

The Role of GM-CSF in the Generation of CD1a Positive Cells *in vitro* by JMML Patients

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Abstract

Objective: Juvenile myelomonocytic leukemia (JMML) is a rare myeloproliferative disorder of childhood. It was recently demonstrated that mononuclear cells from spleen, peripheral blood and bone marrow of the patients with JMML proliferate *in vitro* and population of the cells differentiate within 7 days to the CD1a positive dendritic cells. In the present study, the role of GM-CSF in the proliferation and differentiation of the JMML cells *in vitro* was investigated.

Methods: MNC from patients with JMML was cultured for 7 days in RPMI/10%FCS and 1% penicillin-streptomycin. The phenotype of the cultured cells was determined at day 7 using antibodies against Ki-67, CD1a, CD14 and CD68. GM-CSF concentration was first measured at days 1, 3, 5 and 7 in the supernatants of the cultured mononuclear cells from two patients with JMML. To study the roll of GM-CSF in JMML cultures, GM-CSF activity was neutralized using monoclonal antibody (Ab) against human GM-CSF in different concentrations (0.05 µg/ml, 0.5 µg/ml, 5 µg/ml and 10 µg/ml). The effect of neutralizing antibody was determined at day 7 in treated and untreated cultures by immunostaining using antibodies against Ki-67, CD68, CD1a and CD14. The expression of GM-CSF, GM-CSFR, TNF-α, IFN-γ and SCF was also determined at day 7 in mononuclear cells treated and untreated cultures by RT-PCR.

Findings: GM-CSF neutralization led to decrease of the total cell number. Additionally, neutralization of GM-CSF decreased in dose dependent manner the number of CD1a, CD14 and Ki-67 positive cells in JMML cultures, whereas the percentage of CD68 remained unchanged. The expression of GM-CSF, GM-CSFR, TNF-α and SCF could be demonstrated at the mRNA level in the antibody treated and untreated culture as well.

Conclusion: GM-CSF plays an important role in the proliferation and differentiation of JMML cells into the CD1a and CD14 positive cells.

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Key Words: Juvenile myelomonocytic leukemia; CD1a; Dendritic cell; GM-CSF; Stem cell factor; Suspension cell culture; ELISA; Immunostaining; RT-PCR

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Introduction

Juvenile myelomonocytic leukemia (JMML) is a rare myeloproliferative disorder in childhood. JMML is accompanied with leucocytosis and monocytosis, presence of elevated hematopoietic progenitor cells in the peripheral blood, massive infiltration of various organs causing skin rash, lymphadenopathy, hepatosplenomegaly and damage of lung tissue, which most probably lead to the death of patients^[1,2]. The molecular mechanism involved in the pathogenesis of JMML is not well recognized. The mononuclear cells (MNC) isolated from spleen, peripheral blood (PB) or bone marrow (BM) of patients with JMML show spontaneous growth *in vitro*^[3-6]. The cytokine responsible for this so-called "spontaneous growth" is reported to be GM-CSF. Emanuel et al 1991 have shown that the progenitor cells from JMML patients act hypersensitive to GM-CSF, which is produced by macrophages^[3,7]. They showed that depletion of macrophages from the culture led to the loss of growth property of cells.

Moreover, they have demonstrated that an antibody against GM-CSF also had an inhibitory effect in colony growth in the semi solid medium^[7]. Beside GM-CSF, TNF- α seems to play also an important role in the proliferation of JMML cells. TNF- α neutralizing antibody is able to decrease the proliferation of the JMML *in vitro* semi solid culture^[8, 9].

Furthermore, a tumor necrosis factor α specific ribozyme, which modulates GM-CSF gene expression, leads to decrease of GM-CSF-dependent colony formation and viability of JMML cells *in vitro* culture^[10]. Analysis of GM-CSF receptor (GM-CSFR) showed that expression of GM-CSFR (count per cell) is not altered on the cells by JMML patients and nucleotide sequence analysis of the receptor also showed no novel mutation in the gene^[11-13]. There is, however, evidence that proteins downstream of the GM-CSFR are involved in the pathogenesis of the disease^[14]. Therefore, we previously analyzed the expression of hematopoietic cell phosphatase (HCP), a potential modulator of the GM-CSF-ras signal transduction pathway^[15] and determined the

ratio of intact interferon regulatory factor 1 and 2 (IRF-1, -2), which may contribute to the cell transformation^[16] in patients with JMML.

Our results showed that HCP transcript with or without exon skipping were not expressed differently in hematopoietic cells of patient with JMML compared to the healthy donors and neither tumor suppressor gene IRF-1 nor the oncogene IRF-2 was involved in the pathogenesis of JMML.

Recently it was demonstrated that the MNC isolated from peripheral blood, bone marrow and spleen of the patients with JMML proliferate and differentiate to the CD1a positive cells *in vitro* in RPMI/10%FCS without exogenous growth factors^[6]. In the present study, the role of GM-CSF in the proliferation and differentiation of the JMML cells *in vitro* was investigated. For this aim, the transcript of GM-CSF gene was first measured in the supernatant of the cell cultures using colorimetric Sandwich ELISA. Additionally, the GM-CSF was neutralized using monoclonal antibody against human GM-CSF and the effect of neutralization on the differentiation and proliferation of the JMML-cells was investigated *in vitro*. Furthermore, the expression of GM-CSF, GM-CSFR, TNF- α and SCF was determined in the antibody treated and untreated JMML-cells at mRNA level.

Subjects and Methods

Patients and controls:

MNC from spleen of 2 patients with JMML were analyzed at the time of diagnosis. Clinical data of patients are shown in Table 1. All tissues had been obtained with consent given according to institutional guidelines.

Isolation of Mononuclear Cells:

Mononuclear cells (MNC) were isolated by Ficoll- Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Spleen samples were cut into small pieces and placed in phosphate-buffered saline (PBS). The cells were isolated by scraping, filtering through a

cell strainer (70 µm Nylon, Becton Dickinson) and by Ficoll-Paque.

Cell culture:

MNC from spleen were cultured at a concentration of 1×10^6 /ml in RPMI medium with 10% FCS and 1% penicillin-streptomycin in culture dish. Cultures were incubated for 7 days at 37°C in atmosphere containing 5% CO₂. At day 7 cells in suspension were counted and used for cytopsin preparation.

Immunocytochemistry:

For immunostaining of cultured cells, we used an immunoperoxidase system from DAKO Hamburg, Germany. The used antibodies were CD1a (clone NA1/34), CD14 (clone TÜK4), CD68 (clone KP1) and Ki-67 (code No.A0047).

These antibodies were purchased from DAKO, Germany. The number of positive reaction was determined for Ki-67, CD68, CD1a and CD14 in at least 1000 cells. Ki 67 was used as a proliferation marker. All proliferating cells expressed proliferating associated nuclear protein, Ki-67, whereas this protein was absent in the quiescent cells.

Measurement of GM-CSF in supernatant of cultured MNC:

MNC from spleen of 2 patients with JMML was cultured in RPMI/10%FCS for 7 days. For determination of GM-CSF, supernatant of cultures were centrifuged every second days at 17860 g and 4°C for 2 minutes, transferred to the new sterile tubes, aliquoted and stored at -20°C until used. GM-CSF was then measured using colorimetric Sandwich ELISA kits purchased from R&D Systems GmbH, Germany.

Neutralization of GM-CSF in the cultures:

The hGM-CSF neutralizing monoclonal antibody (hGM-CSF-nmab) was purchased from R&D Systems GmbH, Germany. This antibody has been selected for its ability to neutralize the biological activity of rhGM-CSF (R&D Systems). The ND₅₀ for this lot of antibody was determined to be approximately 0.5-1.5 µg/ml in the presence of 0.5 ng/ml of rhGM-CSF using the factor-dependent human

cell line TF-1 (R&D Systems). Since it was difficult to have enough MNC from PB or BM of the patients, the spleen was used as a source for the isolation of the MNC. MNC from spleen of patient #1 with JMML was cultured at a concentration of 1×10^6 /ml in RPMI/10%FCS in the absence or presence of hGM-CSF-nmab (0.05 µg/ml, 0.5 µg/ml, 5 µg/ml and 10 µg/ml). The antibody was added to the culture once at the initiation time of culture. The cultures were judged daily under microscope.

At day 7, MNC were collected and used for preparation of cytopsin slides. The supernatants of the cultures were collected at days 1, 2, 3, 4, 5, 6 and 7 by centrifugation at 17860 g and 4°C for 2 minutes. Before measurement of GM-CSF concentration in the supernatant of the cell cultures, antigen (GM-CSF)-antibody complex was first removed from the supernatant. Briefly, 300 µl of the supernatant was incubated with Dynabeads M-450 coated with goat anti-mouse IgG (DynaL, Hamburg, Germany), which has been already washed with PBS, to remove the antigen-antibody-complex. After 1 h incubation on ice, the Dynabeads were removed using the Magnetic Particle Concentrator Dynal MPC-E (DynaL, Hamburg, Germany) and the GM-CSF concentration was determined as described above.

Immunocytochemistry analysis of the cells was performed at day 7 of culture on cytopsin as described above.

Polymerase chain reaction (PCR):

RNA from cultured cells was achieved according to the PegGold Trifast Kit (Peglab Biotechnologie GmbH, Erlangen Germany).

One to 5 µg RNA was mixed with 2 pmol hexanucleotide (GibcoBRL/Life Technologies) in 12 µl DEPC. After a denaturation step at 70°C for 10 minutes, RNA was reverse transcribed into cDNA by incubating the 20 µl reaction containing 1 µl dNTP (10 mM each), 2 µl 0.1M DTT, 4 µl 5 x first strand buffer and 1 µl (200 U) superscript II (GibcoBRL/Life Technologies) at 40°C for 60 minutes. Enzyme activity was stopped by a final step at 70°C for 15 minutes. For RT-PCR reaction, cDNA was diluted to 1:10 and 1:100 with sterile water.

For amplification of β -actin, 1 μ l cDNA was used from 1:10 and 1:100 dilution respectively, whereas amplification of other genes was achieved with 1 μ l cDNA from undiluted and 1:10 diluted cDNA solution respectively. cDNA was amplified in a 100 μ l volume containing 10 μ l 10 x PCR-buffer, 3 μ l 50 mM MgCl₂, 2 μ l dNTP (10 mM each), 2 μ l sense primer (20 μ M), 2 μ l antisense primer (20 μ M) and 0.5 μ l (5000U/ml) Taq polymerase (GibcoBRL/Life Technologies).

The nucleotide sequences of the different analyzed genes are listed as follow:

β -actin

sense 5' AAGAGATGGCCACGGCTGCT 3'

antisense 5' TCGCTCCAACCGACTGCTGT 3'

GM-CSF

sense 5' CTGCTGAGATGAATGAAACAG

antisense 5' GTGCTGCTTGTAGTGGCT 3'

GM-CSFR

sense 5' CCAGCAATGTCACCGTACGTT 3'

antisense 5' TGTGGAAGTGGCGGGAACAG 3'

SCF

sense 5' CCCAGGCTCTTTACTCCTGAAG 3'

antisense 5' CTGCCCTTGTAAGACTTGGCTG 3'

TNF- α

sense 5' CAGAGGGAAGAGTTCCCCAG 3'

antisense 5' CCTTGGTCTGGTAGGAGACG 3'

IFN- γ

sense 5' CAGGTCATTCAGATGTAGCG 3'

antisense 5' GCTTTTCGAAGTCATCTCG 3'

To avoid amplifying sequences of the β actin pseudogene, the β -actin primers were chosen from those DNA regions, which are absent in the pseudogene. The primer pairs were located on different exons so that the fragment length was indicative that exclusively cDNA was amplified. The

amplification profile involved one pre-denaturation step at 95°C/3 minutes, denaturation at 95°C/ 45 seconds, annealing at 54°C/30 seconds, elongation at 72°C/45 seconds (35 cycles) followed by 10 minutes at 72°C after the last cycle (PCRExpress, Hybaid, UK). The PCR products were size fractionated by electrophoresis on 1-1.5% agarose gel in 0.5 x TBE (100 mM tris base, 100 mM boric acid and 2 mM EDTA pH 8.3), and stained with ethidium bromide.

Findings

Determination of GM-CSF in supernatant of JMML culture:

MNC from two patients with JMML (pt #1 and pt #2) were cultured for 7 days in RPMI/10%FCS. One of these patients had monosomy 7 whereas the other had normal karyotype (Table 1). GM-CSF was measured every second days in supernatant of JMML-MNC cultured for 7 days. In the supernatants of both cultures, GM-CSF (pt #1 \emptyset 117 pg/ml; pt #2 \emptyset 56 pg/ml) could be determined (Table 2).

The measured concentration of GM-CSF at day 1 in supernatant of *in vitro* culture of patient with normal karyotype was comparable to that of the patient with monosomy 7 (pt #1 76 pg/ml, pt #2 78 pg/ml). But at days 3, 5 and 7 of MNC-cultures, the concentration of GM-CSF in supernatant of the patient with monosomy 7 was lower than that of patient with normal karyotype (Table 2).

Table 1: Patients' characteristics (Data obtained from European Working Group on Childhood MDS)

Patient	Diagnosis	Age (year)	Spleen (cm*)	Liver (cm*)	Karyotype	WBC ‡ ($\times 10^9/l$)	Mono ($\times 10^9/l$)	PB Blast %	BM Blast %
1	JMML	0.3	4	6	Normal	31.0	4.7	4.0	19.0
2	JMML	2.8	3	2	-7 #	22.9	3.8	11.3	6.0

* cm: centimeter below costal margin

‡ WBC: white blood cells; Mono: monocytes

-7: monosomy 7

Table 2: GM-CSF was measured in supernatant of MNC from two JMML patients cultured *in vitro* at days 1, 3, 5 and 7 of culture

Patient	Karyotype	Collection time of supernatant of the <i>in vitro</i> culture (day)	GM-CSF pg/ml
Patient 1	normal	1	76
		3	186
		5	119
		7	87
Patient 2	-7	1	78
		3	64
		5	40
		7	41

Immunocytochemistry analysis of cultured cells from the patient 1 showed that 38% of the cells were Ki-67 positive. The percentage of CD1a and CD68 positive cells was 31% and more than 80% respectively. Immunocytochemistry analysis of cultured cells from the patient 2 showed that 24% of the cells was Ki-67 positive. The percentage of CD1a and CD68 was 8% and more than 80% respectively (data not shown).

Neutralization of GM-CSF in JMML cultures:

Neutralization of GM-CSF was performed in cultured MNC isolated from spleen of patient 1. GM-CSF was neutralized with 0.05 µg/ml, 0.5 µg/ml, 5 µg/ml and 10 µg/ml of monoclonal antibody against human GM-CSF.

The amount of monoclonal antibody was calculated according to the concentration of

GM-CSF in the cultures measured at days 1, 3, 5 and 7, and to the neutralizing capability of antibody demonstrated by R&D Company.

After removal of GM-CSF-Ab-complex with beads-conjugated anti-mouse IgG, GM-CSF concentration was determined in supernatant of untreated as well as treated cultures with 0.5 µg/ml, 5 µg/ml and 10 µg/ml of monoclonal antibody against human GM-CSF at days 1 to 7. The concentration of GM-CSF in supernatant of untreated culture was 100 pg/ml, 103 pg/ml, 100 pg/ml, 132 pg/ml, 78 pg/ml, 46 pg/ml and 24 pg/ml for days 1 to 7, respectively (Table 3). Free GM-CSF could not be measured from day one to day seven in the supernatant of treated cultures with 0.5 µg/ml, 5 µg/ml and 10 µg/ml antibody. The count of the cells was in supernatant of the untreated culture at day 7, 1.15×10^6 /ml,

Table 3: MNC from spleen of patient 1 with JMML were cultured in RPMI/10%FCS and 1% penicillin-streptomycin with or without monoclonal antibody against hGM-CSF in different concentrations for 7 days. The GM-CSF concentration was measured at days 1-7.

mAb against GM-CSF (µg/ml)	Concentration of GM-CSF in supernatants of JMML-cultures at days: (pg/ml)						
	1	2	3	4	5	6	7
0	100	103	100	132	78	46	24
0.5	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-

- = not detectable

Table 4: MNC from spleen of patient 1 with JMML were cultured in RPMI/10%FCS and 1% penicillin-streptomycin with or without monoclonal antibody against hGM-CSF in different concentrations for 7 days. The cells were analyzed with antibodies against Ki-67, CD1a, CD14 and CD68 at day 7 of culture.

Antibody	Concentration of monoclonal antibody against GM-CSF in JMML cultures				
	0	0.05	0.5	5	10
% Ki-67	42	55	43	31	24
% CD1a	45	45	39	28	18
% CD14	24	24	21	16	13
% CD68	>80	>80	>80	>80	>80

whereas it was decreased to $0.28 \times 10^6/\text{ml}$, $0.23 \times 10^6/\text{ml}$ and $0.2 \times 10^6/\text{ml}$, when the cultures were treated with 0.5, 5 and 10 $\mu\text{g}/\text{ml}$ antibody respectively.

Immunocytochemical analysis of the cells at day 7 showed that the percentage of CD68 positive cells remained unchanged. The

percentage of proliferating cells (Ki-67 positive) treated with 0.05 and 0.5 $\mu\text{g}/\text{ml}$ antibody was comparable to that of untreated cultures (approximately 42%). The percentage of proliferating cells reduced to 31% or 24% in cultures treated with 5 $\mu\text{g}/\text{ml}$ or 10 $\mu\text{g}/\text{ml}$ antibody respectively (Table 4, Fig. 1). The

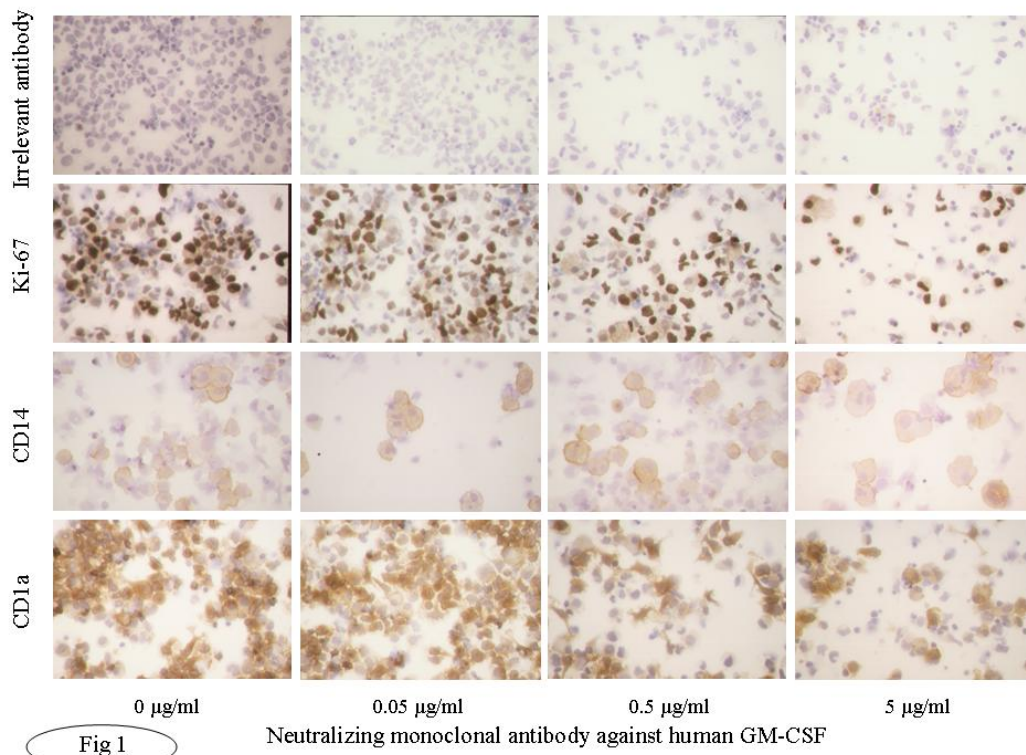


Fig 1. MNC from spleen of patient #1 with JMML was cultured at a concentration of $1 \times 10^6/\text{ml}$ in RPMI/10%FCS and 1% penicillin-streptomycin in the absence or presence of hGM-CSF-nmab (0.05 $\mu\text{g}/\text{ml}$, 0.5 $\mu\text{g}/\text{ml}$ and 5 $\mu\text{g}/\text{ml}$ and 10 $\mu\text{g}/\text{ml}$). The Cells were collected at day 7 of cultures and immunostained with antibodies against proliferation marker Ki-67, CD1a, CD14 and CD68. The nucleus of Ki-67 positive cells and the membrane of CD14 and CD1a positive cells were immunostained brown. Magnification: 40 \times .

percentage of CD1a positive cells remained unchanged in cultures treated with 0.05 $\mu\text{g}/\text{ml}$ antibody compared to the untreated cultures.

The CD1a positive cells decreased to 39%, 28% and 18%, when the cultures were treated with 0.5, 5 and 10 $\mu\text{g}/\text{ml}$ antibody respectively. It was shown that MNC from normal healthy do not differentiate into the CD14 positive cells in abovementioned culture. Interestingly, we could observe that CD14 positive cells could be demonstrated at day 7 in previously some examined PB-MNC (>5%), BM-MNC (>20%) from patients with JMML (Fig. 2, data not shown). To determine the role of GM-CSF in the generation of CD14 positive cells by JMML patients, the phenotype of the cells in untreated and treated cultures was determined using monoclonal antibody against CD14 (Fig. 1). The percentage of CD14 positive cells remained unchanged (24%) in cultures treated with 0.05 $\mu\text{g}/\text{ml}$ antibody

compared to the untreated cultures, whereas it was decreased to 21%, 18% and 13% in cultures treated with 0.5, 5 and 10 $\mu\text{g}/\text{ml}$ antibody respectively (Table 4). RT-PCR analysis was performed with the cells obtained from the untreated and treated cultures with 0.05, 0.5 and 5 $\mu\text{g}/\text{ml}$ antibodies. The results showed that the mRNA for GM-CSF, GM-CSFR, SCF, TNF- α and INF- γ expressed in all examined cultures (Fig. 3).

Discussion

Molecular pathogenesis of JMML is rarely understood. It is known that JMML progenitor cells exhibit spontaneous colony formation *in vitro*. It is believed that selective hypersensitivity of myeloid progenitor cells to the GM-CSF plays a prominent role in the

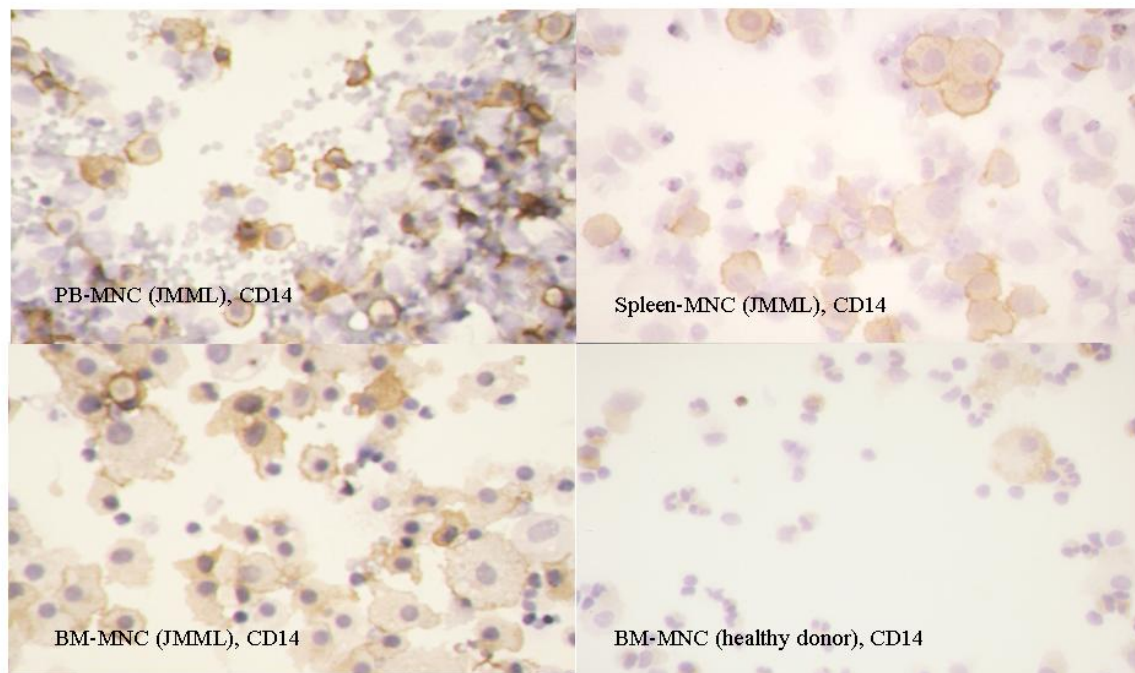


Fig 2

Fig 2: MNC from spleen, peripheral blood and bone marrow of patient with JMML, and MNC from bone marrow of healthy donor were cultured at a concentration of $1 \times 10^6/\text{ml}$ in RPMI/10%FCS and 1% penicillin-streptomycin. The Cells were collected at day 7 of cultures and immunostained with

antibody against CD14. The membrane of corresponding CD14 positive cells was immunostained brown. Magnification: 40 \times .

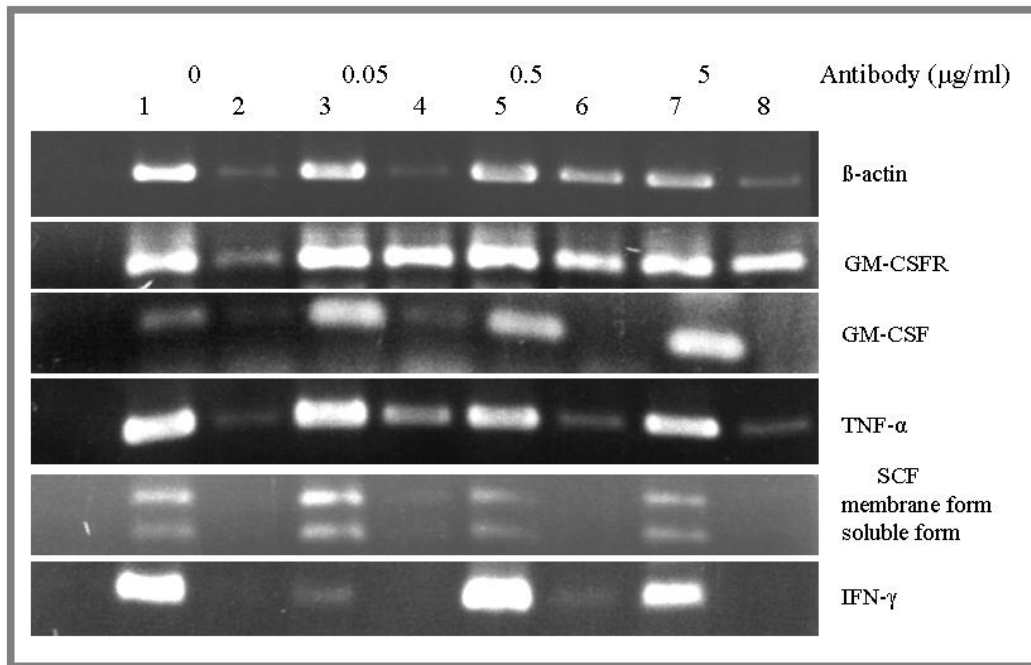


Fig 3

Fig 3: MNC from spleen of patient #1 with JMML was cultured at a concentration of $1 \times 10^6/\text{ml}$ in RPMI/10%FCS and 1% penicillin-streptomycin in the absence or presence of hGM-CSF-nmab (0.05 $\mu\text{g/ml}$, 0.5 $\mu\text{g/ml}$ and 5 $\mu\text{g/ml}$). At day 7 of culture, the RNA was isolated from the cells and cDNA was synthesized. For the RT-PCR reaction, cDNA was diluted to 1:10 and 1:100 with sterile water. For the amplification of β -actin, 1 μl cDNA was used from 1:10 (lanes 1,3,5 and 7) and 1:100 (lanes 2,4,6 and 8) dilutions respectively, whereas the amplification of the GM-CSF, GM-CSFR, SCF, TNF- α and INF- γ genes was achieved with 1 μl cDNA from undiluted (lanes 1,3,5 and 7) and 1:10 diluted (lanes 2,4,6 and 8) cDNA solution respectively.

spontaneous growth of JMML granulocyte-macrophage colony forming units (CFU-GM) in semisolid culture^[3,7] as well as *in vivo*^[17]. A previous study^[4-6] established a culture system of JMML MNC from PB, BM and spleen without exogenous growth factor supplementation. The cultures were characterized by marked adherence of cells and proliferative activity with frequent mitosis and a high Ki-67 index. Remarkably, after 5 days of culture, all JMML specimens featured differentiation into CD1a positive

cells. Since the hematopoietic progenitor cells of JMML patients show a selective hypersensitivity to the GM-CSF, our observation allowed the speculation that the GM-CSF may be involved in the proliferation and differentiation of JMML cells into the CD1a positive cells *in vitro*. To investigate the role of GM-CSF in this culture system, the concentration of GM-CSF was determined in the supernatant of the JMML cultures. As expected, GM-CSF could be detected in the supernatant of both JMML cultures. The

concentration of GM-CSF in the supernatant of the culture from JMML patient with monosomy 7 was lower than from the patient with normal karyotype. The importance of GM-CSF in the generation of CD1a positive cells can be inferred from the culture methods that allowed *in vitro* generation of DC from CD34+ precursors, usually by the sequential use of different cytokines combinations such as GM-CSF, IL-4 and/or TNF-alpha or SCF + IL-3 + IL-6 followed by GM-CSF + IL-4 [18]. Likewise, culture assays from monocytes have been described, using GM-CSF, IL-4 and TNF- α [19]. Since the importance of GM-CSF in the pathophysiology of JMML is unchallenged, the effect of GM-CSF in the proliferation and differentiation of JMML cells into population with DC like features was found out using different concentrations (0.05, 0.5, 5 and 10 $\mu\text{g/ml}$) of monoclonal antibody against human GM-CSF. To control the neutralizing effect of Antibody, GM-CSF concentration was measured every day in supernatant of the treated cultures. The monoclonal antibody at concentrations of 0.5, 5 and 10 $\mu\text{g/ml}$ could neutralize the GM-CSF in all treated JMML cultures as measured by colorimetric Sandwich ELISA. In cultures treated with antibody concentrations of 0.5, 5 and 10 $\mu\text{g/ml}$, the total cell number decreased at day 7 at doses dependent manner. At the same time the percentage of CD68 positive cells remained unchanged, while the GM-CSF neutralizing antibody had decreasing effect at higher concentrations on the proliferation and generation of the cells into CD1a and CD14 positive cells. Although GM-CSF was not detectable in the cultures treated with monoclonal antibody, but its expression in the cells was demonstrable at the mRNA level.

Therefore, it is to speculate that GM-CSF has been produced by macrophages and secreted into the treated culture constitutively. And very low amount of undetectable GM-CSF could be enough for the giving signal to the GM-CSF hypersensitive JMML progenitor cells to differentiate into the CD1a and CD14 positive cells. On the other hand, it was shown that on the cover slips CD1a positive cells

were located in direct neighborhood and often with cell-to-cell contact with macrophages[6].

This observation led to speculate that the binding of the macrophages to the JMML haematopoietic progenitor cells may cause the macrophages to become polarized. The cytoskeleton is then reoriented to align the Golgi apparatus and the microtubule organizing center towards the JMML progenitor cells. GM-CSF secretion is thus specifically directed onto the progenitor cells, as it is the case by release of granules from the cytotoxic cells to the target cells. At the mRNA level the expression of GM-CSFR, TNF- α , SCF and IFN- γ was also demonstrable. Analysis of GM-CSF receptor (GM-CSFR) has shown that the expression of GM-CSFR (count per cell) was not altered on the cells by JMML patients and the nucleotide sequence analysis of the receptor showed also no novel mutation in the gene[11-13]. It was shown that TNF- α neutralizing antibody was able to decrease the proliferation of the JMML in semi solid medium[8,9]. Sawai et al 1996 reported that one possible mechanism for the cell proliferation in JMML is the strong proliferation of GM progenitors induced by haematopoietic factors, in particular SCF[20].

Conclusion

TNF- α neutralizing antibody decreases also in the presented culture system the proliferation and differentiation of JMML progenitor cells into the CD1a positive cells (data not shown). It is obvious that the proliferation and differentiation of the cells to the CD1a positive cells *in vitro* liquid culture is poly factorial. The results in the present study suggest that GM-CSF plays an important role in the proliferation of the JMML cells in liquid culture. Furthermore, the results revealed that the GM-CSF is also important for the differentiation of the JMML progenitor cells into the CD1a and CD14 positive cells *in vitro*.

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References

1. Aricò M, Biondi A, Pui CH. Juvenile myelomonocytic leukemia. *Blood*. 1997; 90(2):479-88.
2. Niemeyer CM, Arico M, Basso G, et al. Chronic myelomonocytic leukemia in childhood: a report of 110 cases. *Blood*. 1997;89(10):3534-43.
3. Emanuel PD, Bates LJ, Castleberry RP et al. Selective hypersensitivity to granulocyte-macrophage colony-stimulating factor by juvenile chronic myeloid leukemia hematopoietic progenitors. *Blood*. 1991;77(5):925-9.
4. Shayan P, Kontny HU, Siebert R, et al. Generation of CD1a positive cells without exogenous growth factors in JMML. *Blood*. 1999;94(Suppl 110a):10.
5. Shayan P, Müller A, Ott C, et al. An *in vitro* culture system as diagnostic tool for JMML. *Leukemia*. 2000;14(5) Suppl: 963.
6. Shayan P. Juvenile myelomonocytic leukaemia (JMML) cells spontaneously differentiate into dendritic-cell like population *in vitro*. *Iran J Pediatr*. 2008; 18(3):213-21.
7. Emanuel PD, Bates LJ, Zhu SW, et al. The role of monocyte-derived hemopoietic growth factors in the regulation of myeloproliferation in juvenile chronic myelogenous leukemia. *Exp Hematol*. 1991;19(10):1017-24.
8. Freedman MH, Hitzler JK, Bunin N, et al. Juvenile chronic myelogenous leukemia multilineage CD34+ cells: Aberrant growth and differentiation properties. *Stem Cells*. 1996;14(6):690-701.
9. Freedman MH, Cohen A, Grunberger T, et al. Central role of tumor necrosis factor, GM-CSF and interleukin I in the pathogenesis of juvenile chronic myelogenous leukaemia. *Br J Haematol*. 1992;80(1):40-8.
10. Iversen PO, Sioud M. Modulation of granulocyte-macrophage colony-stimulating factor gene expression by a tumor necrosis factor α specific ribozyme in juvenile myelomonocytic leukemic cells. *Blood*. 1998;92(11):4263-8.
11. Freeburn RW, Gale RE, Wagner H.M, et al. Analysis of the coding sequence for the GM-CSF receptor alpha and beta chains in patients with juvenile chronic myeloid leukemia (JCML). *Exp Hematol*. 1997;25(4):306-11.
12. Busque L, Gilliland DG, Prchal JT, et al. Clonality in juvenile chronic myelogenous leukemia. *Blood*. 1995; 85(1):21-30.
13. Frankel AE, Lilly M., Kreitman R, et al. Diphtheria toxin fused to granulocyte-macrophage colony-stimulating factor is toxic to blasts from patients with juvenile myelomonocytic leukemia and chronic myelomonocytic leukemia. *Blood*; 1998;92(11):4279-86.
14. Emanuel PD. Juvenile myelomonocytic leukemia and chronic myelomonocytic leukemia. *Leukemia*. 2008;22(7):1335-42.
15. Wütz D, Shayan P, Schmahl GE, et al. Gene expression of the hematopoietic cell phosphatase in juvenile

- myelomonocytic leukemia. Leukemia and Lymphoma. 1999;35(5-6):491-9.
16. Mild GC, Schmahl GE, Shayan P, et al. Expression of interferon regulatory factor 1 and 2 in hematopoietic cells of children with juvenile myelomonocytic leukemia. 1999;35(5-6):507-11.
 17. Lapidot T, Grunberger T, Vormoor J, et al. Identification of human juvenile chronic myelogenous leukemia stem cells capable of initiating the disease primary and secondary SCID mice. Blood. 1996;88(7):2655-64.
 18. Herbst B, Kohler G, Mackensen A, et al. *In vitro* differentiation of CD34+ hematopoietic progenitor cells toward distinct dendritic cell subsets of the Birbeck granule and MHC-positive Langerhans cell and the interdigitating dendritic cell type. Blood. 1996;88(7):2541-8.
 19. Herbst B, Kohler G, Mackensen A, et al. GM-CSF promotes differentiation of a precursor cell of monocytes and Langerhans-type dendritic cells from CD34+ haematopoietic progenitor cells. Br J Haematol. 1998;101(2):231-41.
 20. Sawai N, Koike K, Ito S, et al. Aberrant growth of granulocyte-macrophage progenitors in juvenile chronic myelogenous leukemia in serum-free culture. Exper Hematol. 1996;24(2):116-22.

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