Design and Methods

Generation of human MSC

After obtaining informed consent, adult bone marrow was harvested from healthy donors or from orthopedic patients. Mononuclear cells were isolated using a Ficoll-Paque density gradient (1.077 g/cm^3) and were plated at 1.3 x 10^5/cm^2 in DMEM-low glucose (Invitrogen Corp., Paisley, UK) supplemented with 10% fetal calf serum (FCS; Greiner Bio-one) and Penicillin/Streptomycin (P/S; Invitrogen Corp., Paisley, UK). After 3-4 days the non-adherent cells were removed and medium was refreshed every 3-4 days until the cells reached confluency. The MSC monolayer was detached using trypsin/EDTA (Invitrogen Corp., Paisley, UK) and reseeded at 4,000 cells/cm^2 for expansion. The MSC were characterized by FACS analysis and used in the co-culture experiments at passage 2-5.

Isolation of monocytes

Human PBMC from healthy donors were isolated from buffy coats obtained from Sanquin Blood Supply using a Ficoll-Paque density gradient (1.077 g/cm^3). From the freshly prepared mononuclear cell fraction, CD14 positive monocytes were purified by MACS using CD14 microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Cells were separated with a MACS LS column according to the manufacturer's recommendations.

Differentiation of monocytes

Freshly isolated monocytes (CD14$^+$) were cultured at a concentration of 1.0 x 10^6 cells/well in 6-wells plates in RPMI (Invitrogen Corp., Paisley, UK) containing P/S, L-glutamin (Invitrogen

Corp., Paisley, UK) and 10% FCS supplemented with the growth factors IL-4 (10 ng/ml) and GM-CSF (5 ng/ml) (both from Invitrogen Corp., Paisley, UK) for 6 days, resulting in the generation of iDC ($CD14^-/CD1a^+$) . To examine the effect of MSC on monocyte differentiation, irradiated MSC (60Gy) were added to the culture at a MSC:monocyte ratio of 1:10 as was described before (23, 24). The co-culture experiments were performed in direct cell-cell contact and in a transwell co-culture system (pore size 0.4 μM; Corning Inc., Lowell, MA, USA). In the transwell experiments, MSC were plated in the lower well and monocytes were added to the transwell insert. All experiments were performed in duplicate. In some experiments IL-10 (20 ng/ml), IL-6 (100 ng/ml), anti-IL-10 (2–20 μg/ml) or anti-IL-6 (2.5 μg/ml) (all from R&D systems Europe Ltd., Abingdon, UK) was added. The involvement of secreted factors was further assessed by addition of MSC conditioned medium (MSC-CM) to the monocyte differentiation culture. Conditioned medium was generated by collecting cell-free culture supernatant from MSC alone (MSC-CM), MSC cultured with growth factors (MSC+GF CM) and from the monocyte-MSC co-cultures (MSC+mono CM). In some experiments, IL-6 was depleted from the MSC-CM using the μMACS Streptavidin kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Cell free MSC supernatant was incubated with biotinylated anti-IL-6 (2 μg/ml, BD Biosciences, San Diego, CA, USA) and μMACS Streptavidin microbeads. IL-6 was depleted from the medium using a μMACS column according to the manufacturer's recommendations. MSC-CM was used in the monocyte differentiation in a 2:1 ratio with fresh medium.

FACS analysis

At day 6 the monocyte-derived cell population was harvested from cultures and analyzed for surface marker expression using CD1a-FITC and CD14-PE (BD Biosciences, San Diego, CA,

USA) by flow cytometry. MSC were trypsinized and analyzed for expression of surface markers using CD90-FITC, CD73-PE, CD45-FITC, CD34-PE, HLA-DR-PE, HLA-ABC-FITC, CD80-PE (BD Biosciences, San Diego, USA) and CD105-FITC (Ancell Corp., Bayport, MN, USA). For surface staining, cells were collected and primary antibodies were added and incubated for 30 minutes at 4°C in the dark. Cells were washed with PBS/1%GPO and analyzed using a FACSCanto II (BD Biosciences, San Diego, CA, US). The analysis of the acquired data was done with FlowJo software version 7.6.1 (Tree Star Inc. Ashland, OR, USA). Statistical analysis was performed using the Student t test.

Analysis of cytokines

Cytokine concentrations were measured in cell-free supernatants collected at day 6 from cultures of monocytes with or without MSC. To distinguish between cytokines produced by MSC or monocytes, the cells from the transwell co-cultures were separated at day 6 and cultured further in fresh medium for an additional 2 days. IL-6 protein concentrations were determined in supernatants of these cultures. We also measured the of cytokine concentrations in supernatants of unstimulated MSC and MSC cultures containing IL-4 and GM-CSF. Culture supernatants were stored at -20°C until use. Cytokine concentrations were measured using the Bio-Plex Pro Human Cytokine assay (Bio-Rad laboratories, Inc, Hercules, CA, USA) or by sandwich ELISA (BD Biosciences, San Diego, CA, USA). Statistical analysis was performed using a Student *t*–test and a $p<0.05$ was considered statistically significant.

Gene expression

Total RNA was extracted from MSC and monocyte populations using the RNeasy micro kit (Qiagen GmbH, Hilden, Germany). cDNA was synthesized with Superscript III RT (Invitrogen Corp., Carlsbad, CA, USA). Q-PCR analyses were performed on a StepOnePlus real-time PCR system (Applied Biosystems, Foster city, CA, USA) using Sybr Green. The following primer sets were used:

gene	Forward	reverse
IL-6	5'-TTCAATGAGGAGACTTGCCTG-3'	5'-ACAACAACAATCTGAGGTGCC-3'
IL-10	5'-CCGAGATGCCTTCAGCAGAG-3'	5'-GGTCTTGGTTCTCAGCTTGG-3'
β-actin	5'-AGGCATCCTCACCCTGAAGTA-3'	5'- CACACGCAGCTCATTGTAGA-3'

All data were normalized using β-actin as a reference gene.

Allogeneic mixed lymphocyte reaction assay

Monocytes cultured for 6 days in the presence or absence of MSC in a transwell co-culture, were tested for their ability to stimulate the proliferation of allogeneic T cells. Allogeneic $CD4^+CD25^-$ T cells were isolated from PBMC by MACS (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) using the $CD4^+CD25^+$ regulatory T cell isolation kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). CD4 T cells were isolated from PBMC using a cocktail of biotin-labeled antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCRγ/δ and CD235a and anti-biotin microbeads followed by separation of $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells with CD25 microbeads. The $CD4^+CD25^-$ T cells were stimulated with iDC or the monocyte-derived cells from the monocyte-MSC co-culture (MDC) at various ratios. After four days incubation, cells

were pulsed overnight with 0.5μCi of [^{3}H]-thymidine to determine T cell proliferation. Thymidine incorporation was expressed as mean corrected counts per minute (CCPM).

Statistical analysis

All data represent the average and standard deviation of multiple MSC donors. Unless otherwise specified, statistical analysis was tested by the Student's t test for two groups and by a two-way ANOVA with a Bonferroni posttest for comparison of 3 groups using Prism5 software (GraphPad Software, Inc., CA, USA).