Chapter 2.

Mesenchymal stromal cells isolated from children with systemic juvenile idiopathic arthritis suppress innate and adaptive immune responses.

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Abstract

Background aims: Infusion of mesenchymal stromal cells (MSCs) has been reported to be an effective treatment modality for acute Graft-versus-Host disease, and MSCs haven been considered for use in the treatment of patients with autoimmune diseases. Before contemplating clinical studies with MSCs in patients with systemic juvenile idiopathic arthritis (sJIA), the immunomodulatory capacity of MSCs in this setting needs to be explored. A comparative analysis of bone marrow derived MSCs from children with sJIA and healthy paediatric controls was performed.

Methods: MSCs were successfully expanded from 11 patients with sJIA and 10 controls. The phenotype, differentiation and immunomodulatory capacity of these MSCs were compared. The effect of immunosuppressive drugs on MSC function was also investigated.

Results: MSCs from patients with sJIA and controls showed no differences in their suppressive effect using control peripheral blood mononuclear cells. Furthermore, the suppression of the response of peripheral blood mononuclear cells from patients with sJIA autologous sJIA MSCs and allogeneic control MSCs was comparable. The immunosuppressive effect of both groups of MSCs was diminished in the presence of indomethacin ($p<0.05$). MSCs from patients with sJIA and controls suppressed IL-2 induced natural killer cell activation to a similar extent. In addition, MSCs of patients with sJIA and controls inhibited the differentiation of monocytes to dendritic cells.

Discussion: This is the first explorative study in a significant cohort of patients with sJIA to evaluate the effect of MSCs on adaptive and innate immune responses. The comparable immunosuppressive characteristics of MSCs derived from patients with sJIA to age-matched controls support the potential use of patient derived MSC in the treatment of sJIA.

Introduction

Systemic juvenile idiopathic arthritis (sJIA) is an autoinflammatory disease of unknown aetiology that is characterized by spiking fever, exanthema, anaemia, hepatosplenomegaly and arthritis.(Petty, 2004) The outcome of disease and response to therapy is unpredictable. Improvements in treatment outcome with the use of diverse biological therapies, as anti-interleukin (IL)-1 therapy, have been reported.(Lequerre, 2008; De Benedetti F., 2009; Woo, 2008; Martini, 2006; Beukelman, 2011; Quartier, 2011; Nigrovic, 2011; Yokota, 2008) Despite these observations, the disease progresses and/or requires additional therapy in 27% of children treated with etanercept.(Prince, 2011) Moreover, patients treated with these new drugs may experience adverse effects. Recent data relate the use of tumor necrosis factor (TNF)-blocking agents in patients with JIA and other inflammatory diseases to the development of lymphoma and other cancers in children.(Horneff, 2011; Kwon, 2005; Diak, 2010)

Previous studies in children with refractory JIA undergoing autologous hematopoietic stem cell transplantation following chemotherapy and T cell depletion show a progression free survival of 52% after 5 years.(Farge, 2010) However, high morbidity due to macrophage activation syndrome, viral infections and transplant-related mortality occurred.(Brinkman, 2007) Therefore, new treatment modalities need to be explored for therapy-resistant patients.

Mesenchymal stromal cells (MSCs) are multipotent non-hematopoietic stromal cells with anti-inflammatory and anti-proliferative capacities.(Krampera, 2003; Bocelli-Tyndall, 2009) MSC can be expanded easily from different tissues including bone marrow (BM), and remain genetically stable even after numerous passages.(Bernardo, 2007c) No unique marker has been identified for the isolation of MSCs; however, specific criteria to identify MSC have been accepted.(Dominici, 2006; Horwitz, 2005)

T cell and natural killer (NK) cell proliferation, immunoglobulin production by B cells and dendritic cell maturation are suppressed by MSC in *in vitro* experiments.(Bocelli-Tyndall, 2007; Sotiropoulou, 2006; Traggiai, 2008b) The mechanisms underlying these immunomodulatory functions are not fully understood. However, experimental data suggest that cell-cell contact and soluble factors may both be involved in an overall inhibition of the induction of effector functions.(Beyth, 2005; Gieseke, 2010; Krampera, 2003; Meisel, 2004; Rasmusson, 2005; Selmani, 2008; Uccelli, 2008)

The profound immunomodulatory characteristics of MSCs make them potential candidates for use in the treatment of inflammatory diseases. Clinical trials using allogeneic MSCs in the treatment of steroid resistant acute graft versus host disease (aGvHD) have shown promising results.(Le Blanc, 2008) Moreover, the feasibility and safety of autologous MSC infusions have been demonstrated in phase I clinical trials.(Garcia-Olmo, 2005; Duijvestein, 2010; Sun, 2009; Sun, 2010; Karussis, 2010)

However, the efficacy and safety of MSC treatment has yet to be determined in larger cohort studies.(Dazzi, 2011)

Previously, an aberrant suppressive capacity of MSCs from patients with severe aplastic anaemia was previously reported.(Bacigalupo, 2005) In addition, functional aberrations in synovium-derived MSCs from adults with rheumatoid arthritis have been described, suggesting that MSCs from patients with inflammatory diseases may not be comparable to those derived from healthy donors.(Jones, 2010) Therefore, before contemplating clinical studies of autologous MSC infusions in children with treatment-resistant sJIA, the immunomodulatory properties of MSCs of these patients need to be characterized. To our knowledge this is the first study describing comparable characteristics of MSCs from a relatively large cohort of children with sJIA compared to age-matched healthy controls (HC).

Methods

Expansion of MSCs

Bone marrow (BM) of children with sJIA (n=13) was used to initiate MSC cultures according to a protocol approved by a local ethical committee. Fresh BM was harvested from five patients at time of diagnosis. To expand the study population, frozen bone marrow mononuclear cells (BMMC) from patients with sJIA (n=8) were used for MSC cultures. Classification of sJIA was according to the guidelines from the International League of Associations for Rheumatology.(Petty, 2004) Fresh BM of 10 paediatric donors of an allogeneic hematopoietic stem cell graft was used as HC. Parental and patient informed consent forms were signed for each patient and control.

After Ficoll separation BMMC were plated at a density of $0.16x10^6$ cells/cm^2 in polystyrene culture flasks. Cells were cultured in Dulbecco's modified Eagle medium with Glutamax (DMEM; Invitrogen, Paisley, UK) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin (P/S; Invitrogen) and 10% fetal bovine serum (FBS; VWR International, Bridgeport, NJ, USA). Medium was refreshed every three to four days. Cultures were harvested at 80% confluency by treatment with trypsin (Invitrogen), replated and maintained for maximally six passages at 37˚C and 5% CO_2.

Characterization of MSCs

All MSC cell lines were phenotypically characterized at their second or third passage using antibodies against CD3, CD45, CD86, HLA-DR, CD31, CD34, CD73 and CD90 (all Becton Dickinson Biosciences (BD), San Diego, CA, USA). CD105 was obtained from Ancell Corporation (Bayport, MN, USA). Cells were analyzed on a FACS Calibur flow

Table 1. Clinical characteristics of patients with JIA at the time of BM harvest.

Patient (UPN)	Sex	Age (years)	Disease duration (months)	Active joints (n)	ESR (mm/h)	NSAID[a] (mg/kg/day)	Prednisone (mg/kg/day)	Other immuno-suppressive drugs
1	M	5	7.7	3	19	1.7	-	-
2	F	12	0.3	4	11	2.0	-	-
3	F	7	42.9	2	11	2.9	0.2	Anti-TNF-α
4	F	11	2.4	2	124	2.0	1.0	-
5	M	4	12.9	11	45	2.0	1.0	MTX; Cyclosporin
6	M	11	104.0	11	33	1.7	0.5	-
7	M	7	26.3	32	85	2.0	0.2	Anti-TNF-α
8[b]	M	13	116.7	1	77	1.6	0.3	Azathioprine
9	M	7	74.9	6	46	0.4	0.5	MTX
10[b]	F	5	23.9	8	71	2.0	1.0	MTX; Cyclosporin
11	M	5	0.8	5	91	1.3	-	-
12	F	3	0.5	17	130	1.7	-	-
13	F	1	3.8	10	14	2.5	-	-

ESR, erythrocyte sedimentation rate; NSAID, non-steroidal anti-inflammatory drugs; TNF, tumour necrosis factor; MTX, methotrexate

[a] all patients were treated with indomethacin, except UPN 11 who was treated with diclofenac.

[b] no expansion of MSC was achieved from bone marrow samples of this patient.

cytometer (BD). Mean fluorescence intensity (MFI) was compared with cells stained with isotype-matched negative control antibodies (BD) and with unstained cells.

The osteoblast and adipocyte differentiation potential was evaluated on cells at passage 4-6 as described previously.(Bernardo, 2007b) After 3 weeks, fat vacuoles in adipocytes and calcified depositions in osteoblast were stained with Oil-Red-O (Sigma, St. Louis, MI, USA) or Alizarin Red (MP Biomedicals, Solon, OH, USA), respectively.

Peripheral blood mononuclear cell cultures

To investigate the immunosuppressive effect of MSCs on peripheral blood mononuclear cell (PBMC) proliferation, irradiated (30 Gy) MSCs from patients with sJIA or HC were seeded at 2-fold serial dilutions in flat-bottom 96-well plates. PBMC, isolated by Ficoll separation from buffy coats of healthy blood bank donors (Sanquin, Amsterdam, the Netherlands), were added (100.000 cells/well) after 4 hours. Cells were stimulated with 2 μg/mL phytohemagglutinin (PHA; Murex, Châtillon, France) and cultured at 37˚C and 5% CO_2 for 5 days. Fifty microliters of culture supernatant was harvested at day 4 for cytokine analysis. The concentrations of interferon (IFN)-γ and TNF-α in supernatants were measured using standard protocols of enzyme-linked im-

munosorbent assay kits (Sanquin). Cells were pulsed with ^{3}H-thymidine (1 µCi/well; Perkin Elmer, Wellesley, MA, USA) for the final 16 hours and counted with a β-counter (Perkin Elmer). All cultures were performed in triplicate in RPMI 1640 (Invitrogen) medium with 10% pooled human AB serum (Sanquin) and P/S (total volume 200 µL/well).

To investigate the influence of drugs used in the treatment of sJIA, indomethacin (5 µM; Sigma), dexamethasone (100 ng/mL; Sigma) and the soluble TNF-α receptor adalimumab (10 µg/mL; Abbott Laboratories, Abbott Park, IL, USA) were added at culture initiation.

NK cell cultures

NK cells were isolated from PBMC derived from healthy blood bank donors by negative selection using an NK isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). An NK cell purity greater than 95% was reached. NK cells ($1.0x10^{6}$/well) were stimulated with 30 IU/mL IL-2 (Chiron Corporation, Emeryville, CA, USA). NK cells were co-cultured with or without irradiated MSCs in 24 well plates at an MSC:NK ratio of 1:5 or 1:40 in RPMI 1640 supplemented with 10% human AB serum and P/S.

At day 5, NK cells were harvested and counted and cell marker expression and cytotoxicity were assessed. The expression of CD69 (BD) and NKG2D (Beckman Coulter, Brea, CA, USA) was determined by flow cytometry. Cytotoxicity was determined in a standard 4-hour chromium release assay using various target cells (2.500 cells/well) *i.e.*, Daudi cells, which are only sensitive to killing mediated by activated NK cells, and Epstein-Barr virus transformed B cells (EBV-BLCL) coated with antibodies through pre-incubation with anti-thymocyte globulins (ATG; Genzyme, Cambridge, MA; 50 µg/mL for 20 minutes) to measure antibody dependent cell mediated cytotoxicity (ADCC). Target cells were labelled with 100 µCi sodium-51-chromate (Perkin Elmer) for 1 hour. Effector cells were incubated with target cells at ratios ranging from 40:1 to 0.3:1 in triplicate. Spontaneous and maximum release were determined by incubating target cells with medium or Triton X100 (5%; Merck Chemicals, Darmstadt, Germany), respectively. Subsequently, chromium release in the supernatant was assessed in a β-counter. Specific lysis was determined as follows: (experimental release-spontaneous release) / (maximum release-spontaneous release) x 100%.

Dendritic cell cultures

Monocytes were isolated from PBMC derived from healthy blood bank donors with a positive CD14 selection kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Cell purity greater than 90% was reached in all isolations. Monocytes (0.5×10^{6}) were cultured in 24-well plates for 5 days in RPMI 1640, 10% FCS and 1% P/S supplemented with 800 IU/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) and 40 ng/

mL IL-4 (both from Tebu-Bio, Le Perray en Yvelines, France) to induce dendritic cell (DC) differentiation. At day 5, cells were harvested or similar concentrations of IL-4 and GM-CSF, and CD40-ligand (0.25 ug/mL Beckman-Coulter, Marseille, France) and IFN-γ (500 U/mL, Boehringer, Mannheim, Germany) were added to mature the DC for 2 extra days. All cells harvested on day 5 and 7 were analyzed using flow cytometry for CD14, CD1a, CD80, CD86, and CD163 expression (antibodies from BD). MSC were co-cultured with DC at MSC:DC ratios of 1:5, 1:40 or 1:100.

Statistics

Data were analyzed using GraphPad Prism (LaJolla, CA). For statistic analysis paired and unpaired t-tests were used. *P*-values of <0.05 were considered significant.

Results

Characterization of MSCs

MSCs were successfully expanded from bone marrow (BM) of 11 out of 13 sJIA patients and 10 out of 10 HCs. Age did not significantly differ between the patients and HCs (mean 7.1 years, range 1-12 versus 8.9 years, range 1-19, respectively); 45% of the patients with sJIA and 40% of the controls were female. The characteristics of the patients included in the study are summarized in Table I. Samples from two patients, both frozen BMMC, failed to expand. The cryopreservation time of these samples was 10.2 and 12.3 years, respectively, versus a mean storage time of 7.5 years for the successfully expanded samples (range 2.4-11.1 years). In addition, cultures of these two samples were initiated with relatively low numbers of BMMC ($0.26x10^6$ and $2.8x10^6$). For MSC cultures from frozen BMMC significantly less cells were available compared with cultures from freshly isolated BMMC (mean $3.9x10^6$, range $0.26\text{-}7.56x10^6$ versus mean $33.1x10^6$, range $16\text{-}60x10^6$ BMMC, respectively).

MSCs were characterized by adhesion to plastic and fibroblast-like appearance. In addition, all MSC fulfilled the phenotypic criteria, (i.e., expression of CD73, CD90 and CD105 and no expression of CD34, CD31 and CD45) at passage 2 or 3 (Figure 1A). All MSCs had the ability to differentiate into adipocytes and osteoblasts upon stimulation (Figures 1B-I). However, MSC lines derived from two patients (UPN 2 and 5 in Table I), which were obtained from frozen material, failed to differentiate into the osteoblastic lineage. These two MSC cultures were initiated with relatively low numbers of BMMC ($0.46x10^6$ and $1.4x10^6$, respectively). These MSC were, therefore, not evaluated in the immunomodulatory assays.

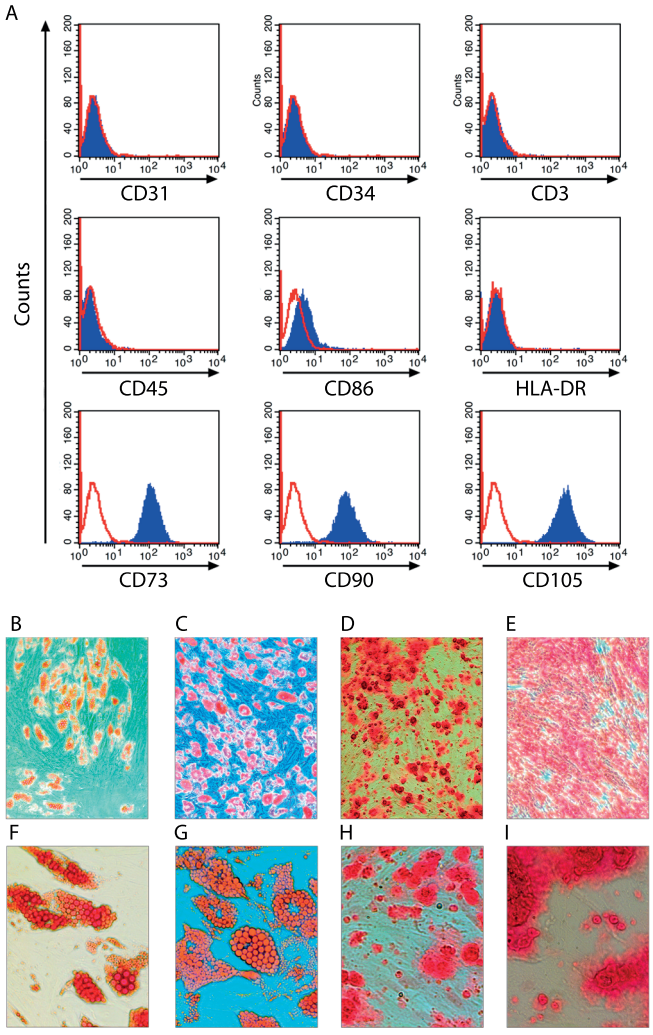

Figure 1. *MSCs of patients with sJIA and healthy controls (HC) show an identical phenotype and differentiation potential.* A) Representative FACS staining of UPN 12 is shown in closed histograms. Open histograms represent the staining intensity with isotype-matched negative control antibodies. B) Differentiation of HC MSC (B,D,F,H) and sJIA MSC (C,E,G,I) to adipocytes (B,C,F,G; stained with Oil-Red-O) and osteoblasts (D,E,H,I; stained with Alizarin Red). Magnification 10x (B,C,D,E) and 40x (F,G,H,I).

Immune modulation of PBMC by MSCs

MSCs down modulated the proliferation of PHA-stimulated PBMC in a dose-dependent manner (Figure 2A). A significant reduction of proliferation was seen at all MSC:PBMC ratios used. No differences were observed between sJIA and HC MSCs investigated in these experiments (Figure 2A). sJIA MSC derived from patients *in vivo* treated with (n=4) and without prednisone (n=4) gave comparable results (data not shown). ^{3}H-thymidine counts did not exceed background levels in MSCs and PBMC co-cultured in the absence of PHA, or in MSCs cultured without PBMC in the presence of PHA.

IFN-γ and TNF-α concentrations were reduced in supernatants of PHA-stimulated HC PBMC co-cultured with MSCs compared with PHA-stimulated PBMC without MSCs. MSCs of HC and patients with sJIA equally suppressed the production of these pro-inflammatory cytokines (Figures 2C/D).

Suppression of autologous PBMC proliferation by sJIA MSCs

PBMC of patients with sJIA were stimulated with PHA and co-cultured either with autologous sJIA MSC or with MSC from two allogeneic HC (Figure 2B). PBMC frozen at the time of BM harvest were used in these experiments. Independent of their origin, MSC suppressed the PBMC proliferation to a similar extent. At an MSC:PBMC ratio of 1:5, the average down-modulating effect of sJIA MSC on autologous sJIA PBMC (Figure 2B) did not differ from their effect on allogeneic HC PBMC (Figure 2A) (25.6% vs. 20.6%; p=0.20).

Influence of immunosuppressive drugs on function of MSCs

Addition of dexamethasone to cultures, but not of indomethacin or adalimumab, to cultures significantly reduced the PHA-induced proliferation of PBMC from HC ($p<0.01$) (Figure 3A). Despite this effect, MSCs added at culture initiation retained their suppressive capacity in the presence of dexamethasone (Figure 3B). The same observation was evident for the production of cytokines (Figures 3C/D).

In contrast, indomethacin interfered significantly with the immunosuppressive activity of MSCs derived from either sJIA or from HC at an MSC:PBMC ratio 1:5 (Figure 3A/B). However, the effect of indomethacin did not reach significance at an MSC:PBMC ratio ranging from 1:10 to 1:80 (data only shown for 1:40). IFN-γ and TNF-α production appeared to be a more sensitive indicator for the interference of indomethacin with the immunosuppressive activity of MSC, because a significant lower decrease of the cytokine production in the presence of the drug ($p<0.05$) was not only observed at an MSC:PBMC ratio of 1:5 but also at 1:40 (Figures 3C/D).

Adalimumab did not affect PBMC proliferation (Figures 3A/B). IFN-γ production, however, was clearly decreased in the presence of adalimumab alone and further diminished when MSCs were added to the culture. The effects of these drugs on the immunomodulatory capacity of MSCs were similar for MSCs derived from patients with sJIA and from HC (data not shown). The mean +/- standerd error of the mean (SEM) percentages of proliferation at a ratio of MSC:PBMC of 1:5 were 38.4% +/- 4.9% and 33.5 +/- 1.9% for HC-MSC and sJIA-MSC, respectively.

Suppression of NK cell activation by MSCs

The expression of CD69 and NKG2D on NK cells was up-regulated after 5 days activation with IL-2 (30 IU/mL). The percentage of $CD69^+$ cells (range 26-70%) and the

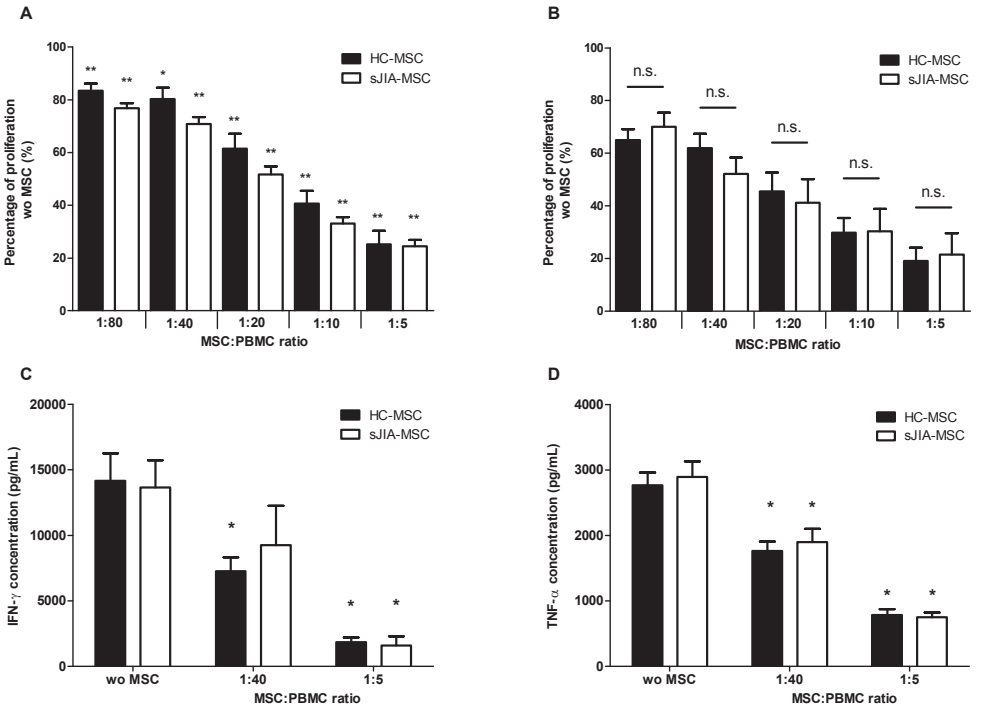

Figure 2. *sJIA and healthy control (HC) derived MSCs suppress activation of patient and control derived PBMC.* sJIA and HC-derived MSCs show no differences in the down modulation of the functional activity of PBMC after PHA stimulation. A) The mean and standard error of the mean (SEM) of proliferation at day 5 are depicted of experiments using HC (n=4) and sJIA (n=8) MSCs as modulators and PBMC of four healthy donors as responders. PHA-induced proliferation in the absence of MSCs was set at 100%. B) Autologous sJIA and allogeneic HC-derived MSCs show no differences in their down-modulating effect on PHA-induced proliferation of sJIA-derived PBMC. The mean and SEM of experiments with six different sJIA PBMC are depicted. The effect of the six autologous sJIA MSCs and of two allogeneic HC MSCs was tested on PBMC of all six patients with sJIA investigated in this experiment. C/D) In co-cultures of PHA-stimulated HC PBMC with sJIA and HC-derived MSCs the (C) IFN-γ and (D) TNF-α concentrations in supernatants taken at day 4 are decreased. The mean concentration of cytokines in cultures of two PBMC donors as responders with or without MSCs from 2 HC and 4 sJIA patients as modulators are shown. *: p <0.05 compared to without (wo) MSC. **: p <0.01 compared to without MSC.

MFI of NKG2D expression (range 150-297), depending on the healthy donor, were set at 100 for NK cells cultured without MSCs to enable comparison amongst different experiments.

After 5 days of co-culture of NK cells with MSCs, the percentage of $CD69^+$ NK cells and the expression of NKG2D were significantly reduced (p<0.05) (Figures 4A/B). No significant differences were observed between MSCs derived from sJIA and HC in down-modulation of IL-2 induced NK cell activation.

The cytotoxic capacity of the cytokine-activated NK cells against Daudi target cells was significantly suppressed after co-culture with MSCs. In contrast, ATG-mediated ADCC of NK cells against EBV-BLCL was not significantly impaired, indicating that the intrinsic cytolytic potential of the NK cells was not affected (Figures 4C-F).

MSCs inhibit the differentiation of monocytes to dendritic cells

The monocyte population isolated by positive CD14 selection contained less than 1% CD1a-positive cells. Upon combined GM-CSF and IL-4 exposure, monocytes differentiated towards immature $CD1a^+/CD14^-$ DC (Figure 5A). MSCs strongly inhibited this differentiation, as shown by retainment of $CD14^+$ monocytes accompanied by a reduced appearance of $CD14^-CD1a^+$ DC, in a dose-dependent manner (Figure 5B/C). A similar pattern in CD14 and CD1a expression was seen after 2 additional days of

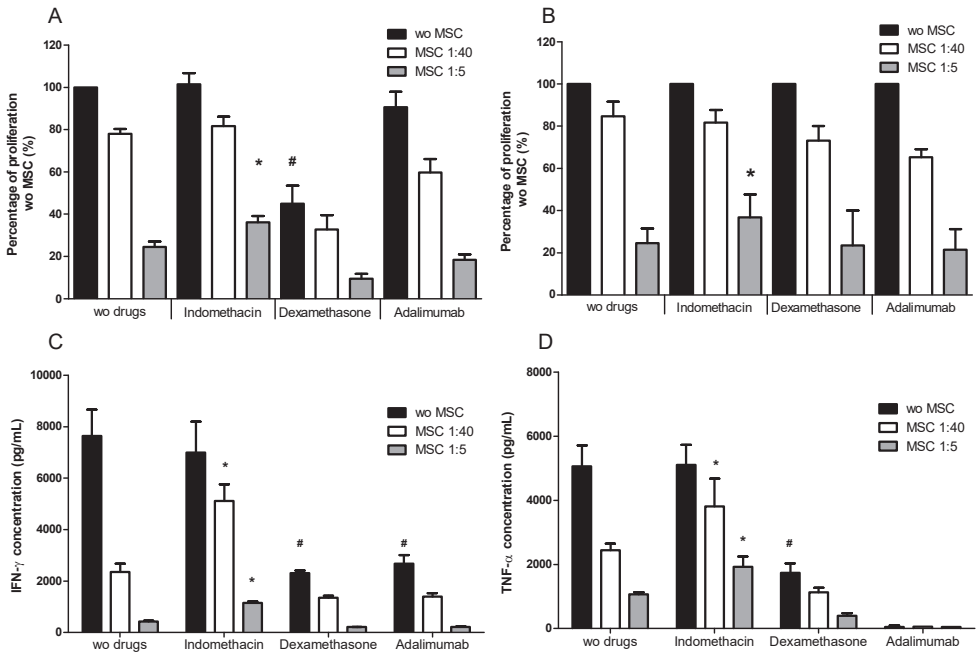

Figure 3. *Indomethacin decreases the immunosuppressive effect of MSCs.* A/B) PHA-induced proliferation of healthy control PBMC in co-cultures with or without MSCs and in the presence or absence of different drugs is shown. A) The proliferation in the culture condition without drugs and without MSCs was set at 100%. B) The proliferation in the culture conditions without MSC but with drug was standardized to 100% for each of the drugs separately. The mean and SEM of 6 different MSC preparations (sJIA = 3; HC = 3) are depicted. C/D) Concentrations (mean +/- SEM) of (C) IFN-γ and (D) TNF-α in the culture supernatants of healthy control PBMC stimulated with PHA and cultured in the absence or presence of MSCs with or without drugs are depicted. One experiment, representative for MSCs derived from 2 HC and 2 patients with sJIA, is shown. *: $p<0.05$ compared with the corresponding MSC:PBMC ratio without drugs; #: $p<0.05$ compared with the condition without MSC and without drugs.

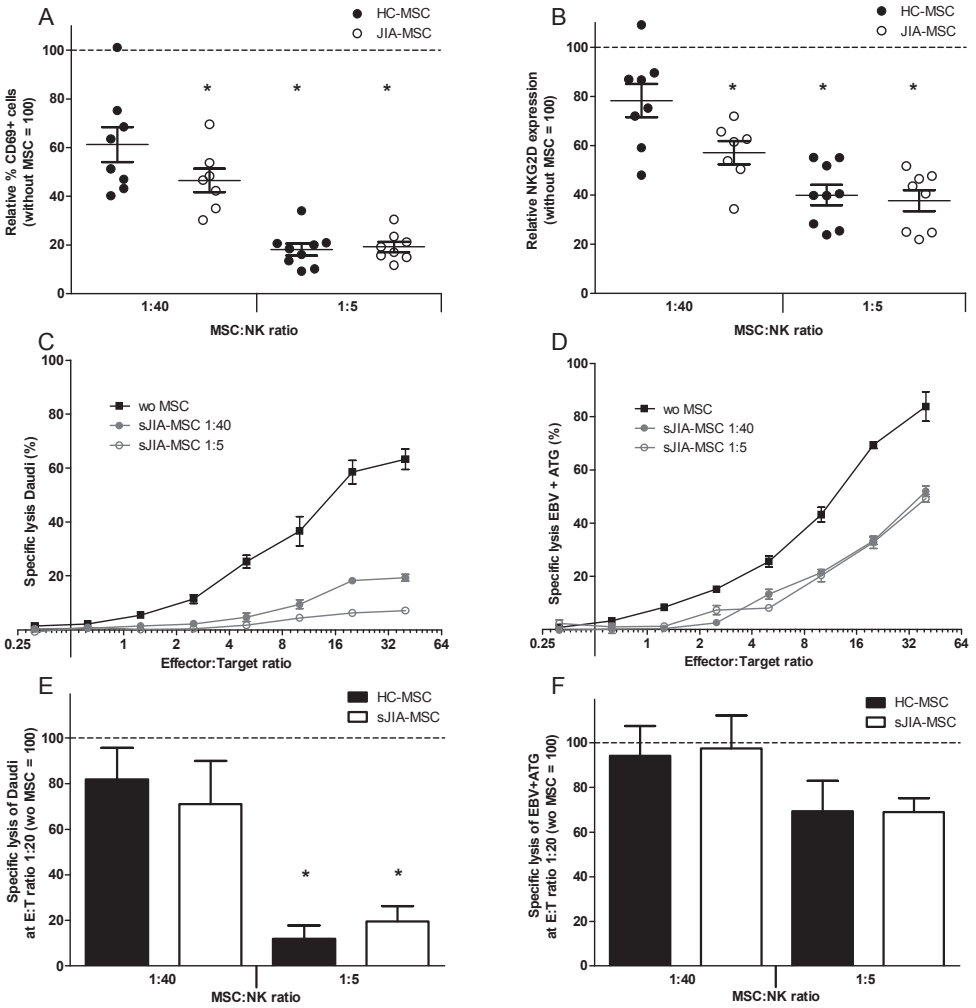

Figure 4. *sJIA derived MSCs suppress NK cell activation and cytotoxity during co-cultures.* MSCs were co-cultured with NK cells during 5 days in the presence of IL-2 (30 IU/mL). At day 5, activation and cytotoxicity of NK cells were determined using flow cytometry and a chromium release assay, respectively. A/B) The relative percentage of CD69 positivity (A) and the relative MFI of NKG2D expression (B) on NK cells isolated from PBMC of different healthy donors are depicted after co-culture of NK cells of three different donors with MSCs from six patients with sJIA and seven allogeneic healthy controls (HC) at MSC:NK cell ratios of 1:40 and 1:5. Percentage of CD69 positivity and MFI of NKG2D expression of NK cells cultured for 5 days with IL-2 in the absence of MSC was set at 100. The mean +/- SEM is indicated by the horizontal lines. *: $p<0.05$ compared to without MSC. C/D) The killing of (C) Daudi and (D) ATG mediated ADCC of EBV-BLCL by NK cells cultured with or without (wo) MSCs of one sJIA patient is shown. E/F) At an effector:target (E:T) ratio of 1:20, the killing of (E) Daudi and (F) ATG coated EBV-BLCL by NK cells co-cultured with MSCs at the indicated MSC:NK ratios is expressed relative to the killing by NK cells cultured without MSC which was set at 100; The effect of MSCs derived from five patients with sJIA and five HC was analyzed. The bars represent the mean +/- SEM. *: $p<0.05$ compared to without MSCs.

exposure to GM-CSF, IL-4, CD40-ligand and IFN-γ in order to further mature the DC. Monocytes co-cultured with MSC acquired CD163 expression (relative MFI +/- SEM 8.8 +/- 0.3 on day 0) on day 5 (12.7 +/- 1.8 without MSCs and 47.3 +/- 3.5 with HC MSCs or 39.4 +/- 1.6 with sJIA MSC) (data not shown). On day 7, the expression of CD80 and CD86 on the total population was significantly (p<0.01 for CD80 and p<0.05 for CD86) lower in co-cultures with MSCs (Figure 5D). Comparable results were obtained for either $CD1a^+$ or $CD14^+$ cells (data not shown). MSCs of sJIA patients and HC did not differ in their effect on DC differentiation.

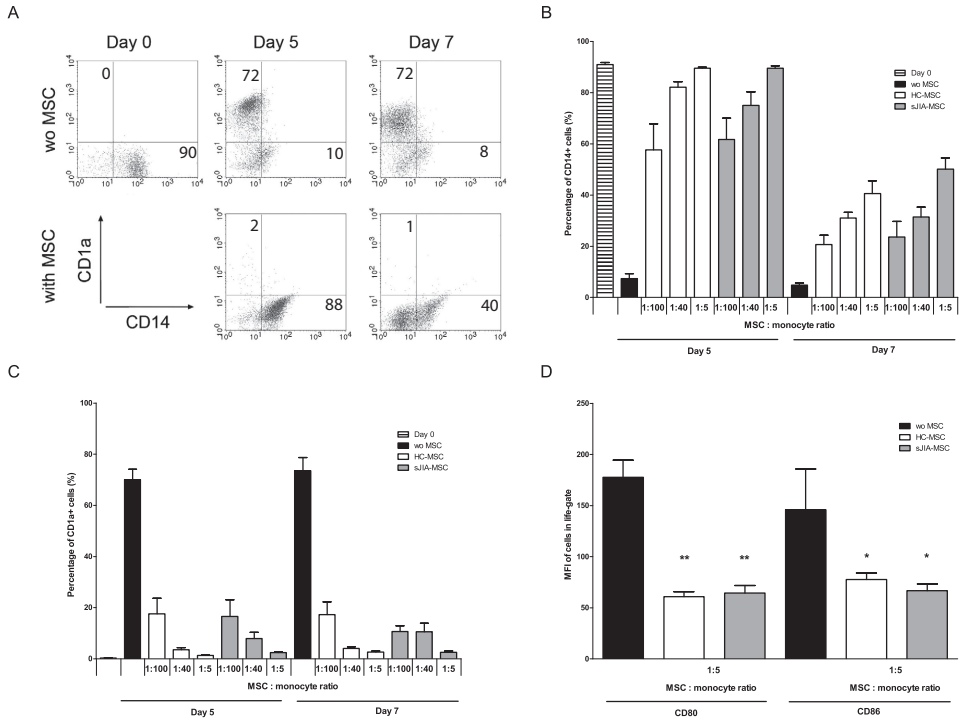

Figure 5. *MSCs of patients with sJIA interfere with differentiation of monocytes to dendritic cells.* Monocytes in co-culture with MSC in the presence of GM-CSF, IL-4, CD40 ligand and IFN-γ show impaired maturation to dendritic cells (DC). Cells were gated on forward scatter/side scatter life-gate. A) Representative FACS plots of combined CD14 and CD1a staining on day 0, day 5 and day 7 are presented. The numbers in plots represent the percentage of gated cells in the different quadrants reflecting $CD1a^+CD14^-$ DC and $CD1a^-CD14^+$ monocytes, respectively. Monocytes were cultured in the absence or in the presence of MSCs at a MSC:monocyte ratio 1:5. B/C) Mean and SEM of the percentage (B) $CD14^+$ monocytes and (C) $CD1a^+$ DC are depicted. Results were obtained using isolated monocytes from four healthy adult donors co-cultured with allogeneic MSCs generated from BM of three healthy controls (HC) and four patients with sJIA. D) The CD80 and CD86 expression on the total population is significantly decreased in co-cultures with sJIA and HC MSC at day 7. *: p <0.05 compared to without (wo) MSC. **: p <0.01 compared to without MSC.

Discussion

In this study, the biological and functional characteristics of bone marrow derived MSCs from patients with sJIA were compared with those from paediatric HC. Determination of the immunomodulatory capacity of sJIA-derived MSCs is an essential step before considering the administration of autologous MSCs in patients with sJIA. Previously, aberrant immunomodulation was reported for BM-derived MSCs from severe aplastic anaemia patients.(Bacigalupo, 2005) In addition, synovial fluid-derived MSCs from patients with rheumatoid arthritis had a decreased differentiation capacity compared to MSCs from synovial fluid of patients with osteoarthritis.(Jones, 2010)

The use of autologous MSCs in the treatment of sJIA is preferred to allogeneic MSCs for three reasons. Firstly, knowledge on the administration of allogeneic MSCs in children is limited to immunocompromised patients, such as hematopoietic stem cell transplantation recipients with steroid-resistant aGvHD.(Le Blanc, 2008) Secondly, mice models indicate the occurrence of immune rejection and specific immunological memory induction after administration of allogeneic MSCs in an immunocompetent setting which would disallow a repetitive infusion of MSCs.(Nauta, 2006; Zangi, 2009) Third, more recent data in patients treated for renal graft rejection demonstrate that primed T cells are able to lyse allogeneic MSCs.(Crop, 2011)

Systemic JIA is considered an autoinflammatory disease caused by an inadequate suppression of innate immunity.(Vastert, 2009) Therefore, we evaluated the *in vitro* immunomodulatory capacities of MSCs not only by focusing on T cell function in a model of PHA-stimulated PBMC,(Samuelsson, 2009; Bacigalupo, 2005; Aggarwal, 2005) but also by investigating the effect on NK cells(Spaggiari, 2006) and monocytes. In both adaptive and innate experimental systems, the immunomodulatory effects of MSC of patients and healthy controls were indistinguishable, in accordance with previous reports on patients with autoinflammatory diseases.(Bocelli-Tyndall, 2007; Bernardo, 2009; Larghero, 2008) However, in these studies, only the effect of MSC on lymphocyte proliferation was investigated. We have shown a decreased cytolytic function of NK cells after co-cultivation with MSCs of patients with sJIA and HC. In addition, MSCs of patients and HC inhibited the differentiation of monocytes towards DC, as illustrated by the generation of a low percentage of CD1a-positive cells and decreased levels of CD80 and CD86 expression. In contrast, monocytes cultured with MSCs acquired expression of CD163, a scavenger receptor associated with the suppressive type 2 macrophage.(Sica, 2006)

To enable inclusion of a substantial number of patients, not only fresh BM but also long-term frozen BM of patients with sJIA was applied to culture MSCs. The expansion of MSC was successful for six of eight frozen BM samples. Previously, the use of 1-week cryopreserved BM for the expansion of MSCs was compared to

fresh BM.(Haack-Sorensen, 2007; Casado-Diaz, 2008) In these studies, no differences were observed in expansion or differentiation capacity. However, we found an aberrant osteoblast differentiation in the MSCs started from frozen BM of two sJIA patients. The absence in osteoblast differentiation could be explained by the relatively low numbers of BMMC at the initiation of MSC cultures compared with these studies, in which they used 20 or $100x10^6$ BMMC, respectively.(Casado-Diaz, 2008; Haack-Sorensen, 2007) Nevertheless, a possible influence of the disease itself or the long-term use of drugs can not formally be excluded based on our study results. In our study, no differences in immunomodulatory capacity were found between MSCs derived from cryopreserved and fresh BM. This is in contrast to results reported by Samuelson *et al*.(Samuelsson, 2009) describing a variable effect of cryopreservation.

Despite unsuccessful expansion of MSCs from frozen BM in two patients, the expansion of MSCs from frozen material is a promising tool for further research on rare paediatric diseases.

An important issue in administration of MSCs to patients is the continuation of disease-specific drugs. Previously, calcineurin inhibitors and indomethacin were shown to suppress the immunomodulatory effect of MSCs, whereas mycophenolic acid has a synergistic effect with MSCs.(Aggarwal, 2005; Buron, 2009) Consistent with previous data(Rasmusson, 2005) our findings indicate that indomethacin suppresses the immunomodulatory function of MSCs. All of our patients were treated with non-steroidal anti-inflammatory drugs (NSAID) before aspiration of BM. Apparently, *in vivo* exposure of MSCs to NSAID does not interfere with the suppressive effect of MSCs after *ex vivo* expansion. Dexamethasone, on the other hand, although having a suppressive effect on PBMC proliferation by itself, did not affect the suppressive effect of MSCs, consistent with a previous report.(Buron, 2009) Adalimumab, a TNF-α antagonist, did not affect PBMC proliferation, but significantly decreased PHA-induced production of IFN-γ. Interestingly, despite the low IFN-γ production in cultures containing adalimumab, MSCs retain their capacity to reduce proliferation and IFN-γ secretion in this condition.

It is relevant to evaluate whether patient-derived cells, independent of the course and actual activity of the disease and past or present medication, are susceptible to suppression by MSC. Therefore, we determined the effect of patient derived MSCs on PHA-driven T cell proliferation using autologous PBMC. Patient-derived PBMC were equally suppressed by MSCs compared to healthy control derived PBMC. Our data further show a comparable suppression of T cell responses by allogeneic and autologous MSCs, implying a comparable susceptibility of patient and control PBMC to MSCs.

Our *in vitro* study did not address the route of administration of these expanded MSCs, and the optimal use remains to be determined. Most reported clinical studies

of MSC treatment have used intravenous infusions to control systemic inflammatory diseases, and this would also seem to be a logical choice for the future use in children with sJIA. In contrast, however, oligo-articular disease or target joints might benefit from local intra-articular administration. Future prospective clinical trials should address these issues.

In conclusion, this is the first study describing that MSCs derived from a cohort of patients with sJIA have similar immunosuppressive capacities compared to age-matched HC in assays focused on adaptive (T cell) and innate (NK cell and monocyte) immunity. Therefore, the results of this study support the use of autologous MSCs in clinical trials of patients with sJIA.

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