Mesenchymal stromal cells in pediatric disease : pathophysiology and treatment

Calkoen, Friso Gerhard Jan

Chapter 4.

Gene-expression and *in vitro* function of mesenchymal stromal cells are affected in juvenile myelomonocytic leukemia.

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Calkoen FGJ, Vervat C, Vijfhuizen L, Eising E, 't Hoen PAC,
van den Heuvel-Eibrink MM, Egeler RM, van Tol MJD, Ball LM.

Abstract

An aberrant interaction between hematopoietic stem cells and mesenchymal stromal cells has been linked to disease and shown to contribute to the pathophysiology of hematologic malignancies in murine models. Juvenile myelomonocytic leukemia is an aggressive malignant disease affecting young infants. Here we investigated the impact of juvenile myelomonocytic leukemia on mesenchymal stromal cells. Mesenchymal stromal cells were expanded from bone marrow samples of patients at diagnosis (n=9) and after hematopoietic stem cell transplantation (n=7; from 5 patients) and from healthy children (n=10). Cells were characterized by phenotyping, differentiation, gene expression analysis (of controls and samples obtained at diagnosis) and in vitro functional studies assessing immunomodulation and hematopoietic support. Mesenchymal stromal cells from patients did not differ from controls in differentiation capacity nor did they differ in their capacity to support in vitro hematopoiesis. Deep-SAGE sequencing revealed differential mRNA expression in patient-derived samples, including genes encoding proteins involved in immunomodulation and cell-cell interaction. Selected gene expression normalized during remission after successful hematopoietic stem cell transplantation. Whereas natural killer cell activation and peripheral blood mononuclear cell proliferation were not differentially affected, the suppressive effect on monocyte to dendritic cell differentiation was increased by mesenchymal stromal cells obtained at diagnosis, but not at time of remission. This study shows that active juvenile myelomonocytic leukemia affects the immune response related gene expression and function of mesenchymal stromal cells. In contrast, the differential gene expression of hematopoiesis-related genes could not be supported by functional data. Decreased immune surveillance might contribute to the therapy resistance and progression in juvenile myelomonocytic leukemia.

Introduction

The bone-marrow (BM) niche represents the supportive environment for hematopoietic stem cells (HSC).(Mendez-Ferrer, 2010; Morikawa, 2009) Mesenchymal stromal cells (MSCs), being precursors to osteoblasts, adipocytes and chondrocytes and a cellular constituent of the niche, are crucial for maintenance of quiescent HSC. (Sugiyama, 2006b) MSCs, or differentiated sub-populations of these cells, are used *in vitro* as a model for the BM microenvironment. Soluble factors as well as direct cell-to-cell contact have been described to play a role in normal MSC-HSC interaction. (Greenbaum, 2013; Wang, 2010)

Hematopoietic malignancies such as leukemia originate in the BM. Although leukemic blast cells can be detected throughout the body during disease, the leukemic stem cells are thought to remain in the BM, and more specifically in the hematopoietic stem cell niche.(Ayala, 2009) It is widely accepted that malignant cells have a negative impact on the normal hematopoiesis causing anemia and thrombocytopenia. However, the effect of the malignant cells on the BM microenvironment has not been studied extensively.

Recent studies in mice have demonstrated that myeloid neoplasms affect the normal niche structure.(Schepers, 2013; Zhang, 2012b; Arranz, 2014) These alterations contribute potentially to the formation of the leukemic niche in which leukemic stem cells are difficult to target by conventional chemotherapy or irradiation.(Kurtova, 2009) Studies describing MSC characteristics in human myeloproliferative neoplasms are mostly limited to adult patients, demonstrating conflicting results with regard to genetic abnormalities, gene-expression and MSC function.(Zhao, 2012b; Raaijmakers, 2012; Klaus, 2010; Flores-Figueroa, 2008)

Juvenile Myelomonocytic Leukemia (JMML) is an aggressive leukemia occurring in young children, predominantly in infants between 0 and 4 years of age. Patients usually present with hepatosplenomegaly, fever and monocytosis.(Niemeyer, 1997) Monosomy 7 is the most common karyotype abnormality detected in 25% of cases, and numerous leukemogenic mutations have been identified mainly involving the RAS-RAF-ERK pathway, *e.g. PTPN11*, *K-RAS* and *c-CBL*.(Niemeyer, 2008; de Vries, 2010) Hematopoietic stem cell transplantation (HSCT) is the standard first line treatment. Unfortunately, the one year relapse rate ranges between 30 and 50%.(Locatelli, 2005; Locatelli, 2015)

We hypothesize that the aggressive and therapy resistant characteristics of JMML may point towards an altered BM microenvironment based on previous experimental data suggesting support of a neoplasm by the stromal compartment.(Zhang, 2012b; Arranz, 2014) In the current study we aimed to identify stromal factors involved in the support of JMML. MSCs of patients with JMML were obtained at diagnosis and after

HSCT and expanded from BM. These patient derived MSCs were compared to healthy pediatric donor derived MSCs by investigating their capacity of immunomodulation and hematopoietic support and their gene expression profiles.

Methods

A detailed version of the Methods section is available online at the Haematologica website.

Patients

Children referred to our center for HSCT were included in this study according to a protocol approved by the institutional review board (P08.001). BM of 9 consecutive children with JMML was collected prior to treatment initiation. In addition, BM after HSCT was collected from 5 of these 9 children. The patients were classified following previously described criteria.(de Vries, 2010; Loh, 2011) BM samples were sent to the EWOG-MDS-reference center in Freiburg, Germany for mutation analysis. BM samples of healthy pediatric HSCT donors (n=10) were used as control group (HC). Informed consent was obtained from all parents. This study was conducted according to the Declaration of Helsinki.(World Medical Association, 2013)

MSC expansion and characterization

MSC were expanded from fresh BM and characterized as previously described. (Calkoen, 2013a) Monosomy 7 by FISH and chimerism by variable number of cytosine adenine (CA) repeat analysis were determined in expanded MSC before and after HSCT, respectively.(Lankester, 2010; Bronkhorst, 2011) MSC gene expression and function was investigated using MSCs obtained at passage 2-3 and 3-5, respectively.

Functional assays

The effect of MSCs on proliferation of peripheral blood mononuclear cells (PBMCs) was investigated in co-cultures stimulated with phytohemagglutinin (PHA) by measuring ^{3}H-thymidine incorporation.

The suppressive effect of MSC on NK cell activation was determined by stimulation of purified NK cells with IL-2 in the absence or presence of MSCs for 5 days. NK cells were harvested and activation was measured by flow cytometry investigating DNAM-1, NKp30 and NKp44 expression.

To evaluate the effect of MSCs on antigen presenting cells, monocytes were isolated from PBMCs and cultured with growth factors for 5 or 7 days to differentiate towards

immature dendritic cells (DC) or mature DC, respectively. Cells were phenotyped for the expression of CD14 and CD1a after co-culture.

Short-term co-culture assays with MSCs and hematopoietic progenitor cells (HPC) were performed to determine the supportive capacity of MSCs for HPCs maintenance and differentiation. Therefore, HPCs were isolated from healthy transplant donors using CD34 positive selection. Proliferation (day 7) and differentiation (day 7 and 14) were assessed using ^{3}H-thymidine incorporation and flow cytometry, respectively.

To determine a direct effect of MSCs on HPC differentiation into colony-forming-units (CFU), MSCs were added to freshly purified HPCs in methylcellulose containing growth factors and cultured for 14 days (CFU-assay).

Gene expression

Total RNA was isolated from MSCs and mRNA was profiled using Deep-SAGE (serial analysis of gene expression) sequencing using Illumina technology.(Mastrokolias, 2012) Data were mapped against the UCSC-hg19 reference genome using Bowtie, with permission of one mismatch and only retaining unique mappings.

Expression of genes of interest was validated using independent biological samples by RT-qPCR after generation of cDNA using the listed primers (Supplementary Table 1), as previously described.(Mastrokolias, 2012) Expression levels were calculated relative to the average expression of two housekeeping genes.

Statistical analysis

Mann-Whitney and Wilcoxon matched-pairs signed rank tests were performed to compare different groups in functional assays and in the validation of mRNA expression. Differential gene expression analysis was performed in R, using EdgeR, Globaltests and Limma data analysis packages.(Robinson, 2010; R Development Core Team, 2012; Smyth, 2005) Correction for multiple testing was performed according to Benjamini and Hochberg.(Hochberg, 1990) STRING software was used for analysis of protein interactions.(Franceschini, 2013) Adjusted *p*-values <0.05 were considered statistically significant.

Results

Patients

MSCs were successfully expanded from all 9 patients with JMML (Table 1) at diagnosis and 10 healthy controls (HC). In addition, 7 BM samples collected after HSCT, derived from 5 of the 9 JMML patients included, were used for MSC expansion (Table 1). These MSCs were of patient origin as tested by CA-repeat analysis. Median age

at diagnosis was 2.1 years (range 0.5 - 3.5), whereas the age of healthy controls ranged between 1.1 and 16.4 years (median 7.4). Children with different genetic mutations were included as shown in Table 1. Monosomy 7, present at diagnosis in two children, was not detected in the MSCs at diagnosis.

MSC expansion and characterization

All expanded MSC populations fulfilled the criteria proposed by Horowitz *et al*. (Horwitz, 2005), *i.e.* differentiation towards adipocytes and osteoblasts, a characteristic phenotype ($CD73^+$, $CD90^+$, $CD105^+$, HLA-DR^- and no expression of lineage markers) and adherence to plastic. Representative examples of differentiation of

Table 1. Patient characteristics

UPN	Sex	Age at diagnosis (months)	Chromosomal abnormalities and gene mutation(s)	Donor and graft type	Conditioning	MSC post HSCT (time BM sample, months after HSCT)	Relapse (months after HSCT)
JMML001	F	8	None	MUD/ PBSC	Thio, Flu, Treo, ATG	Yes (6^1)	No
JMML002	M	9	c-CBL	MUD/ BM	Flu, Bu, ATG	Yes (4^1)	No
JMML003	M	32	RAS (exon 1 c35 G>A)	MUD/ BM	Bu, Cy, Mel, ATG	Yes (3^1)	No
JMML004	M	35	PTPN11 (exon 3 c182 A>T)	IRD/BM	Bu, Cy, Mel	Yes (4^1, 7^1, 17^2)	Yes (12, 17)
JMML005	F	26	Monosomy 7 + NF1 gene: (2033delC en R416x)	MUD/ BM	Bu, Cy, Mel, ATG	No	No
JMML006	F	6	None	MUD/ CB	Flu, Treo, ATG	No	No
JMML007	M	43	PTPN11 gene (exon 13 1508 G>C 503 G>A	MUD/ CB	Flu, Bu, ATG	Yes (5^2)	Yes (5)
JMML008	F	35	PTPN11 gene (exon 13 1504 T>C 502 S>P)	MUD/ BM	Bu, Cy, Mel, ATG	No	No
JMML009	M	12	Monosomy 7/RAS	MUD/ PBSC	Flu, Bu, ATG	No	No

Donor: MUD: matched unrelated donor; IRD: identical related donor. Graft type: BM: bone-marrow; CB: cord blood; PBSC: peripheral blood stem cells. Conditioning: Thio: thiotepa; Flu: fludarabine; Treo: treosulfan; ATG: anti-thymocyte globulin; Bu: busulfan; Cy: cyclophosphamide; Mel: melphalan. [1]: Bone-marrow (BM) sample after HSCT was obtained after remission induction; [2]BM sample after HSCT was obtained at relapse.

MSCs prior to and after HSCT are shown in Figure 1A-B. Besides these criteria, MSCs have been described to suppress PHA-induced PBMC proliferation. MSCs derived from patients and controls did not differ in their immunosuppressive capacity of PHA-induced PBMC proliferation (Figure 1C).

Hematopoietic support

MSCs have been described to play an important role in the tight regulation of hematopoiesis in the bone marrow. We investigated the capacity of JMML-MSCs derived at diagnosis to support hematopoiesis *in vitro*. HPC proliferation induced by SCF and Flt3-L was enhanced in the presence of MSCs and dependent on the HPC : MSC ratio (Figure 2A). JMML-MSCs and healthy control MSCs did not differ in this respect. Despite increased HPC proliferation, the percentage of $CD34^+$ cells after 14 days of

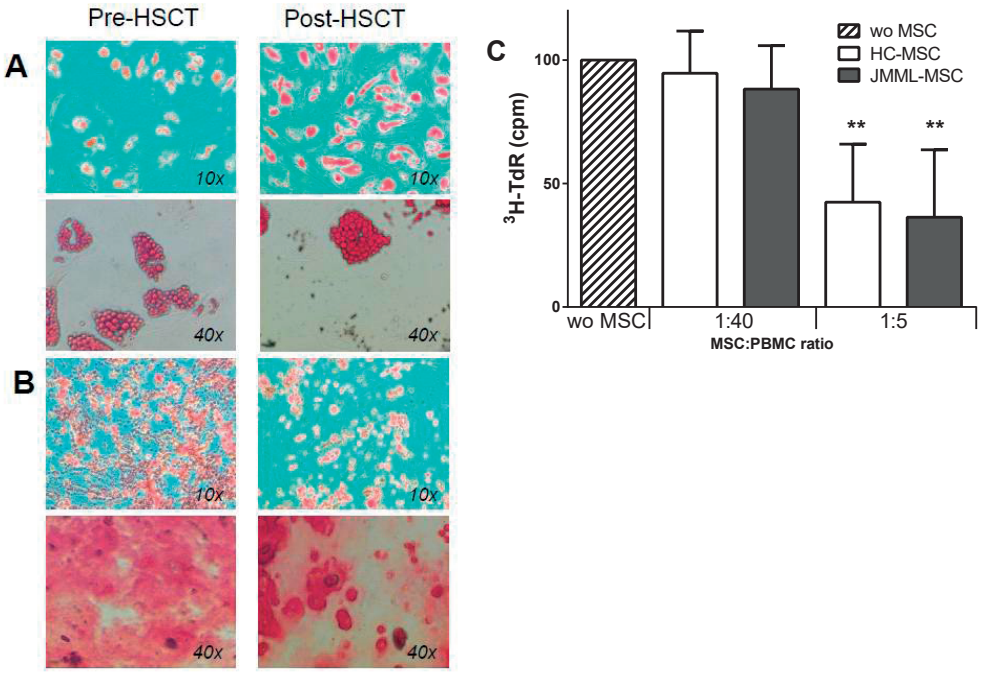

Figure 1. *Expansion and characterization of MSCs from JMML patients at diagnosis and post-HSCT and healthy controls.* MSCs were expanded and differentiation capacity towards adipocytes (A) and osteoblasts (B) was evaluated. MSCs were cultured with differentiation factors for 3 weeks and Oil Red O and Alizarine Red was used to stain fat and calcium deposition, respectively (examples are representative for MSCs of 9 JMML patients at diagnosis and 5 JMML patients after HSCT). C. Healthy Control (HC)-MSCs and JMML-MSCs showed a comparable dose-dependent suppressive effect on PBMC proliferation after PHA stimulation. Boxes indicate the mean proliferation at the indicated PBMC:MSC ratio relative to the proliferation induced without (wo) MSCs. Error bars represent standard deviation. Experiments were performed with n=7 JMML-MSCs and n=4 HC-MSCs. Statistics were performed using Mann-Whitney tests: **: $p<0.01$.

culture was significantly better maintained in the presence of MSCs (Figure 2B). Cells that lost CD34 expression expressed lineage markers such as CD14. The frequency of $CD14^+$ cells amongst $CD45^+$ lineage-committed cells was significantly higher after 7-14 days of culture of $CD34^+$ cells with MSCs (Figure 2C). The supportive effect by MSCs was comparable between MSCs of JMML patient and MSCs of healthy controls.

To evaluate the effect of MSCs on functional differentiation of HPCs, MSCs were added to semi-solid cultures of isolated HPCs in methylcellulose containing growth-factors. Both the total number of CFU and the different types of colonies increased in the presence of MSCs (Figure 2D). The percentage of $CD45^+CD235a^-$ cells was increased after culture with MSCs suggesting support of differentiation and proliferation of myeloid cells at the cost of $CD45^-CD235a^+$ erythroblasts in this culture condition (Figure 2E). Indeed, also in this culture configuration the percentage of monocytes ($CD14^+$ cells) was significantly increased in the presence of both control as well as JMML-MSCs (Figure 2F). Also in these differentiation assays, no difference was observed between JMML-MSCs and healthy control MSCs.

Gene expression analysis

Because the functional tests executed so far did not reveal differences between JMML and healthy control (HC) MSCs, we explored possible differences in mRNA expression profiles by performing Deep-SAGE sequencing, a next generation sequencing-based approach with higher sensitivity than traditional microarrays (*de novo* JMML n=8; HC n=8).(Hoen 't, 2008) The median number of obtained reads that fulfilled quality control criteria was $15.9x10^6$ reads (range $11.4x10^6$ - $30.6x10^6$). A median of 65.6% of all reads aligned uniquely to the reference genome (range 59.3% - 68.4%). The percentage of the aligned reads mapping to an annotated exon was 84.5% (range: 74.7% - 86.3%).

The differentially expressed genes (n=162; $p<0.001$) are listed in Supplementary Table 2. A heat-map of the top 100 differentially expressed genes shows clustering of the JMML and HC derived MSCs (Supplementary Figure 1). After correction for multiple testing, in total 43 genes were differentially expressed between JMML and HC-MSCs (criterion: false discovery rate (FDR) < 0.05, see Supplementary Table 2) The top 6 genes (Dickkopf-1 (*DKK1*), ectonucleotide pyrophosphatase/phosphodiesterase 2 (*ENPP2*), DEAD (Asp-Glu-Ala-Asp) box helicase 17 (*DDX17*), monooxygenase DBH-like 1 (*MOXD1*), Adenomatosis Polyposis Coli Down-Regulated 1-Like (*APCDD1L*) and tumor necrosis factor alpha-induced protein 2 (*TNFAIP2*)), that were expressed at intermediate to high levels (> 100 reads per million sequenced reads, in both patients and controls) were selected for RT-qPCR validation (Figure 3).

In addition, three out of the 162 differentially expressed genes were previously reported to be involved in MSC function and were validated using RT-qPCR (*CXCL12,*

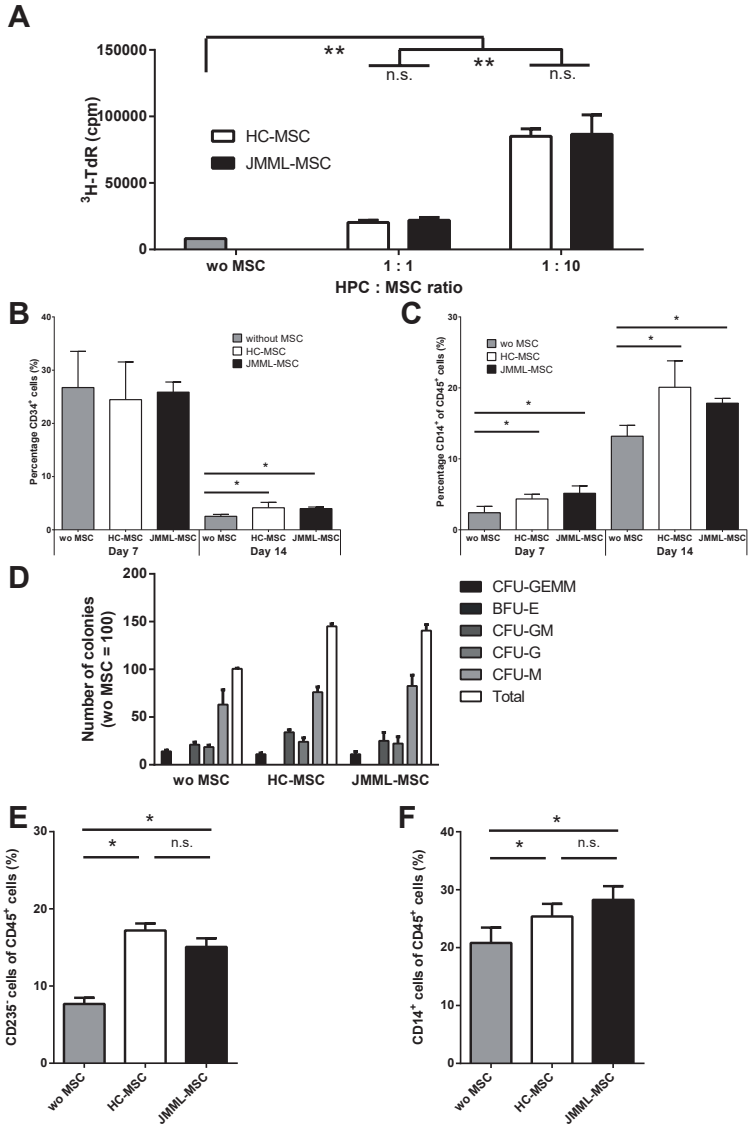

Figure 2. *JMML-MSCs support the proliferation and differentiation of HPCs in vitro.* A. Both in the presence of JMML patient and healthy control (HC) derived MSCs the proliferation of $CD34^+$ cells (HPCs) was increased after 7 days of culture. MSCs alone did not show ^{3}H-thymidine incorporation (data not shown). B. HPCs lost the expression of CD34 after 14 days of culture; however, in the presence of MSCs (HPC : MSC ratio 1:5) the decline in CD34 expression was diminished. C. HPCs acquired lineage markers, *e.g.* CD14, but no differences were seen between JMML-MSCs and HC-MSCs. D. A significantly (p<0.05) increased number of colonies was seen in CFU-assays in the presence of MSCs (HPC : MSC ratio 1:60). E-F. Cells harvested after colony formation contained increased percentages of CD235a negative and CD14 positive cells within the $CD45^+$ cell population. Boxes indicate the mean and error bars represent standard deviation. Experiments were performed with n=4 JMML-MSCs and n=2 HC-MSCs. Statistics were performed using Mann-Whitney tests: *: p<0.05; **: p<0.01; n.s.: non-significant.

CXCR7 and *IL-6*, Figure 3G, 3F and 3H, respectively*). CXCL12* (Figure 3G), previously reported to be of importance in HSC - MSC interaction and mobilization of HSCs, was found to be significantly decreased in JMML-MSCs.(Sugiyama, 2006b) Whereas the commonly involved receptor *CXCR4* was not differentially expressed, expression of the alternative receptor *CXCR7* was significantly decreased in JMML-MSCs (Figure 3F). String analysis of the top differentially expressed genes (data not shown) revealed that many of the genes upregulated in JMML-MSCs are associated with the IL-1 superfamily (*IL-1*β, *IL-6* (Figure 3H), *PTHLH*, *CLU*, *ATF3*, *PENK*, *RGS3* and *RGS16*). *DKK1* expression (Figure 3B), related with osteolysis, was also increased. In contrast, expression of genes in the leptin pathway was decreased (*LEP*, *LEPR*, *KISS1*, *SLC25A27*, *RXRA* and *CBLB*). Results of gene-ontology (p<0.001 after Holms correction and at least 100 covariates) are listed in the Supplementary Table 3. Pathways related to immune responses and protein ubiquination, involved in cell regulation of cellular interaction,(Hammond-Martel, 2012) were predominantly affected.

Normalization of gene expression in JMML-MSCs after HSCT

Genes of interest, identified by Deep-SAGE, were studied in patient samples after HSCT. BM obtained at remission was available for MSC expansion of 4 patients, 5 samples in total. RT-qPCR was used to study gene expression in these samples. *ENPP2*, *DDX17*, *CXCR7*, *CXCL12* and *TNFAIP2* expression was decreased in JMML-MSCs at diagnosis, however, expression was restored to the level of HC-MSC in samples after HSCT (Figure 3A, D, F, G, I). *DKK1*, *MOXD1* and *IL-6* expression was increased in JMML-MSCs at diagnosis, but normalized in JMML-MSCs post-HSCT (Figure 3B, C, and H). *APCDD1L* (Figure 3E), a paralog of the WNT inhibitor *APCDD1*,(Shimomura, 2010) was the only gene studied, of which the abnormal (increased) expression at diagnosis remained unchanged after HSCT. BM at time of relapse post HSCT was only available in two patients and therefore not included in the gene-expression analysis.

Immunomodulation

Differential expression of *IL-6* and other genes in the IL-1 superfamily suggests a differential effect of JMML patients derived MSCs on the innate immune system. Escape from NK cell surveillance is an important survival mechanism in tumorigenesis. However, HC-MSCs and JMML-MSCs derived from BM obtained at diagnosis suppressed NK cell activation to a similar extent (Figure 4A).

The suppressive effect of MSCs on monocyte to dendritic cell (DC) differentiation has been described to be IL-6 dependent.(Melief, 2013) Therefore, we analyzed the monocyte to DC differentiation in the absence and presence of MSCs at different MSC : monocyte ratios. The addition of both sources of MSCs resulted in decreased differentiation to immature DCs as measured by a lower percentage of cells express-

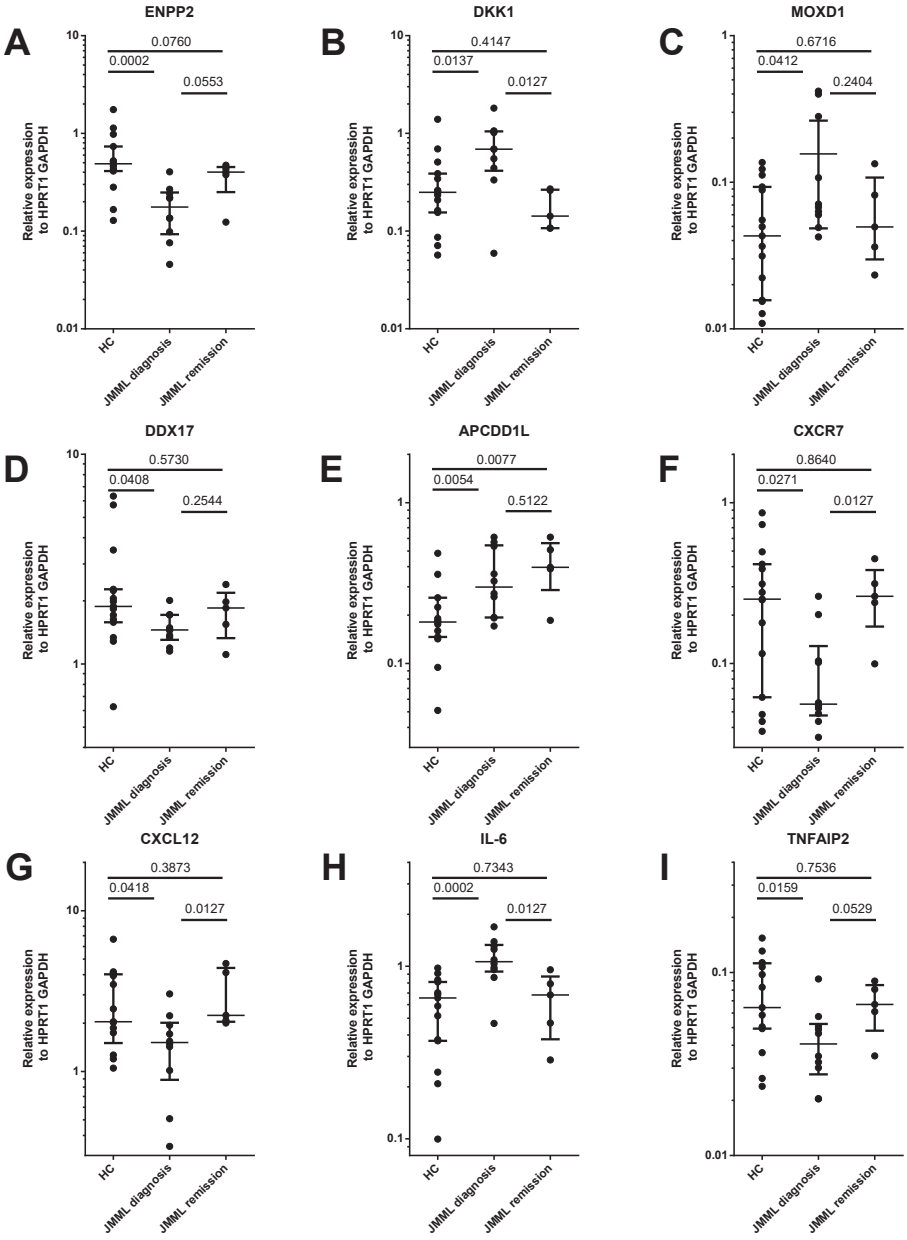

Figure 3. *Gene expression of differentially expressed genes in MSCs of JMML patients at time of diagnosis and after HSCT.* Genes identified by Deep-SAGE sequencing were validated by RT-qPCR in JMML-MSCs expanded from bone-marrow samples obtained prior to (n=9) and after HSCT at time of remission (n=5) and in MSCs derived from healthy donors (HC, n=10). Expression levels were expressed relative to that of the average of the housekeeping genes *HPRT1* and *GADPH*. The results are indicated as median (horizontal line) and interquartile range (whiskers) on a logarithmic scale. Statistics were performed using Mann-Whitney tests.

ing the DC marker CD1a on day 5 of co-culture; these cells remained $CD14^+$ (Figure 4B-C). JMML-MSCs had a significantly stronger suppressive effect on monocyte to immature DC differentiation than HC-MSCs. This was supported by the observation that CD163 expression on $CD14^+$ cells, a marker associated with anti-inflammatory monocytes and macrophages, was higher after co-culture with JMML-MSCs (MFI: 49.3 +/- 7.4 SEM) compared to co-culture with HC-MSCs (MFI: 26.6 +/- 4.7 SEM; $p = 0.03$, data not shown).

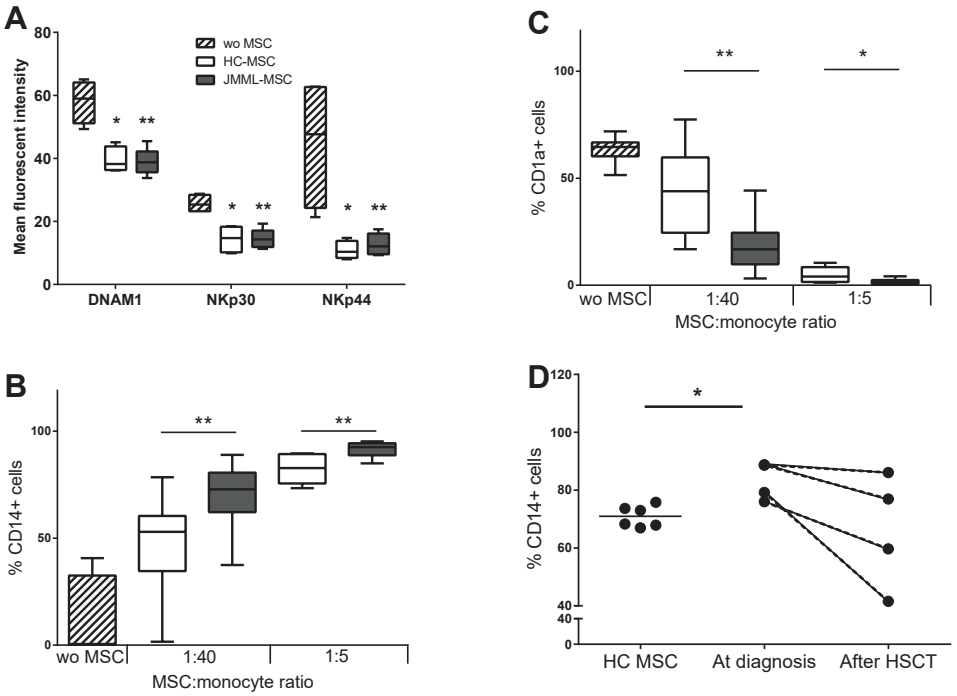

Figure 4. *JMML-MSCs expanded from bone-marrow at diagnosis have an increased suppressive effect on monocyte to immature dendritic cell differentiation but not on NK cell activation.* A. NK cell activation induced by IL-2 (30 IU/mL), measured by mean fluorescence intensity (MFI) of staining for DNAM-1, NKp30 and NKp44 expression on day 5, was suppressed in the presence of MSCs of healthy controls (HC) and JMML patients. MSC: NK cell ratio 1:5 (JMML-MSCs n=4 and HC-MSCs n=4). B-C. An increased suppressive effect of JMML-MSCs was observed on the differentiation of monocytes ($CD14^+$ cells, B) to immature dendritic cells ($CD1a^+$ cells, C) during 5 days of stimulation with IL-4 and GM-CSF (JMML-MSCs n=6, grey boxes, and HC-MSCs n=6, white boxes). D) JMML-MSCs derived from bone-marrow after hematopoietic stem cell transplantation (HSCT) showed a trend towards a lower suppressive effect on the monocyte to immature DC differentiation than JMML-MSCs derived from bone-marrow obtained at diagnosis. Paired samples of JMML-MSCs at diagnosis and after HSCT (n=2) and HC-MSCs (n=3) were investigated using monocytes from two donors as target populations (MSC : monocyte ratio 1:40). Results in panel A-C are depicted as mean (horizontal line) with interquartile distance (boxes) and range (whiskers). Statistics were performed using Mann-Whitney tests in panel A-D. *: $p<0.05$; **: $p<0.01$; wo: without MSC.

The differential suppressive effect of JMML-MSCs compared to HC-MSCs disappeared after two additional days in culture with growth-factors (IFN-γ and CD40-L) to support the differentiation of immature DCs to mature DCs (data not shown).

Gene-expression, *i.e. IL-6*, in MSCs obtained after HSCT was comparable to healthy controls. Therefore, the effect of JMML-MSCs expanded from BM after HSCT on monocyte to immature DC differentiation was studied. The percentage of $CD14^+$ cells in co-cultures of monocytes with JMML-MSCs derived from BM obtained after HSCT during remission was variable and not different from that in co-cultures with HC-MSCs. In addition, MSC derived during remission showed a trend to a lower percentage of $CD14^+$ cells compared to the percentage observed in co-cultures with JMML-MSC at diagnosis.(Figure 4D).

Discussion

A genetic mutation in the RAS-pathway is identified in 90% of JMML patients.(de Vries, 2010) However, the etiology of therapy resistance and the high relapse rate after HSCT remains unknown. The aggressive nature of the disease in combination with the high burden of malignant cells may lead to a disturbance of the BM microenvironment.

As a model for the BM microenvironment, we used BM derived MSCs. In our study, MSCs of JMML patients had a comparable differentiation capacity compared to MSCs of healthy controls. In contrast to previously reported studies in adult MDS, adipocyte and osteoblast differentiation was not adversely affected.(Zhao, 2014) This is in line with the unaffected differentiation capacity of MSCs derived from BM of children with MDS.(Calkoen, 2015) However, gene expression analysis revealed increased *DKK1* expression in JMML derived MSCs. *DKK1*, related with osteolysis and *WNT* signaling, has been described to be upregulated in MSCs derived from patients with multiple myeloma.(Corre, 2007) Identification of this protein, also expressed by multiple myeloma cells,(Qian, 2007) led to therapeutic strategies using antibodies or vaccination against *DKK1* with a beneficial effect in murine models.(Heath, 2009; Qian, 2012)

The control group differed from the patients regarding age at BM collection. However, data were validated using selected controls aged 1 to 4 years (n=4). We did not observe an age dependent effect on gene-expression in our patient or control MSCs.

In this study, the suppressive effect of JMML-MSCs on differentiation from monocytes to immature DCs was significantly stronger compared to MSCs of healthy controls. The increased *IL-6* expression by JMML-MSCs suggests a causal relation because IL-6 has been described to be essential in the suppressive mechanism.(Melief, 2013) Previously, pediatric MDS derived MSCs showed an even stronger increase in

IL-6 expression, whereas no effect on monocyte to DC differentiation was observed. (Calkoen, 2015) Inhibition of differentiation towards professional antigen presenting cells might contribute to the escape of JMML cells from the immune system and might explain the usually progressive course of this disease. NK cells are involved in the innate defense against malignant transformed cells. Whereas IL-6 production has been reported to suppress NK cell cytotoxicity against neuroblastoma and lymphoblastoid cells, facilitating tumor escape,(Xu, 2013; Tanner, 1991) the suppressive effect of MSCs on NK cell activation was comparable between JMML-MSCs and HC-MSCs. In previous studies, we demonstrated a correlation between the suppressive effect of MSCs on NK cell activation marker expression, *i.e.* DNAM-1, NKp30 and NKp44, and cytotoxicity.(Calkoen, 2013a)

JMML clinically presents with anemia and thrombocytopenia in combination with monocytosis and leukocytosis. Previously, the misbalance in hematopoiesis in patients with hematologic malignancies was suggested to be caused by factors excreted by malignant cells.(Aoyagi, 1994) In addition, disturbed support of hematopoiesis by MSCs might contribute to dysplasia in these patients. *CXCL12* expression was decreased in JMML-MSCs as was also previously shown in studies on MSC from adult CML and pediatric ALL patients.(van den Berk, 2014; Zhang, 2012b) The CXCL12-CXCR4 interaction is an important mechanism in hematopoietic support and decreased *CXCL12* expression has been linked to dysplasia in mice and adult chronic myeloid leukemia.(Zhang, 2012b; Arranz, 2014) Our *in vitro* hematopoiesis experiments did not reveal differences in support of proliferation and differentiation of HPCs between JMML and control derived MSCs. Both JMML and healthy control MSCs have a strong supportive effect on hematopoiesis involving multiple pathways, *e.g.* CXCL12, G-CSF and SCF. Therefore, aberrant expression of one of these molecules could be compensated by other pathways in our *in vitro* experiments. In this study, we used *in vitro* and polyclonal expanded MSCs and as a consequence differences *in vivo* between healthy controls and JMML derived MSCs might be lost during culture. However, Zhang *et al.* demonstrated sustained decreased expression of *CXCL12 in vivo and* in *in vitro* expanded MSCs.(Zhang, 2012b) Further *in vivo* characterization of the MSCs in mice models modelling the hematopoietic niche with human MSCs, such as previously used in *e.g.* multiple myeloma, might help to identify more subtle differences.(Groen, 2012)

The different gene expression profile observed in JMML-MSCs versus HC-MSCs is more likely the consequence than the cause of the disease, with JMML cells interacting with the stromal compartment. This conclusion is supported by the observation that after allogeneic HSCT, in which hematopoietic progenitor cells become of donor origin, gene expression profiles in MSCs normalized in paired samples analysis. Of

note, the MSCs after HSCT remain of patient origin, suggesting that the aberrant MSC gene expression pattern before HSCT is induced by JMML cells.

In conclusion, our data demonstrate an effect of JMML cells on MSCs during active disease. *In vitro* hematopoietic support and maintenance of CD34+ HPCs by JMML-MSCs were not affected despite differential gene expression. However, immunosuppression was affected via a stronger decrease of monocyte to immature DC differentiation by JMML-MSC.

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Supplementary data

Methods for online publication

Patients

Children referred to our center for HSCT were included in this study according to a protocol approved by the institutional review board (P08.001). Bone marrow of 9 children with JMML was collected prior to treatment initiation. In addition, bone marrow after HSCT was collected from 5 of these 9 children. The patients were classified following the criteria described by Loh *et al*.(Loh, 2011) Bone marrow samples were sent to the JMML-reference center in Freiburg, Germany for genetic analysis. Bone marrow samples of healthy pediatric hematopoietic stem cell donors (n=10) were used as control group (HC). Informed consent was obtained from the children and/or their parents or guardians. This study was conducted according to the Declaration of Helsinki.(World Medical Association, 2013)

MSC expansion and characterization

MSCs were expanded and characterized as previously described.(Calkoen, 2013a) Briefly, bone marrow mononuclear cells (MNC) obtained after Ficoll separation were cultured in DMEM (Invitrogen, Paisley, UK) containing 100 U/mL penicillin/100 µg/mL streptomycin (P/S; Invitrogen) and 10% (v/v) fetal bovine serum (FBS; VWR International, Bridgeport, NJ, USA). Non-adherent cells were removed by refreshing medium twice weekly. Upon reaching confluence MSC were harvested and passaged for further expansion. Phenotype (CD73, CD90, CD105 positive; CD3, CD31, CD34, CD45, CD86, HLA-DR negative) and differentiation capacity towards osteoblasts and adipocytes were investigated at passage 2-3 and 5-7, respectively. All but anti-CD105 (Ancell Corporation Bayport, MN) antibodies were derived from Becton Dickinson Biosciences (BD), San Diego, CA, USA.

To investigate common chromosome abnormalities in MSCs and malignant cells, interphase fluorescence in situ hybridization (FISH) for chromosome 7 was performed on MSC from patients with known monosomy 7 using the Vysis LSI D7S486/CEP7 (Abbott Laboratories, Abbott Park, IL,USA) probe.(Bronkhorst, 2011) Chimerism of MSCs obtained after HSCT (donor or recipient origin) was studied by variable number of cytosine adenine (CA)-repeat analysis in MSCs cultured from bone marrow harvested after HSCT as previously described.(Lankester, 2010) MSC function was investigated using MSCs obtained at passage 3 to 5 and MSC gene expression was analyzed using MSCs obtained at passage 2 to 3.

Immunomodulatory assays

Effect of MSCs on PHA-induced PBMC proliferation

The effect of MSCs (30-Gy irradiated) on proliferation of peripheral blood mononuclear cells (PBMC) obtained from adult bloodbank donors (100.000 cells/well) after stimulation with phytohemagglutinin (PHA, PeproTech, London, UK, 2 µg/mL) was analyzed at MSC : PBMC ratios of 1:5 and 1:40. MSCs and PBMC were co-cultured in RPMI P/S, 10% (v/v) fetal calf serum (FCS) for 5 days with the addition of ^{3}H-thymidine (1 µCi/well; Perkin Elmer, Wellesley, MA, USA) for the last 16 hours to measure proliferation using a β-counter (Perkin Elmer). Experiments were performed in triplicate.

Effect of MSCs on NK cell activation

The suppressive effect of MSCs on NK cell activation was determined using NK cells isolated from PBMC of bloodbank donors with manual MACS cell separation technology and negative selection (Miltenyi Biotec, Bergisch Gladbach, Germany). NK cell purity ($CD56^{+}CD3^{-}$ cells, using anti-CD3-PerCPC5.5 and anti-CD56-APC (Immunotech, Marseille, France)) was over 95%. NK cells (0.1 x 10^{6}/well) were cultured with (0.02 x 10^{6}/well) or without MSCs in the presence of IL-2 (30 IU/mL, Chiron Corporation, Emeryville, CA, USA) for 5 days. NK cells were harvested and activation was measured by flow cytometry investigating the mean fluorescence intensity (MFI) of DNAM1 (BD), NKp30 (Immunotech) and NKp44 (Immunotech) expression using PE-labelled antibodies.

Effect of MSCs on monocyte differentiation

To evaluate the effect of MSCs on antigen presenting cells, monocytes were isolated from PBMC of blood bank donors using positive CD14 selection (Miltenyi Biotec) and cultured with IL-4 (40 ng/mL) and GM-CSF (800 IU/mL) (both from Tebu-Bio, Le Perray en Yvelines, France) for 5 days to differentiate towards immature dendritic cells (DC). Cells were harvested or cultured for 2 additional days with IL-4, GM-CSF, IFN-γ (500 U/mL, Boehringer, Mannheim, Germany) and CD40-ligand (0.25 µg/mL Beckman-Coulter, Marseille, France) to generate mature DC. Cells were phenotyped by flow cytometry for the expression of CD14 and CD1a (both antibodies from BD) on day 0, day 5 and day 7 after co-culturing of monocytes and MSC at MSC : monocyte ratios 1:5 or 1:40 or after culturing monocytes without MSCs.

In vitro hematopoietic support of MSCs

Short-term co-culture assays with MSCs and hematopoietic progenitor cells (HPC) were performed to determine the supportive capacity of MSCs for HPC maintenance and differentiation. Therefore, HPC were isolated from remaining material of G-CSF

mobilized stem cell grafts from healthy transplant donors using CD34 positive selection (Miltenyi). After purification, >90% of selected cells expressed CD34. To investigate the effect of MSCs on proliferation of $CD34^+$ cells, short-term cultures of 1000 CD34 selected cells/well without or with MSCs (MSC : $CD34^+$ cell ratios 1:1 and 10:1) were performed in Stemspan medium (H3000, StemCell Technologies, Vancouver, Canada) with addition of 1% P/S, stem cell factor (SCF, 100 ng/mL, StemCell Technologies) and Flt3-ligand (Flt3-L, 100 ng/mL, StemCell Technologies), because SCF and Flt3-L are not produced by MSCs. To investigate the effect of MSCs on differentiation of $CD34^+$ cells, cultures were initiated with $10x10^3$ $CD34^+$ cells at a $CD34^+$ cell : MSC ratio of 1:5. Half of the culture medium was refreshed with the addition of growth factors on day 4, 7 and 11. Proliferation (day 7) and differentiation (day 7 and 14) were assessed using ^{3}H-thymidine during the last 16 hours or flow cytometry, respectively. Antibodies used for flow cytometry were anti-CD13-PE, anti-CD14-FITC, anti-CD33-APC, anti-CD34-PE, anti-CD45-FITC, anti-CD45-Percpc5.5, anti-CD163-PE and anti-CD235a-PE (all antibodies from BD).

To determine a direct effect of MSCs on HPC differentiation into colony-forming units (CFU), MSC (30.000 per dish) were added to freshly purified HPC (500 cells/dish) in methylcellulose containing SCF, GM-CSF, IL-3 and EPO (H4434 StemCell Technologies), and cultured for 14 days (CFU-assay). Colonies were scored by two independent observers according to standard guidelines for the definition of CFU-GEMM, BFU-e, CFU-GM, CFU-G and CFU-M. Results are depicted as the average of duplicate dishes. After scoring of colonies in the CFU-assay, cells were harvested and phenotyped for the expression of CD45, CD14 and CD235a by flow cytometry as described above.

Gene expression

Total RNA was isolated from MSCs obtained at passage 2 to 3 using a Qiagen RNeasy Minikit (Qiagen, Hilden, Germany). mRNA was profiled using Deep-SAGE sequencing using Illumina technology.(Mastrokolias, 2012) CATG was added to the 5' end of the 17 base pair sequences obtained. Data were mapped against the UCSC hg19 reference genome using Bowtie for Illumina (version 1.1.2) with the permission of one mismatch and suppression of reads if more than one best match existed. Tags aligned to the same gene were summed for further analysis. Gene information was added to the sequences with the biomaRt package in R (version 2.16.0).

Expression of genes of interest was validated using independent biological samples by RT-qPCR after generation of cDNA (cDNA synthesis kit, Roche, Basel, Switzerland) using the listed primers (Supplementary Table S1), as previously described. (Mastrokolias, 2012) Expression levels were calculated relative to expression of the housekeeping genes *GAPDH* and *HPRT1*.

Statistical analysis

Graphpad 6 (Prism, La Jolla, CA) was used for data-analysis. Mann-Whitney and Wilcoxon matched-pairs signed rank tests were performed to compare different groups in functional assays. Validation of gene-expression amongst the different groups was compared using Mann Withney tests. Differential gene expression analysis was performed using the following data analysis packages in R (version 2.15.0): EdgeR (version 3.2.4) for data normalization, Globaltests (version 5.12.0) for gene-ontology, and Limma (version 3.16.7) for correction of multiple testing performed according to Benjamini and Hochberg.(Robinson, 2010; R Development Core Team, 2012; Smyth, 2005; Hochberg, 1990) STRING version 9.1 software was used for analysis of protein interaction (string-db.org).(Franceschini, 2013) Adjusted *p*-values <0.05 were considered statistically significant.

Supplementary Table 1. Primer design for RT-PCR

ENPP2-forward	CAGCATCATCACCAGCTGTC
ENPP2-reverse	ATTGCAGCTCTCCTCGTTGT
DKK1-forward	TCCGAGGAGAAATTGAGGAA
DKK1-reverse	CCTGAGGCACAGTCTGATGA
MOXD1-forward	TGCTGAGTGGTCGATTCAAG
MOXD1-reverse	TGCAGGGAAGAGGAAGAAGA
DDX17-forward	TCACAGAGCTCTAGCCAGCA
DDX17-reverse	CAGTCTGCCCCATGTAACCT
APCDD1L-forward	GCAGCTCAGCTTTCCTGAGT
APCDD1L-reverse	CCCGGGAAAACTGGATTTAT
CXCR7-forward	GGCTATGACACGCACTGCTA
CXCR7-reverse	CTCATGCACGTGAGGAAGAA
CXCL12-forward	AGAGCCAACGTCAAGCATCT
CXCL12-reverse	CTTTAGCTTCGGGTCAATGC
IL-6-forward	GAAAGCAGCAAAGAGGCACT
IL-6-reverse	TTTCACCAGGCAAGTCTCCT
TNFAIP2-forward	CCTATTGCCGTGACAGGTTT
TNFAIP2-reverse	CTCCAGAAGGAGTGCAGGAC
HPRT1-forward	TGACACTGGCAAAACAATGCA
HPRT1-reverse	GGTCCTTTTCACCAGCAAGCT
GAPDH-forward	GGCCTCCAAGGAGTAAGACC
GAPDH-reverse	AGGGGAGATTCAGTGTGGTG

Supplementary Table 2. Differential expression between healthy control and JMML MSC

Ensembl gene id	Gene symbol	*p*-value	FDR	Average expression Healthy control	Average expression JMML	Analyzed[1]	Function[2]
ENSG00000130592	LSP1	4.67E-10	8.14E-06	1483	479	No available primer set	Adhesion
ENSG00000136960	ENPP2	5.65E-10	8.14E-06	365	168	Yes	Cell proliferation, chemotaxis
ENSG00000127951	FGL2	2.02E-09	1.94E-05	4	111	<100	Unknown
ENSG00000125851	PCSK2	1.91E-07	1.38E-03	206	7	<100	Protein processing
ENSG00000185215	TNFAIP2	3.52E-07	1.71E-03	479	154	Yes	Inflammation
ENSG00000138135	CH25H	3.57E-07	1.71E-03	66	6	<100	Cholesterol and lipid metabolism
ENSG00000010610	CD4	5.18E-07	2.13E-03	85	11	<100	Immunology
ENSG00000125538	IL1B	8.59E-07	3.09E-03	3	56	<100	Inflammation
ENSG00000230750	SDAD1P2	9.63E-07	3.09E-03	0	10	<100	Pseudogene
ENSG00000164764	SBSPON	1.88E-06	5.38E-03	1	89	<100	Polysaccharide binding
ENSG00000107984	DKK1	2.05E-06	5.38E-03	268	783	Yes	WNT inhibitor
ENSG00000079931	MOXD1	2.41E-06	5.79E-03	130	414	Yes	Copper binding, dopamine activity
ENSG00000065485	PDIA5	4.44E-06	9.31E-03	757	1729	No function	Unknown
ENSG00000226920		4.52E-06	9.31E-03	9	0	<100	Unknown
ENSG00000100201	DDX17	6.27E-06	1.20E-02	3361	1300	Yes	RNA processing
ENSG00000198768	APCDD1L	6.68E-06	1.20E-02	683	1265	Yes	Membrane protein
ENSG00000183092	BEGAIN	7.44E-06	1.26E-02	1	24	<100	Neuronal cell body
ENSG00000143882	ATP6V1C2	1.01E-05	1.62E-02	406	710	No	ATPase
ENSG00000123500	COL10A1	1.23E-05	1.79E-02	142	4	<100	Ossification

Supplementary Table 2. Differential expression between healthy control and JMML MSC (continued)

Ensembl gene id	Gene symbol	*p*-value	FDR	Average expression Healthy control	Average expression JMML	Analyzed[1]	Function[2]
ENSG00000138735	PDE5A	1.24E-05	1.79E-02	279	667	No	Phosphodiesteras
ENSG00000108950	FAM20A	1.39E-05	1.91E-02	1248	461	No function	Unknown
ENSG00000082684	SEMA5B	1.73E-05	2.14E-02	0	14	<100	Axon growth
ENSG00000079215	SLC1A3	1.77E-05	2.14E-02	312	79	<100	Neurotransmission
ENSG00000181195	PENK	1.78E-05	2.14E-02	1547	18076	Based on one outlier	Neuropeptide activity
ENSG00000133106	EPSTI1	2.40E-05	2.76E-02	219	63	<100	Epithelial stroma interaction
ENSG00000239648	MTND1P3	2.69E-05	2.98E-02	127	24	<100	Pseudogene
ENSG00000206066	IGLL3P	3.00E-05	3.14E-02	0	9	<100	Pseudogene
ENSG00000234745	HLA-B	3.05E-05	3.14E-02	0	12	<100	Immunology
ENSG00000196139	AKR1C3	3.23E-05	3.21E-02	423	114	No	Aldo/keto reductase
ENSG00000118515	SGK1	3.55E-05	3.41E-02	1083	1929	No	Stress response
ENSG00000170323	FABP4	3.83E-05	3.49E-02	1	76	<100	Fatty acid metabolism
ENSG00000120885	CLU	4.15E-05	3.49E-02	2981	24019	Based on one outlier	Protein binding
ENSG00000163975	MFI2	4.17E-05	3.49E-02	138	522	No	Glycoprotein
ENSG00000114423	CBLB	4.37E-05	3.49E-02	535	244	No	Proto-oncogene
ENSG00000003137	CYP26B1	4.40E-05	3.49E-02	3	43	<100	Lipid metabolism
ENSG00000198113	TOR4A	4.52E-05	3.49E-02	63	186	<100	Protein folding
ENSG00000254366		4.58E-05	3.49E-02	11	0	<100	Unknown
ENSG00000170801	HTRA3	4.59E-05	3.49E-02	171	57	<100	Protease
ENSG00000164484	TMEM200A	5.36E-05	3.85E-02	359	671	No function	Unknown

Supplementary Table 2. Differential expression between healthy control and JMML MSC (continued)

Ensembl gene id	Gene symbol	*p*-value	FDR	Average expression Healthy control	Average expression JMML	Analyzed[1]	Function[2]
ENSG00000227825	SLC9A7P1	5.36E-05	3.85E-02	5	0	<100	Pseudogene
ENSG00000255198	SNHG9	5.48E-05	3.85E-02	0	21	<100	RNA gene
ENSG00000196352	CD55	7.09E-05	4.78E-02	142	281	No	Complement activation
ENSG00000104332	SFRP1	7.12E-05	4.78E-02	105	795	No	WNT pathway
ENSG00000095380	NANS	8.43E-05	5.53E-02	1821	2588	No	
ENSG00000248874	C5orf17	8.97E-05	5.54E-02	8	0	No	
ENSG00000236318		9.17E-05	5.54E-02	0	12	No	
ENSG00000118855	MFSD1	9.37E-05	5.54E-02	1048	1467	No	
ENSG00000161980	POLR3K	9.42E-05	5.54E-02	477	703	No	
ENSG00000079385	CEACAM1	1.03E-04	5.89E-02	0	16	No	
ENSG00000046604	DSG2	1.04E-04	5.89E-02	6	0	No	
ENSG00000154188	ANGPT1	1.12E-04	5.95E-02	1424	668	No	
ENSG00000171819	ANGPTL7	1.14E-04	5.95E-02	1	46	No	
ENSG00000166592	RRAD	1.15E-04	5.95E-02	80	174	No	
ENSG00000101000	PROCR	1.15E-04	5.95E-02	379	1050	No	
ENSG00000106823	ECM2	1.16E-04	5.95E-02	1290	542	No	
ENSG00000078725	BRINP1	1.20E-04	6.05E-02	1544	510	No	
ENSG00000109323	MANBA	1.30E-04	6.47E-02	607	948	No	
ENSG00000091483	FH	1.59E-04	7.73E-02	539	762	No	
ENSG00000117152	RGS4	1.63E-04	7.73E-02	1369	3147	No	
ENSG00000104412	EMC2	1.67E-04	7.73E-02	1010	1378	No	

Supplementary Table 2. Differential expression between healthy control and JMML MSC (continued)

Ensembl gene id	Gene symbol	*p*-value	FDR	Average expression Healthy control	Average expression JMML	Analyzed[1]	Function[2]
ENSG00000140750	ARHGAP17	1.67E-04	7.73E-02	649	918	No	
ENSG00000187720	THSD4	1.70E-04	7.73E-02	85	209	No	
ENSG00000106809	OGN	1.72E-04	7.73E-02	4	57	No	
ENSG00000010278	CD9	1.77E-04	7.78E-02	266	748	No	
ENSG00000145908	ZNF300	1.80E-04	7.78E-02	45	8	No	
ENSG00000119899	SLC17A5	1.86E-04	7.78E-02	246	370	No	
ENSG00000116337	AMPD2	1.87E-04	7.78E-02	919	1268	No	
ENSG00000185803	SLC52A2	1.87E-04	7.78E-02	1303	1840	No	
ENSG00000171067	C11orf24	1.89E-04	7.78E-02	1874	2677	No	
ENSG00000117616	RSRP1	1.97E-04	8.02E-02	1894	730	No	
ENSG00000131732	ZCCHC9	2.01E-04	8.04E-02	369	188	No	
ENSG00000143333	RGS16	2.07E-04	8.17E-02	50	311	No	
ENSG00000165409	TSHR	2.13E-04	8.29E-02	66	10	No	
ENSG00000197406	DIO3	2.21E-04	8.50E-02	1	37	No	
ENSG00000103342	GSPT1	2.35E-04	8.90E-02	1312	1835	No	
ENSG00000138834	MAPK8IP3	2.42E-04	9.05E-02	325	137	No	
ENSG00000228300	C19orf24	2.51E-04	9.20E-02	246	376	No	
ENSG00000112096	SOD2	2.68E-04	9.66E-02	247	446	No	
ENSG00000127418	FGFRL1	2.84E-04	9.98E-02	933	1639	No	
ENSG00000137497	NUMA1	2.96E-04	9.98E-02	919	444	No	
ENSG00000108602	ALDH3A1	3.01E-04	9.98E-02	3	53	No	
ENSG00000250569	NTAN1P2	3.01E-04	9.98E-02	5	0	No	

Supplementary Table 2. Differential expression between healthy control and JMML MSC (continued)

Ensembl gene id	Gene symbol	*p*-value	FDR	Average expression Healthy control	Average expression JMML	Analyzed[1]	Function[2]
ENSG00000163798	SLC4A1AP	3.06E-04	9.98E-02	425	588	No	
ENSG00000036672	USP2	3.08E-04	9.98E-02	21	63	No	
ENSG00000164414	SLC35A1	3.15E-04	9.98E-02	110	45	No	
ENSG00000145020	AMT	3.15E-04	9.98E-02	145	41	No	
ENSG00000183421	RIPK4	3.15E-04	9.98E-02	17	68	No	
ENSG00000184465	WDR27	3.15E-04	9.98E-02	303	114	No	
ENSG00000104549	SQLE	3.31E-04	1.02E-01	710	1004	No	
ENSG00000157168	NRG1	3.36E-04	1.02E-01	325	106	No	
ENSG00000170498	KISS1	3.36E-04	1.02E-01	5	0	No	
ENSG00000231290	APCDD1L-AS1	3.37E-04	1.02E-01	56	122	No	
ENSG00000185633	NDUFA4L2	3.39E-04	1.02E-01	642	1629	No	
ENSG00000163682	RPL9	3.55E-04	1.05E-01	56	13	No	
ENSG00000164344	KLKB1	3.56E-04	1.05E-01	10	0	No	
ENSG00000101843	PSMD10	3.64E-04	1.05E-01	1141	1499	No	
ENSG00000136826	KLF4	3.65E-04	1.05E-01	2246	996	No	
ENSG00000103196	CRISPLD2	3.67E-04	1.05E-01	749	223	No	
ENSG00000149547	EI24	3.90E-04	1.10E-01	732	988	No	
ENSG00000112981	NME5	4.01E-04	1.12E-01	169	62	No	
ENSG00000102471	NDFIP2	4.07E-04	1.13E-01	357	515	No	
ENSG00000099889	ARVCF	4.14E-04	1.14E-01	495	237	No	
ENSG00000162804	SNED1	4.20E-04	1.14E-01	621	246	No	
ENSG00000123572	NRK	4.28E-04	1.14E-01	31	3	No	

Supplementary Table 2. Differential expression between healthy control and JMML MSC (continued)

Ensembl gene id	Gene symbol	*p*-value	FDR	Average expression Healthy control	Average expression JMML	Analyzed[1]	Function[2]
ENSG00000227502	LINC01268	4.28E-04	1.14E-01	5	0	No	
ENSG00000132688	NES	4.40E-04	1.16E-01	58	179	No	
ENSG00000163347	CLDN1	4.44E-04	1.17E-01	22	77	No	
ENSG00000184924	PTRHD1	4.54E-04	1.17E-01	1405	1880	No	
ENSG00000198682	PAPSS2	4.57E-04	1.17E-01	2291	3753	No	
ENSG00000164106	SCRG1	4.77E-04	1.20E-01	1223	2416	No	
ENSG00000143248	RGS5	4.78E-04	1.20E-01	63	221	No	
ENSG00000229729		4.88E-04	1.20E-01	5	0	No	
ENSG00000148450	MSRB2	4.89E-04	1.20E-01	360	182	No	
ENSG00000187514	PTMA	4.96E-04	1.21E-01	210	60	No	
ENSG00000139874	SSTR1	5.04E-04	1.21E-01	5	35	No	
ENSG00000147408	CSGALNACT1	5.05E-04	1.21E-01	53	238	No	
ENSG00000184408	KCND2	5.38E-04	1.28E-01	7	0	No	
ENSG00000114857	NKTR	5.65E-04	1.33E-01	623	302	No	
ENSG00000170266	GLB1	5.76E-04	1.33E-01	1040	1386	No	
ENSG00000105248	CCDC94	5.76E-04	1.33E-01	1	9	No	
ENSG00000155363	MOV10	5.80E-04	1.33E-01	180	80	No	
ENSG00000105426	PTPRS	5.92E-04	1.34E-01	401	210	No	
ENSG00000196616	ADH1B	5.94E-04	1.34E-01	18	238	No	
ENSG00000130600	H19	5.99E-04	1.34E-01	92	452	No	
ENSG00000136244	IL6	6.14E-04	1.36E-01	1658	2495	Yes	
ENSG00000125430	HS3ST3B1	6.21E-04	1.37E-01	122	49	No	

Supplementary Table 2. Differential expression between healthy control and JMML MSC (continued)

Ensembl gene id	Gene symbol	*p*-value	FDR	Average expression Healthy control	Average expression JMML	Analyzed[1]	Function[2]
ENSG00000110328	GALNT18	6.31E-04	1.38E-01	90	192	No	
ENSG00000179091	CYC1	6.43E-04	1.39E-01	2716	3739	No	
ENSG00000174109	C16orf91	6.45E-04	1.39E-01	220	310	No	
ENSG00000129250	KIF1C	6.49E-04	1.39E-01	945	1228	No	
ENSG00000254187		6.58E-04	1.39E-01	0	5	No	
ENSG00000261857	MIA	6.65E-04	1.40E-01	8	62	No	
ENSG00000179403	VWA1	6.78E-04	1.41E-01	227	96	No	
ENSG00000251349	MSANTD3-TMEFF1	6.78E-04	1.41E-01	275	388	No	
ENSG00000087494	PTHLH	6.92E-04	1.42E-01	17	62	No	
ENSG00000185608	MRPL40	6.98E-04	1.43E-01	799	1038	No	
ENSG00000258498	DIO3OS	7.05E-04	1.43E-01	4	72	No	
ENSG00000171813	PWWP2B	7.41E-04	1.49E-01	159	69	No	
ENSG00000150627	WDR17	7.72E-04	1.54E-01	1	15	No	
ENSG00000102760	RGCC	7.75E-04	1.54E-01	56	127	No	
ENSG00000231231	LINC01423	7.84E-04	1.55E-01	72	8	No	
ENSG00000121039	RDH10	7.96E-04	1.56E-01	136	290	No	
ENSG00000154174	TOMM70A	8.16E-04	1.59E-01	814	1047	No	
ENSG00000184232	OAF	8.20E-04	1.59E-01	188	294	No	
ENSG00000177542	SLC25A22	8.33E-04	1.59E-01	277	391	No	
ENSG00000186469	GNG2	8.38E-04	1.59E-01	108	196	No	
ENSG00000145337	PYURF	8.47E-04	1.59E-01	2593	3524	No	

Supplementary Table 2. Differential expression between healthy control and JMML MSC (continued)

Ensembl gene id	Gene symbol	*p*-value	FDR	Average expression Healthy control	Average expression JMML	Analyzed[1]	Function[2]
ENSG00000021762	OSBPL5	8.56E-04	1.59E-01	171	75	No	
ENSG00000156042	CFAP70	8.59E-04	1.59E-01	87	12	No	
ENSG00000153291	SLC25A27	8.60E-04	1.59E-01	175	59	No	
ENSG00000197635	DPP4	8.62E-04	1.59E-01	179	397	No	
ENSG00000151929	BAG3	8.90E-04	1.63E-01	658	870	No	
ENSG00000145901	TNIP1	9.05E-04	1.65E-01	1059	1366	No	
ENSG00000103449	SALL1	9.11E-04	1.65E-01	0	9	No	
ENSG00000239382	ALKBH6	9.16E-04	1.65E-01	52	11	No	
ENSG00000137965	IFI44	9.20E-04	1.65E-01	155	62	No	
ENSG00000229563	LINC01204	9.53E-04	1.70E-01	5	0	No	
ENSG00000113643	RARS	9.71E-04	1.71E-01	1951	2540	No	
ENSG00000145476	CYP4V2	9.81E-04	1.71E-01	194	87	No	
ENSG00000151376	ME3	9.84E-04	1.71E-01	118	45	No	
ENSG00000144476	ACKR3	9.89E-04	1.71E-01	618	231	Yes = CXCR7	
ENSG00000107159	CA9	9.97E-04	1.71E-01	3	23	No	

p-value of the average of HC and JMML-MSC; average reads expressed as the number of reads per 10^6 reads analyzed

[1] Analyzed: this column indicates if the gene was further analyzed using RT-qPCR, based on FDR, expression threshold >100 copies and function.

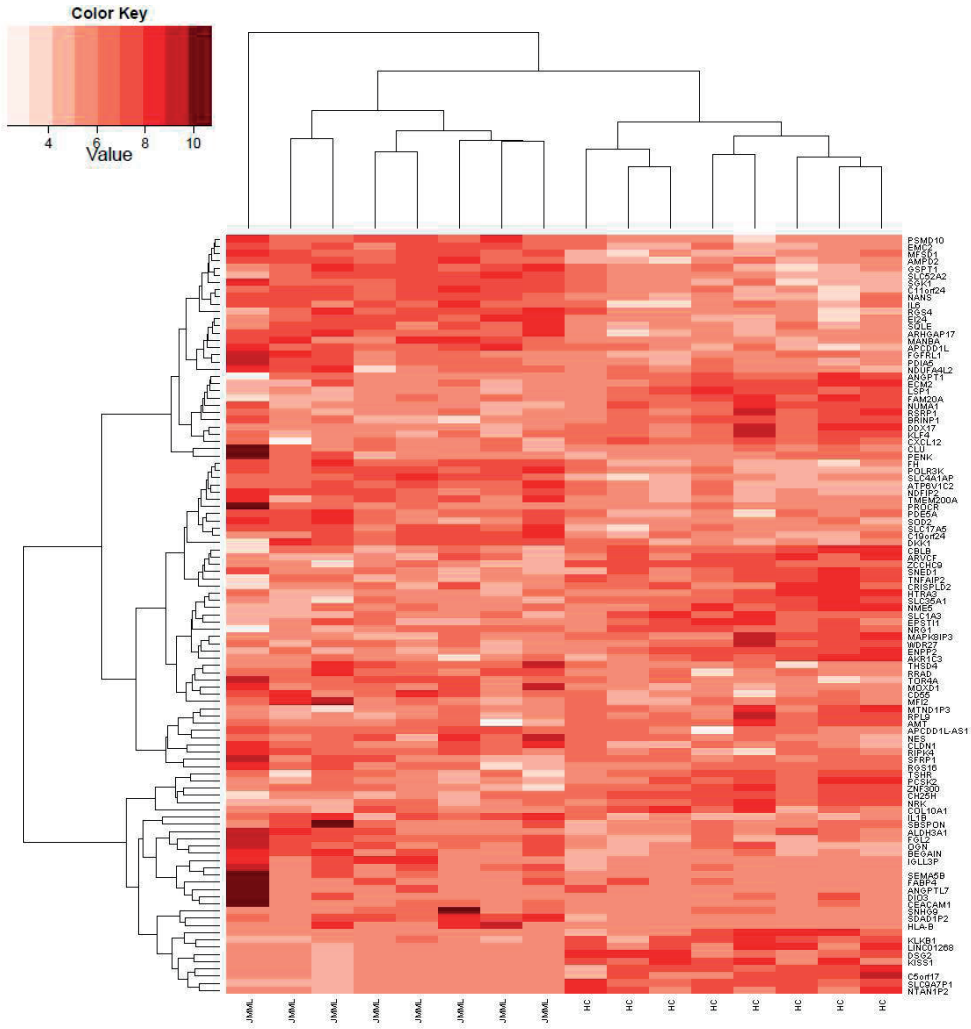
[2] Function: a short discription, not intended to be exhaustive, of the function based on gene-ontology and www.genecards.org

HC: healthy control; JMML: juvenile myelomonocytic leukemia; FDR: false discovery rate with threshold $p<0.05$

Supplementary Table 3. GO-term analysis

GO-term	Holm's correction	Alias	p-value	Covariates
GO:0002252	0.0001	immune effector process	8.65E-09	305
GO:0002253	0.0005	activation of immune response	3.27E-08	235
GO:0050778	0.0005	positive regulation of immune response	3.29E-08	283
GO:0007186	0.0006	G-protein coupled receptor signaling pathway	3.98E-08	520
GO:0043254	0.0011	regulation of protein complex assembly	6.57E-08	152
GO:0007005	0.0015	mitochondrion organization	9.59E-08	201
GO:0051091	0.0015	positive regulation of sequence-specific DNA binding transcription factor activity	9.66E-08	161
GO:0002250	0.0027	adaptive immune response	1.67E-07	148
GO:0002460	0.0028	adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	1.77E-07	133
GO:0035270	0.0037	endocrine system development	2.33E-07	120
GO:0002449	0.0038	lymphocyte mediated immunity	2.42E-07	113
GO:0002443	0.0040	leukocyte mediated immunity	2.51E-07	148
GO:0016567	0.0041	protein ubiquitination	2.60E-07	513
GO:0031396	0.0043	regulation of protein ubiquitination	2.70E-07	160
GO:0051260	0.0061	protein homooligomerization	3.84E-07	205
GO:0031625	0.0083	ubiquitin protein ligase binding	5.30E-07	134
GO:0044389	0.0083	small conjugating protein ligase binding	5.30E-07	134
GO:0031398	0.0089	positive regulation of protein ubiquitination	5.64E-07	121

GO: gene-ontology



Supplementary Figure 1. *JMML and healthy control (HC) MSCs have a different gene-expression profile.* In this heat-map the 100 differentially expressed genes detected by Deep-SAGE sequencing are depicted demonstrating clustering of healthy control (HC) derived bone-marrow (BM) MSC (n=8) and MSCs derived from BM of JMML patients at diagnosis (n=8). Darker colors correspond with increased expression.