induced IFN- γ production from PBMC.

(A) PBMC from 6 normal donors (ND) were stimulated with irr allo PBMC, irr autol LCL or pp65-peptides in the presence or absence of MSC for 5 days. Cells were then analyzed by ELISPOT to determine the frequency of IFN- γ spot forming cells (SFC). (B) Representative FACS plot of intracellular IFN- γ staining from PBMC gated on CD3⁺ T-cells compartment to either EBV (5.10% without MSC vs 5.27% with MSC) or CMV (28.9% vs 31.3%).

and counted. We found that CTL expanded equally well in the absence or presence of MSC (2.66-fold $\pm$ 0.30, mean $\pm$ SD, without MSC vs 2.45-fold $\pm$ 0.46 with MSC). To determine if MSC affected expansion of virus-specific CTL within these cultures, we stained CTL lines with EBV- and CMV-pentamers (n=3 and 2, respectively) and found that the percentage of pentamer-positive cells was unaffected by the presence of MSC. Representative pentamer stainings of T-cells recognizing epitopes from EBV peptides from 2 donors and CMV pp65 peptides from 2 donors are shown in figure 3a and b, respectively.

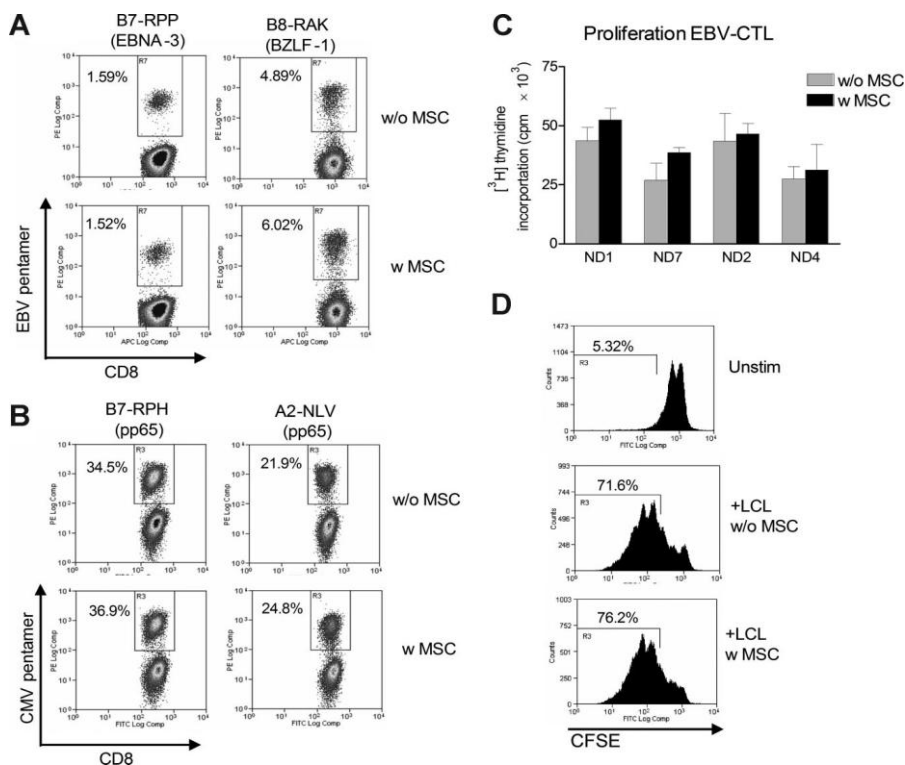


Figure 3. MSC have no effect on expansion of EBV or CMV-specific CTL.

Established EBV-CTL (A) or CMV-CTL (B) were stimulated with irr autologous LCL or pp65-pulsed LCL, respectively, in the presence or absence of MSC for 5 days and stained with virus-specific pentamers. Representative FACS plot for stainings with 2 EBV-pentamers and 2 CMV-pentamers gated on CD3⁺CD8⁺ T-cells are shown. (C) Four EBV-CTL lines were stimulated with irr autol LCL in the presence or absence of MSC for 5 days and analyzed for ^3H -thymidine incorporation. (D) Representative histogram from one of the EBV-CTL lines (gated on CD3⁺ T-cells) showing similar dilution of CFSE dye after stimulation with LCL when cultured with or without MSC.

As noted above, we found that MSC partially inhibited proliferation of PBMC in response to autologous LCL. However, MSC had no effect on proliferation of established EBV-CTL lines in response to autologous LCL in any of the 4 donors analyzed (figure 3c). To further confirm this finding, we stained EBV-CTL from 2 donors with CFSE and stimulated the cells with LCL in the presence or absence of MSC. As shown in the representative histograms in figure 3d, MSC had no effect on the proliferation of EBV-CTL as assessed by CFSE dye dilution.

MSC do not suppress IFN- γ production in established virus-specific CTL.

We next investigated the effect of MSC on antigen-induced cytokine secretion in established virus-specific CTL lines. CTL that had been stimulated with their cognate antigen (LCL or pp65-pulsed LCL) in the presence or absence of third-party MSC for 5 days were analyzed for IFN- γ secretion using Elispot assays. As demonstrated in figure 4a, the presence of MSC in CTL cultures had no effect on the frequency of T-cells secreting IFN- γ in response to EBV and CMV antigens. In contrast, MSC had a suppressive effect on IFN- γ production from allospecific CTL generated from PBMC stimulated with irradiated allogeneic PBMC for 4 rounds (mean 57% suppression). Additionally, EBV-CTL lines and CMV-CTL lines were further analyzed by intracellular IFN- γ . Again, MSC did not affect the ability of established virus-specific CTL to produce IFN- γ , either in CD4⁺ or CD8⁺ T cells. Representative FACS plots of CD3⁺CD8⁺ and CD3⁺CD4⁺ cells in one EBV-CTL line and CD3⁺CD8⁺ cells in one CMV-CTL line are shown in figure 4b (CD4⁺ fraction too low in CMV-CTL line for analysis).

To be certain that MSC have no effect of IFN- γ production from virus-specific T-cells within these cultures, we next co-stained 3 EBV-CTL lines and 2 CMV-CTL lines after antigenic stimulation in the presence or absence of third-party MSC with pentamers and intracellular IFN- γ . As shown in figure 4c, IFN- γ production from pentamer-positive CTL recognizing epitopes from EBV and CMV in response to stimulation with their cognate antigen was not suppressed by MSC.

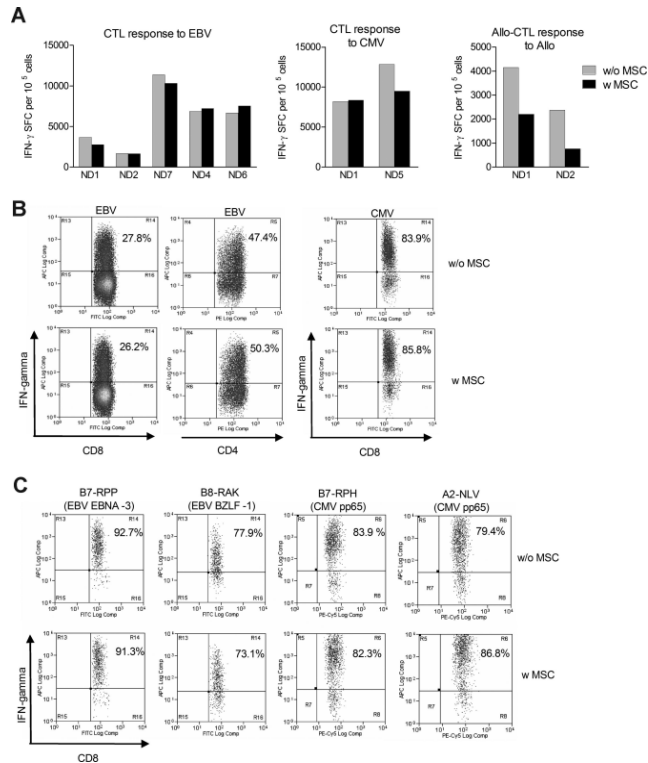


Figure 4. MSC do not affect IFN- γ production from EBV or CMV-specific CTL.

(A) Proportion of EBV-CTL (n=6), CMV-CTL (n=2) or allo-CTL (n=2) producing IFN- γ in response to irradiated autologous LCL, pp65-pulsed LCL or irradiated allogeneic PBMC, respectively, in the presence or absence of MSC were analyzed with ELISPOT. (ND=normal donor) (B) Representative FACS plots of intracellular IFN- γ staining of EBV-CTL gated on CD3⁺CD8⁺ or CD3⁺CD4⁺ T-cells and CMV-CTL gated on CD3⁺CD8⁺ T-cells that were stimulated with corresponding viral antigen in the presence or absence of MSC. (C) Representative stainings of EBV- and CMV-pentamer positive cells producing IFN- γ in response to viral antigens in CTL cultured in the presence or absence of MSC.

MSC do not inhibit cytotoxic killing of EBV or CMV-targets by virus-specific CTL.

Previous studies have shown that MSC suppress cytotoxic killing of allogeneic targets by alloreactive T-cells⁶ and we next examined how the cytotoxic activity of virus-specific CTL is affected by MSC. We found that EBV-CTL that had been cultured in the presence MSC for 5 days and during the cytotoxicity assay killed autologous LCL equally well as EBV-CTL cultured without MSC (figure 5a). Similar experiments

performed with CMV-CTL showed that MSC did not suppress the ability of CMV-CTL to lyse pp65-pulsed autologous LCL (figure 5b). The lysis of allogeneic targets and HSB-2 cells was low, confirming that the observed cytotoxicity was EBV-specific or CMV-specific and MHC-restricted. To control for lysis of third-party MSC by virus-specific CTL during the stimulation, we examined whether ^{51}Cr labeled MSC that had been cultured with cold LCL or pulsed with pp65 were killed by CTL. MSC alone were not killed by CTL and neither were MSC cultured with LCL or pulsed with pp65 antigen (figure 5c). These data demonstrate that MSC have little effect on the cytotoxic T-cell effector functions of established viral-specific T-cells.

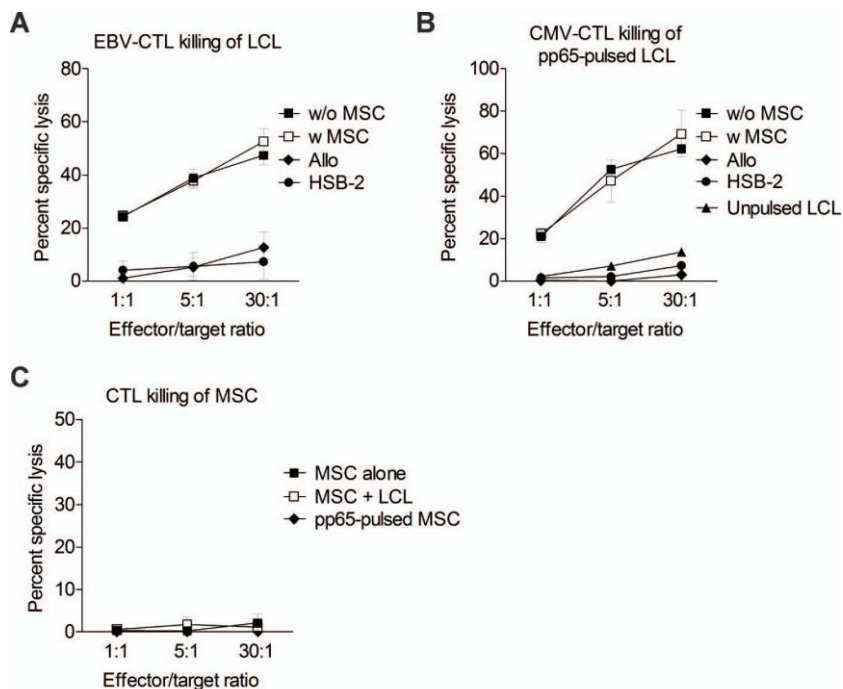


Figure 5. MSC have no effect on cytolytic killing of virally infected target cells by virus-specific CTL.

(A) EBV-CTL or (B) CMV-CTL cultured with EBV or CMV antigens, respectively, for 5 days in the presence or absence of MSC were analyzed for cytolytic activity of LCL or pp65-pulsed LCL, respectively, by a standard ^{51}Cr release assay. Allogeneic LCL and HSB-2 cells were used to control for MHC-restricted killing. (C) Negligible killing of MSC cultured together with LCL or pulsed with pp65 peptides by virus-specific CTL. The bars show the mean specific lysis by (A) 5 different EBV-CTL, (B) 2 different CMV-CTL lines and (C) 2 EBV-CTL and 2 CMV-CTL. The error bars illustrate the SEM.

MSC suppress proliferation of both naïve and memory T-cells.

In the present study we only used cells from CMV- and EBV-seropositive donors, simply because virus-specific responses generally are undetectable in vitro in seronegative donors due to low precursor frequency. Thus, the measured virus-specific T-cells are predominantly of memory phenotype. In contrast, normal donor allospecific T-cells reside mainly in the naïve T-cell compartment²⁶, although a small number of alloreactive T-cells may cross-react with conventional antigens presented on self MHC²⁷ and thereby have acquired a memory phenotype. We next examined if the differential effects of MSC on viral-specific and alloreactive T-cells are due to differential effects of MSC on naïve and memory T-cells. We separated CD3⁺ T-cells from 2 normal donors into CD45RA⁺ and CD45RO⁺ fractions and assayed proliferation after stimulation with allogeneic dendritic cells (DC) or PHA in the presence or absence of third-party MSC. We found that MSC suppressed allo- or mitogen-induced proliferation in both CD45RA⁺ naïve and CD45RO⁺ memory T-cells (figure 6A). This indicates that the differential effects of MSC on antiviral and alloreactive T-cell effector functions are unlikely to be due to preferential suppression of naïve T-cells by MSC.

Phenotype of virus- and allo-stimulated PBMC and MSC

MSC have been suggested to be involved in the generation of CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Treg)^{5,7,22}, and we examined if the differential suppressive properties of MSC on viral- and allospecific T cells could be explained by that MSC have different effects on the differentiation Tregs in these settings. We found that the proportion of CD25^{high}Foxp3⁺ in CD4⁺ T cells was significantly increased in unstimulated PBMC in the presence of MSC, but that MSC had no effect on Treg expansion in either CMV-specific or allo-stimulated PBMC (figure 6b). However, we found that the proportion of T-cells with regulatory phenotype was markedly higher in general in the allostimulated setting than in CMV-stimulated cells. In line with these findings, we observed that the cytokine profile of CMV- and allostimulated PBMC was different with respect to production of anti- and proinflammatory cytokines. Although IL-10 was produced in similar levels in both settings, IFN- γ was secreted at

much higher levels from CMV-stimulated cells (figure 6c) The mean IFN- γ /IL-10 ratio was 12.2 (range 6.2-39.9) and 10.6 (3.3-39.8) in response to pp65 in the absence and presence of MSC, respectively, whereas the corresponding figures for allostimulated cells were 1.1 (range 0.64-1.8) and 0.91 (0.62-1.5). Thus, a higher proportion of Tregs along with a cytokine milieu with suppressive character in allostimulated cells might make these cells more assessable to suppression by MSC.

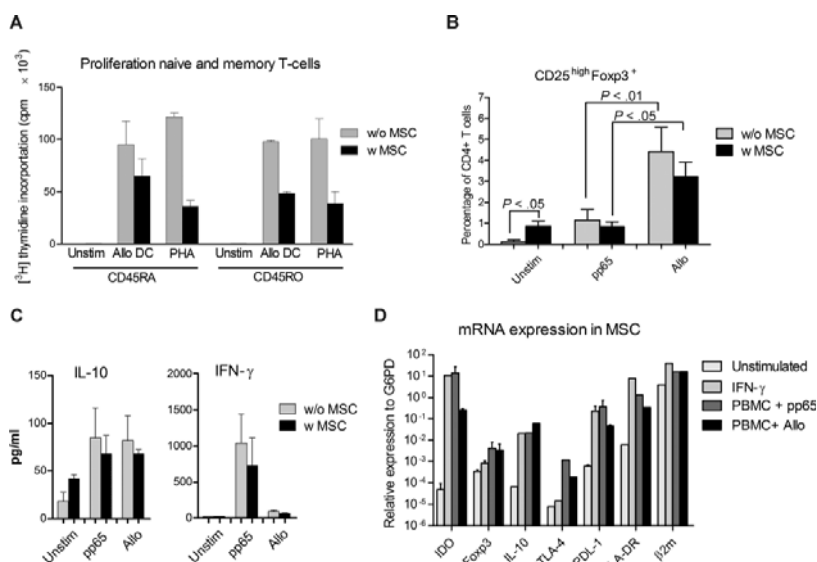


Figure 6. Effect of MSC on naïve and memory T cells and phenotype of T cells and MSC under different culture conditions.

(A) CD3⁺ T-cells were separated into CD45RA⁺ and CD45RO⁺ subsets and stimulated with allo DC or PHA in the presence or absence of MSC. Proliferation was analyzed by ^3H -thymidine incorporation. Bars illustrate the mean proliferation from 2 different normal donors and the error bars show the SEM. (B) Percentage of CD25^{high}Foxp3⁺ cells in CD4⁺ T-cells after stimulation with pp65 peptides or allogeneic PBMC for 5 days in the presence or absence of MSC (n=6). The difference in expression of CD25 and Foxp3 between the different culture conditions was analyzed by Friedman test followed by Dunn's multiple comparison tests. (C) IFN- γ and IL-10 levels measured in supernatants from PBMC cultured with indicated stimulation with or without MSC for 5 days (n=6). (D) mRNA expression in MSC cultured in transwell system with PBMC stimulated with pp65 peptides or allogeneic PBMC or with 100 U/ml IFN- γ for 3 days, analyzed with quantitative RT-PCR.

Comparison of other phenotypic markers in virus and allo-stimulated PBMC showed that MSC had no effect on the proportion of CD4⁺ or CD8⁺ T cells or expression of CD25, CD69 and PD-1 on CD3⁺ T cells (n=6, data not shown).

We further examined how the expression of genes implicated in suppression was affected in MSC cultured with CMV-stimulated or allo-stimulated PBMC. We found that mRNA expression of IDO was profoundly upregulated in MSC after culture with both allo and virus-stimulated PBMC, as well as with 100 U/ml IFN- γ (figure 6d). Furthermore, MSC cocultured with CMV- and allostimulated PBMC increased the expression of IL-10, Foxp3, CTLA-4 and PDL-1 mRNA. Concerning molecules involved in antigen-presentation and costimulation, we found that expression of HLA-DR was increased by both stimulations, whereas expression of β 2m, which is constitutively expressed in MSC, was less affected. No significant expression of CD80 or CD86 was induced by any of the stimulations (data not shown). FACS analysis confirmed an increased surface expression of MHC class II on MSC and that low levels of IDO could be detected intracellularly after CMV- and allostimulation (data not shown).

In vivo administration of MSC in patients with GVHD does not inhibit CMV-specific T-cell responses.

Having demonstrated that MSC do not appear to suppress virus-specific T-cell functions in vitro, we next examined the effect of MSC on CMV-specific T-cell responses in vivo. We monitored immunity to CMV in 2 pediatric patients who received third-party MSC for steroid-refractory acute GVHD. Importantly, in both patients, immunosuppression was not changed during the period of study. Pentamer analysis for CMV-specific CD8⁺ T-cells recognizing epitopes of pp65 showed that both patients had a significant population of pentamer-positive cells prior to MSC infusion and that these persisted after MSC infusion at the time points when the GVHD had responded (figure 7a and b). Additionally, we analyzed IFN- γ production from PBMC in response to pp65 peptides at varying time points post infusion. Both patients had a significant number of IFN- γ producing cells prior to MSC infusion, although lower than normal CMV-positive donors, and these were retained at 1 week, 1 month and 3 months post MSC infusion (figure 7c). There was no response to pp65 from 3 normal

CMV-negative donors, supporting that the IFN- γ production induced by the pp65 peptides is CMV-specific (figure 7c). These data indicate, at least in these two SCT patients, that CMV-specific T-cells were not affected by the infusion of third-party MSC. Neither of these patients had detectable EBV-pentamer populations and both had been treated with rituximab, precluding generation of LCL and hence assessment of EBV-specific immunity.

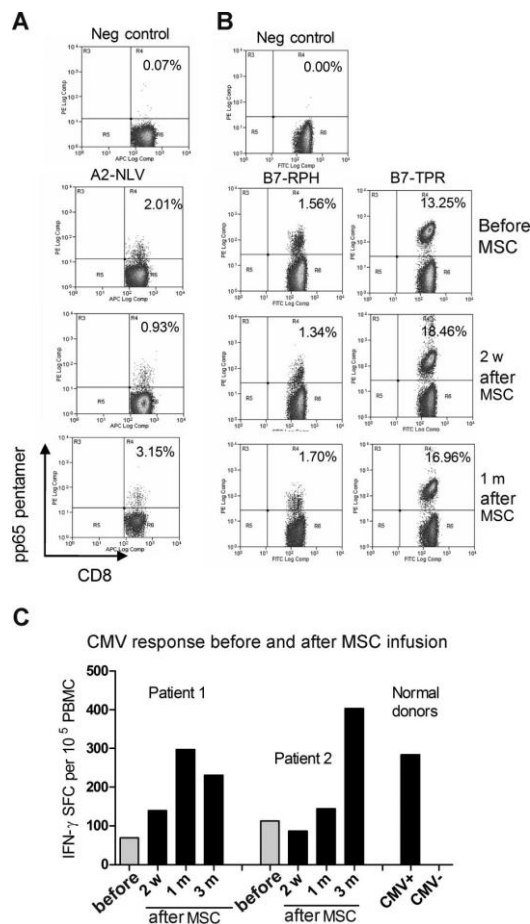


Figure 7. Anti-CMV T-cell immunity is preserved in vivo after MSC-infusion.

PBMC from patient 1 (A) and 2 (B) were analyzed with pentamers for CMV-specific CD8⁺ T-cells recognizing epitopes of pp65 pre and post MSC-infusion. (C) Frequency of IFN- γ producing PBMC from 2 patients in response to pp65 peptides before and 2 weeks, 1 month and 3 months after MSC-infusion were analyzed with ELISPOT. IFN- γ response to pp65 in PBMC from 3 normal CMV-positive donors and 3 normal CMV-negative donors were used as controls.

Discussion

Infusion of MSC appears a promising therapy for acute GVHD, but the mechanisms for the therapeutic effects are still unclear. As MSC inhibit alloresponses *in vitro*, it is likely that MSC have an immunosuppressive effect on alloreactive T-cells. However, if this immunosuppressive effect is non-specific, administration of MSC to immunocompromized patients could also suppress immune responses to infectious pathogens, resulting in increased susceptibility to infectious complications. We have examined how MSC affect virus-specific T-cell effector functions, both *in vitro* and *in vivo*, and found that MSC have little inhibitory effect on viral T-cell immunity. In contrast, alloreactive T-cells are highly susceptible to suppression by MSC. There are a few published reports to date on how MSC affect immunity to pathogens. Potian et al found that third-party party MSC do not suppress proliferation induced by recall antigens, such as *Candida* and tetanus toxin¹⁰. In contrast, Maitra et al found that MSC suppress IFN- γ production in response to tuberculin purified protein derivative²³ in Elispot assays. Our experience is that MSC adhere to the wells and prevent effector cells from interacting with the antibodies, so such assays are difficult to interpret and these studies were performed with MSC were plated in high numbers (effector to MSC ratios of 1:1 and 1:2). Another study showed that PBMC cultured with herpes simplex virus had reduced cytolytic activity against P815 mastocytoma cell line if MSC were added to the cultures²⁹. However, they did not test the cytolytic killing of viral-infected targets, which makes it difficult to conclude that MSC have an effect on viral-specific T-cell lysis. We used established virus-specific CTL lines and found that MSC had no effect on cytolytic killing of cells presenting viral antigens.

We found that MSC have an immunosuppressive effect on proliferation of PBMC stimulated with LCL as a source of EBV-antigen. This is consistent with the study by Sundin et al showing that MSC suppressed proliferation of PBMC in response to EBV particles²⁴. In contrast, we found no suppression of proliferation in established EBV-CTL lines. This could be explained by that PBMC contain several different cell types with extensive specificities, whereas the EBV-CTL lines predominantly consist of T-cells specific for immunodominant EBV-antigens. When examining the expansion of EBV-specific T-cells using pentamer-staining we found that PBMC that had been cultured

with LCL in the presence of MSC contained a similar percentage of EBV pentamer-positive T-cells as PBMC cultured in the absence of MSC. This indicates that proliferation of EBV-specific T-cell clones with reactivity against immunodominant EBV-antigens are not influenced by MSC. Furthermore, MSC had no effect on the ability of established EBV-CTL to proliferate and produce IFN- γ in response to EBV antigens and to lyse EBV-infected targets.

We found that the effector functions of CMV-specific immunity were not affected by MSC. In contrast, Sundin et al showed that MSC suppressed proliferation of PBMC in response to infectious CMV particles²⁴. Previous studies have shown that crude CMV preparations induce proliferation of lymphocytes even in CMV seronegative individuals, suggesting that this may not be entirely CMV-specific²⁵. Thus, these conflicting results may reflect the fact that the CMV preparations used by Sundin et al provide a polyclonal activation of lymphocytes, whereas pp65-peptides exclusively stimulate CMV-specific T-cells. We here chose to use peptides or cells constitutively presenting viral peptides as opposed to viral particles to control for infection of MSC and to ensure that the effects on virus-specific T-cell responses were studied.

One potential explanation for the differential effects of MSC on anti-viral and alloreactive T-cell responses is that naïve and memory T-cells are affected differently. However, we found that MSC suppress proliferation of both CD45RO⁺ memory and CD45RA⁺ naïve T-cells in response to allogeneic DC or PHA. This is consistent with the study by Krampera et al, who showed that MSC inhibited proliferation and IFN- γ production in both naïve and memory T-cells specific for a minor histocompatibility antigen²⁸. Thus, differential immunosuppressive effects on naïve and memory T-cells are unlikely to account for the absence of suppression of virus-specific T-cells, at least not when based on expression of CD45RA and CD45RO.

Another difference between allogeneic and viral stimulation of T-cells in our experimental set up is that we used exogenous peptides to study the response to CMV, whereas alloantigens were endogenously presented. The fact that T-cell responses to EBV-antigens, which are at least partly endogenously processed and presented, suggests the route of presentation also does not explain the differential effects of MSC

on alloreactive and virus-specific T-cell responses. Nonetheless, peptides could potentially be taken up and loaded on MHC molecules on MSC, rendering them susceptible to killing by CMV-specific CTL if the third-party MSC and responder T-cells share MHC alleles. Thus, our observation that MSC had no effect on suppression of CMV-induced T-cell effector functions could potentially be explained by that MSC presenting pp65-peptides in the context of MHC were lysed and thereby could not to exert an inhibitory effect on T-cell effector functions. Indeed, Horwitz et al showed that MSC retrovirally transduced to express neomycin were killed by T-cells in vitro and that infused neomycin-expressing MSC could not be detected in paediatric patients undergoing immunotherapy for osteogenesis imperfecta²⁹. Further, it has been demonstrated that IFN- γ -treated MSC can process and present influenza protein to influenza-specific MHC class II-restricted T-cell hybridomas³⁰. However, human MSC, in contrast to murine MSC, do not appear to be primed for an antigen-presenting phenotype when cultured at high cell-culture densities³¹. The MSC cultures in this study were plated at high densities and we found that MSC pulsed with pp65-peptides are not killed by CMV-specific T-cells. Similarly, it was recently shown that MSC pulsed with EBV-peptides are resistant to lysis by peptide-specific MHC class I-restricted EBV-CTL³². The viral and alloinduced responses described here presumably differ in contribution of CD8⁺ and CD4⁺ T-cells, but we found that MSC had no suppressive effect on either EBV-specific T-cell subset, indicating that the observed differences in MSC-mediated inhibition is not explained by differential effects on CD4⁺ and CD8⁺ T-cells.

Although several potential mediators of MSC-mediated suppression of lymphocyte responses have been proposed, the mechanisms by which MSC exert their effects remain largely unclear. It has been suggested that suppression by MSC is mediated by secretion of IFN- γ by inducing production of IDO, thereby depleting tryptophan which is essential for T-cell proliferation^{33,34}. We found that both CMV- and allostimulated PBMC induce an upregulation of IDO in MSC, along with other factors involved in immunosuppression, indicating that the incapacity of MSC to suppress CMV-specific T-cells is not due to lack of IDO production. It has further been proposed that MSC may implement their inhibitory effect by inducing generation of T-cells with regulatory functions^{5,7,35}. Indeed, we observed that MSC induced an upregulation of Foxp3 in

CD4⁺ T-cells in the absence of any other stimulation, whereas this effect of MSC was not found in CMV and allo-stimulated T-cells. However, PBMC stimulated with allogeneic cells showed a markedly higher proportion of CD25^{high} Foxp3⁺ T-cells in general compared to CMV-stimulated cells, which possibly could render these cells more susceptible to suppression by MSC. The mechanisms by which MSC suppress immune responses need to be further elucidated in order to understand why MSC show differences in their ability to regulate immunity.

We have demonstrated that MSC do not appear to suppress virus-specific T-cell functions *in vitro*, but clinically the most important question is whether MSC have effects on virus-specific immunity in patients. As a first step to addressing the clinical relevance of our findings *in vivo*, we examined anti-CMV T-cell immunity in 2 patients who received MSC for steroid-refractory GvHD. We found that both the number of CMV-pentamer specific T-cells and CMV-induced IFN- γ production was preserved after MSC-infusion at time-points at which GvHD had clinically and/or histologically responded. Clearly, data from such a limited number of patients need to be interpreted with caution and we plan to study this further when more samples from patients who fulfil the criteria have been collected. Further, it should be noted that in such patients anti-CMV responses are blunted compared to normal donors by virtue of ongoing immunosuppression and further data are needed on the effect of MSC on anti-viral responses in immunocompetent individuals. Nonetheless, these results are encouraging and consistent with our *in vitro* data and recent clinical data where no excess of viral infections was observed after MSC-infusion³⁶, suggesting that MSC have little immunosuppressive effect on anti-CMV T cell responses. Given that MSC are used in clinical settings where patients are highly susceptible to infections, which are the major cause of treatment failure in GvHD, these data are of great clinical significance. If our findings are confirmed in larger numbers of patients, MSC may represent an advance over existing therapeutic modalities for GvHD, which are uniformly associated with a high risk of infectious complications.

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