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ORIGINAL ARTICLE

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Rapid generation of antigen-presenting cells from leukaemic blasts in acute myeloid leukaemia

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Abstract The ability of acute myeloid leukaemia (AML) cells to acquire dendritic cell (DC)-like characteristics in vitro with a rapid culture method based either on the phorbol ester PMA or calcium ionophores has been studied in comparison to conventional AML-DC cultures with the cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF), tumour necrosis factor-alpha (TNF-α), interleukin-3 (IL-3), SCF, FLT3-L and IL-4. In all AML patients, antigen-presenting cells (APC) could be generated from leukaemic cells in 2 days by incubation with PMA or calcium ionophore (A23187 or ionomycin) in the presence as well as in the absence of IL-4. In 30 out of 36 patients APC could be generated after 2 weeks of culture in cytokine-enriched medium. AML-APC cultured with PMA or calcium ionophores immunophenotypically and functionally were at a more mature stage than those cultured in cytokine-enriched medium. The most mature APC were generated by calcium ionophore A23187 plus IL-4, as evidenced by the higher expression of CD40, CD80, CD86 and HLA-DR. Autologous T cell mediated cytotoxicity towards AML blast cells in vitro was observed in 2 cases tested. The persistence of cytogenetic abnormalities confirmed the leukaemic origin of the AML-APC. The generation of AML-APC was possible from freshly isolated as well as cryopreserved material. Our data show that generation of sufficient AML-APC by A23187 plus IL-4 is feasible, for vaccination purposes, in approximately 70% of AML specimens, offering a time-saving and cost-effective approach in preparing anti-leukaemia vaccines.

Keywords Acute myeloid leukaemia · A23187 · Calcium ionophore · Cytotoxicity · Dendritic cell

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Introduction

Although intensive chemotherapy including stem cell transplantation has improved treatment outcome in acute myeloid leukaemia (AML), at best 40% to 50% of AML patients can be cured. The presence of residual leukaemic cells after intensive chemotherapy is likely to contribute to relapse of the disease [22, 23]. Immunotherapy could provide a more effective approach to controlling or eradicating minimal residual disease. A promising immunotherapeutic approach in leukaemia is to utilise tumour antigen bearing antigen-presenting cells (APC) that present these antigens to T cells to generate autologous anti-leukaemic responses [17, 33]. The most potent APC are dendritic cells (DC). DC are thought to derive from $CD34^+$ haematopoietic progenitor cells, and can be generated in vitro by using combinations of cytokines including granulocyte-macrophage colony-stimulating factor (GM-CSF), tumour necrosis factor-alpha (TNF-α) and interleukin-4 (IL-4) [3, 25, 27]. DC can also be generated from monocytes [29]. An alternative approach in AML could be the generation of morphologically, phenotypically and functionally potent APC from malignant leukaemic cells to conserve the characteristic cytogenetic and phenotypic aberrations of the latter that may be of relevance for antigen presentation. Such an approach has been shown feasible in chronic myeloid leukaemia using GM-CSF, TNF-α and IL-4 [5, 12]. Recently, the generation of DC from AML blasts has also been proven successful. Addition of GM-CSF, TNF-α and IL-4 to the culture

medium has been used combined with CD40L, stem cell factor (SCF), FLT3 ligand (FLT3-L) or transforming growth factor-beta (TGF-β) in various combinations and concentrations [2, 4, 6, 7, 8, 16, 18, 26].

Activation of protein kinase C (PKC) is one of the major events in the IL-4, TNF-α and CD40 ligand-mediated signalling pathway. The phorbol ester PMA can bypass this receptor-mediated pathway by directly activating some of the PKC isoforms. Incubation of normal $CD34^+$ haematopoietic progenitor cells with PMA showed DC differentiation in about 50% of the cells; the remaining cells were found to be apoptotic [11, 21]. It has also been shown that calcium-mobilising agents such as the calcium ionophores (CI) A23187 and ionomycin induce the differentiation of monocytes and $CD34^+$ haematopoietic progenitor cells into DC [10, 19]. The acquisition of dendritic features upon CI incubation occurs far more rapidly than after incubation with cytokines. Not only monocytes and $CD34^+$ haematopoietic progenitor cells differentiate into DC after incubation with CI, but this has also been shown for chronic myeloid leukaemia (CML) and, more recently, for AML cells [13, 20, 34]. Incubation of CML cells with A23187 for 20 h resulted in the expression of CD40, CD80, CD86 and CD83 as well as in the upregulation of HLA-DR and the adhesion molecule CD54 [13, 20]. In AML, approximately 50% of the cells displayed DC morphology and significantly upregulated CD40 and CD86 upon A23187 [34]. These observations suggest that incubation with PMA and calcium mobilisation might provide a powerful and cost-effective approach in rapidly generating potent leukaemic DC vaccines.

In this study, the generation of DC from AML cells in vitro, with emphasis on the use of PMA and CI, was analysed with a conventional cytokine culture method as reference. Our data show that the generation of AML-APC with A23187 and IL-4 alone provided the most mature APC. This method could be used for vaccination purposes in approximately 70% of AML specimens, providing a time-saving and cost-effective approach in preparing anti-leukaemia vaccines.

Materials and methods

Patient samples

Peripheral blood or bone marrow mononuclear cells from 37 AML patients were isolated by density centrifugation using Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden). All samples were drawn after patients' informed consent at the time of collection. Patient characteristics have been presented in Table 1. The cells were cultured immediately, or cryopreserved at a controlled rate in liquid nitrogen using medium containing RPMI-1640 (Gibco, Life Technologies, Paisley, U.K.), 20% fetal bovine serum (FBS; Gibco) and 10% dimethylsulphoxide (Merck, Darmstadt, Germany). Cryopreserved material was rapidly thawed and washed in RPMI-1640 supplemented with 40% FBS. The cells were resuspended in RPMI-1640, 40% FBS for 45 min at 37°C, and then resuspended in culture medium as described below.

AML-APC culture

The cells were cultured in RPMI-1640 supplemented with 20% FBS, 100 IU/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco) at a concentration of 0.5×10^6 cells/ml in 6-well plates (Corning Costar, Corning, N.Y.). APC differentiation was induced by various kinds of cocktails. All cytokines were recombinant human material. In the cytokine culture method, the following cytokines were used: GM-CSF (250 IU/ml, specific activity 1×10^7 IU/mg protein; Pepro Tech, Rocky Hill, N.J.), TNF-α (50 IU/ml, spec. act. 1×10^8 IU/mg protein; Boehringer Mannheim Biochemica, Mannheim, Germany), IL-3 (20 ng/ml, spec. act. 1×10^6 IU/mg protein; Pepro Tech), SCF (50 ng/ml, spec. act. 1×10^5 IU/mg protein; Pepro Tech) and FLT3-L (50 ng/ml, spec. act. 2×10^5 IU/mg protein; Pepro Tech). All cytokines were present in the culture medium during the whole period. At day 7, IL-4 (250 IU/ml, spec. act. 2×10^6 IU/mg protein; Pepro Tech) was added. In case of an overgrown culture, wells were split and fresh medium and cytokines were added. The cells were harvested at day 14.

In a non-cytokine culture setting, cells were cultured for 2 days in culture medium supplemented with the phorbol ester PMA (10 ng/ml; Sigma, St. Louis, Mo.) and TNF-α (500 IU/ml), or with one of the two tested CI A23187 (375 ng/ml, Sigma) and ionomycin (150 ng/ml, Sigma). The different culture conditions were coded as follows: (1) cytokines; (2) PMA, TNF-α; (3) A23187; and (4) ionomycin. Addition of IL-4 (250 IU/ml) to culture methods 2, 3 and 4 was designated as "a" (method 2a, 3a and 4a). Culture methods are summarised in Table 2.

Cytokine concentrations in method 1 were chosen according to a two-step $CD34^+$-derived DC culture system, as described by Rosenzwajg et al. [28] and the AML-DC culture system as described by Robinson et al. [26]. Optimal concentrations of PMA, TNF-α, A23187 and ionomycin were assessed by dose titration experiments in AML-APC cultures (data not shown). Adherent cells were harvested by incubating the wells with 5 mM ethylene diamine tetraacetic acid (EDTA; Sigma) in RPMI-1640, 20% FBS for 15 min at 37°C.

Optimisation of AML-APC culture by sequential addition of cytokines and A23187

Various combinations of cytokines and A23187 were tested. The concentrations used were similar to those described above. First, cells were incubated for 2 days with GM-CSF, IL-3, SCF and FLT3-L in co-culture with A23187 and IL-4 (method EXP-1). Second, cells were preincubated for 5 days with cytokines followed by 2 days incubation with A23187 and IL-4. Tested cytokine combinations were IL-3, SCF and FLT3-L (method EXP-2) combined with GM-CSF without (method EXP-3) and with TNF-α and IL-4 (method EXP-4). Third, to mimic the physiological differentiation steps of DC described by Santiago-Schwarz, cytokines were added sequentially during the 5-day preincubation period [30]. This culture condition was named EXP-5: preincubation with TNF-α for 1 day to upregulate the GM-CSF receptor and to induce selective apoptosis of non-DC-progenitors, followed by expansion with GM-CSF, IL-3, SCF and FLT-3 in combination with IL-4 for 4 days followed by 2 days incubation with A23187 and interferon-gamma, IFN-γ (1,000 IU/ml, spec. act. 2×10^7 IU/mg protein; Strathmann Biotech, Hannover, Germany) for the last 24 h. All culture strategies are summarised in Table 2.

Cell number and viability

Cell number and viability before and after culture were determined using the trypan blue dye exclusion test (0.2 g/ml; Merck). The percentage of necrotic, viable and apoptotic cells was determined by incubation with Syto-16 (3 nM; Molecular Probes, Eugene, Ore.), PSC833 (2 μM; a kind gift from Novartis, Basle, Switzerland) and 7-amino-actinomycin D (7-AAD; ViaProbe, Pharmingen, San Diego, Calif.) for 45 min at 37°C and flow-cytometric analysis as described by Schuurhuis et al. [32].

Table 1 Patient characteristics. Patients are listed according to FAB classification. Percentages of blasts were scored in May-Grünwald-Giemsa stained cytospin preparations of Ficoll-density separated samples

Patient no.	Age (yr)	FAB	Material	Blasts (%)	Cytogenetics
1	34	M0	Bone marrow	82	Inv(3), del(7)
2	61	M0	Blood	78	Various, +6, +8
3	37	M0	Blood	91	Normal
4	72	M0	Blood	41	Normal
5	86	M1	Bone marrow	91	N.T.
6	75	M1	Bone marrow	92	Normal
7	55	M1	Bone marrow	86	Del(9) (q11q22)
8	76	M1	Bone marrow	82	Normal
9	52	M1	Bone marrow	82	Der(1) t(1; ?) (q32; ?), der(3) t(1; 3) (q32; q25)
10	46	M2	Blood	97	+8, +13
11	63	M2	Bone marrow	64	Normal
12	69	M2	Bone marrow	35	Normal
13	66	M2	Bone marrow	91	Normal
14	33	M2	Bone marrow	63	Normal
15	40	M3	Bone marrow	46	t(15; 17)
16	51	M3	Bone marrow	93	t(15; 17)
17	42	M4	Bone marrow	60	Del(9) (q11q22)
18	44	M4	Bone marrow	80	Normal
19	41	M4	Blood	43	N.T.
20	71	M4	Bone marrow	86	Add(8) (q24), +14, der(14; 14) (q10; q10)
21	64	M4	Bone marrow	98	Normal
22	46	M4eo	Bone marrow	68	Inv(16)
23	57	M5a	Bone marrow	94	t(11; 16)
24	65	M5a	Bone marrow	56	Normal
25	69	M5a	Bone marrow	84	No metaphases
26	76	M5a	Bone marrow	96	No metaphases
27	37	M5a	Bone marrow	95	Normal
28	46	M5a	Bone marrow	97	t(9; 11) (p22; q23)
29	60	M5b	Bone marrow	85	N.T.
30	71	M5b	Blood	99	Normal
31	70	M5b	Bone marrow	95	Normal
32	68	M6	Bone marrow	78	Normal
33	74	M6	Bone marrow	86	Normal
34	62	RAEB-t	Bone marrow	19	Normal
35	72	RAEB-t	Bone marrow	85	Normal
36	61	Not classified	Bone marrow	84	N.T.
37	74	Not classified	Bone marrow	88	45, X

N.T.: not tested

Table 2 Culture conditions

Culture method	Additives during culture
1	Day 0–14: IL-3, SCF, FLT3-L, GM-CSF, TNF-α. Day 7–14: IL-4
2 (a)	Day 0–2: PMA, TNF-α, (IL-4)
3 (a)	Day 0–2: A23187, (IL-4)
4 (a)	Day 0–2: ionomycin, (IL-4)
EXP-1	Day 0–2: IL-3, SCF, FLT3-L, GM-CSF. Day 0–2: A23187, IL-4
EXP-2	Day 0–7: IL-3, SCF, FLT3-L. Day 5–7: A23187, IL-4
EXP-3	Day 0–7: IL-3, SCF, FLT3-L, GM-CSF. Day 5–7: A23187, IL-4
EXP-4	Day 0–7: IL-3, SCF, FLT3-L, GM-CSF, TNF-α, IL-4. Day 5–7: A23187
EXP-5	Day 0–7: TNF-α. Day 1–5: IL-3, SCF, FLT3-L, GM-CSF, IL-4. Day 5–7: A23187. Day 6–7: IFN-γ

Morphology

Cytospin slides were prepared before and after each culture, stained by the May-Grünwald-Giemsa method and evaluated by light microscopy [17].

Immunophenotypic analysis by flow cytometry

Flow-cytometric analysis was performed before and after culture. Prior to labelling with monoclonal antibodies (mAb), the cells were incubated with human immunoglobulin (60 mg/ml; CLB, Amsterdam, the Netherlands). Triple colour analysis was performed by FACSCalibur (Becton Dickinson, San Jose, Calif.). Results were analysed using CellQuest software (Becton Dickinson).

The following mAb were used: FITC-labelled CD14 (Becton Dickinson), CD1a (CLB) and CD54 (Dako, Glostrup, Denmark), PE-labelled CD40 (Immunotech, Marseille, France), CD80 (Becton Dickinson) and CD83 (Immunotech), PerCP-labelled CD34 (Becton Dickinson) and anti-HLA-DR (Becton Dickinson), and biotin-labelled CD86 (Pharmingen). CD86 biotin-containing samples were subsequently stained using streptavidin PerCP (Becton

Dickinson). Isotype controls used were FITC-labelled IgG1 (Dako) and IgG2b (CLB), PE-labelled IgG1 (Becton Dickinson) and IgG2b (Dako) and PerCP-labelled IgG1 and IgG2a (both from Becton Dickinson). Results were calculated as percentage positive cells compared to the appropriate isotype control. Necrotic cells were excluded from the flow-cytometric analysis using 7-AAD-staining.

Definitions for evaluation of culture by immunophenotyping

A definition for AML-APC was formulated according to a working definition for DC proposed by Hart based on the morphologic, immunophenotypic and functional properties of DC [17]. An APC was defined as a cell with DC-like morphology, a bright expression of CD40, increased or acquired expression of CD54, expression of CD80, CD86, CD83 and HLA-DR and an increased T cell stimulating capacity as compared to control non-cultured blast cells.

The relative APC yield was calculated to evaluate the number of APC cultured. The number of viable cells after culture was divided by the number of viable cells before culture, and multiplied by the percentage of $CD40^{bright}/CD54^{bright}$ cells. CD40 expression has been reported on DC at all stages of their maturation [17, 31]. In previous experiments, we found the upregulation of CD40 and CD54 to be most consistent in APC culture (data not shown).

The mean fluorescence index (MFI) was determined to measure the fluorescence intensity. The MFI is defined as the ratio of the mean channel peak fluorescence intensity of the specific antibody and the mean channel peak fluorescence intensity of its isotypic control.

Mixed leukocyte reaction

The ability of the cultured cells to stimulate T cells was tested in an allogeneic mixed leukocyte reaction (MLR). Peripheral blood mononuclear cells (PBMC) were isolated from a buffy-coat from one donor and used as responder cells. Non-cultured leukaemic cells or cultured cells (1×10^5 viable cells) were suspended in 1 ml RPMI-1640, 10% FBS and 50 μM β-mercaptoethanol (Sigma) and irradiated at 30 Gy. These cells were added in several ratios to a constant number of PBMC (5×10^4 per well) in a 96-well round-bottomed plate (Costar), co-cultured for 5 days and then pulsed for 5 h with 0.4 μCi [^{3}H]thymidine per well (Amersham Pharmacia Biotech, Buckinghamshire, U.K.). Co-cultures were harvested onto a fibreglass filter mat and analysed for incorporation of [^{3}H]thymidine in a liquid scintillation counter (Wallac, Turku, Finland). All ratios were tested in triplicate. Only minimal [^{3}H]thymidine uptake was found in AML blast cells and AML-APC.

Only one PMBC donor was used to ensure uniformity. A second PBMC donor was tested simultaneously in a few cases to determine whether the observed responses were not specific for one donor.

Autologous cytotoxicity assay

To examine T cell mediated cytotoxicity, autologous T cells were isolated by magnetic cell sorting from de novo AML samples from 2 patients (patients 22 and 27). T cells were labelled with CD28 (CLB) and isolated by goat-anti-mouse-microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Isolated T cells were activated by CD3 (2 μg/ml; CLB) and expanded for 14 days with IL-2 (10 IU/ml, spec. act. 10^7 IU/mg protein; Strathmann Biotech, Hannover, Germany) and IL-7 (5 ng/ml, spec. act. 5×10^7 IU/mg protein; Strathmann Biotech) in RPMI-1640/10% FBS. Expanded T cells were harvested and stimulated by AML-APC in a 5:1 ratio in combination with IL-2 (10 IU/ml) and IL-7 (5 ng/ml) or cultured with IL-2 and IL-7 alone for another 5 days in RPMI/10% human AB serum (Biowhittaker, Walkersville, Md.).

Cytotoxicity was analysed by a flow-cytometric assay based on the method described by Godoy-Ramirez et al. [15]. Unstimulated and APC-stimulated T cells (effector cells; E) were harvested and co-cultured with AML blasts (target cells; T) for 6 h at E/T ratios of 20:1, 10:1, 5:1 and 2.5:1. After co-culture, effector T cells and target AML blasts were stained by a specific T cell marker (CD5; Becton Dickinson) and an AML blast-specific marker (CD34 or CD13; Becton Dickinson). Syto16/7-AAD staining was performed to detect early apoptosis and secondary necrosis [32]. All E/T ratios were tested in duplicate.

Detection of leukaemic origin of cultured cells

In two cases (patients 10 and 16) cultured cells were sorted to determine the leukaemic origin of the APC. In patient 10 (trisomy 8 and 13) APC were cultured by method 1, in patient 16 [t(15;17)] by method 3a. Cultured cells were labelled with CD54 FITC (Dako) and CD40-PE (Immunotech). $CD40^{bright}/CD54^{bright}$ cells were selected using the FACS-Vantage technique (Becton Dickinson). Collected cells were spun onto glass slides. For trisomy 8, cytospin preparations were fixed in 70% ethanol (Merck). Interphase fluorescence in situ hybridisation (FISH) was then performed, as described previously [24, 36] using a probe specific for the chromosome 8 centromere (pJM128; provided by the Academic Medical Centre, Amsterdam, the Netherlands). Non-fixed slides of selected cells from patient 16 were used for the detection of t(15;17). Immunostaining of the PML/RARα fusion protein was performed using the mAb PG-M3 (Santa Cruz Biotechnology, Calif.) as described previously, with minor modifications [14]. The NB4 cell line and the HL60 cell line were used as a positive and negative control, respectively.

Statistical analysis

The statistical significance of the relation between the phenotype and functional capacity, and the relation between immunophenotypical data from two separate sets of experiments was determined by Pearson's correlation coefficient (one-tailed). The paired sample Student's *t*-test (two-tailed) was used to compare the results of different culture methods. *P*-values of <0.05 were regarded as significant.

Results

Cytokine culture

AML blast cells from 36 patients were cultured using the combination of cytokines described in Materials and methods (method 1). After 2 weeks of culture, cell morphology and immunophenotype were evaluated. At the start of culture, leukaemic cells from 25 patients expressed CD40 with a low fluorescence intensity. The median percentage of CD40 positive cells in the total patient group was 23% (range: 0% to 82%). The expression of CD40 was upregulated during culture in 33 out of 36 patients. Cells with dendritic morphology could be detected in 30 out of 36 cases. In these patients, the cultured cells were found to be $CD40^{bright}$ and $CD54^{bright}$. This distinct subpopulation could not be found before culture. Fig. 1 shows the results of immunophenotypic analysis before and after culture. CD40, CD54 and the costimulatory molecule CD80 were significantly upregulated. No significant changes in the expression of CD1a, CD83, CD86 and HLA-DR were found.

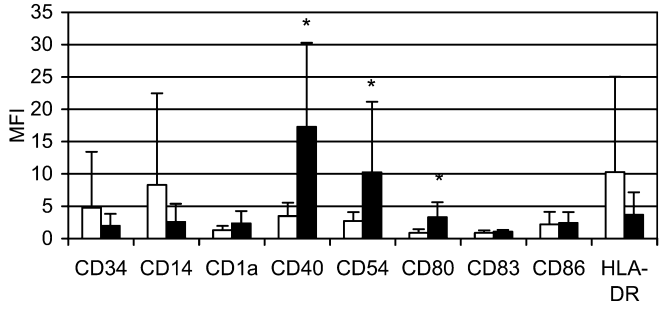

Fig. 1 Immunophenotype of AML cells before and after culture in cytokine-enriched medium according to method 1. Mean MFI and corresponding standard deviation of data obtained from 36 patients are depicted. *Open bars*: before culture, *filled bars*: after culture. **P*-values are as follows: CD40: $P = 0.003$; CD54: $P = 0.05$; CD80: $P = 0.008$

Comparison of AML-APC culture methods

Four different culture conditions (methods 1, 2a, 3a and 4a) were tested simultaneously in 15 patients. Five of these patients (patients 5, 10, 18, 23 and 25) were also tested using the non-cytokine methods without additional IL-4 (methods 2, 3 and 4).

Culture characteristics, morphology and immunophenotypic analysis

The morphology of the AML-APC derived in all culture conditions was similar to that described by Hart, e.g. veiled cells of increased size [17]. AML-APC cultured in cytokine-enriched medium (method 1) showed a higher cytoplasm/nucleus ratio (Fig. 2A) as compared to the AML-APC culture in the non-cytokine setting (Fig. 2B).

An example of an immunophenotypic analysis is shown in Fig. 3 for culture methods 1 and 3a. The APC culture with cytokines resulted in two distinct cell populations. The subpopulation with no to only weak expression of the antigens tested represents the undifferentiated AML blast cells. The second subpopulation with bright expression represents the AML-DC. Results of the immunophenotypic analysis of 4 culture methods are depicted in Table 3. Non-cytokine culture methods without additional IL-4, tested in 5 patients, resulted in a lower expression of the tested antigens than in culture with IL-4 (except for CD54 with methods 3 and 4; data not shown). CD40 and CD54 were significantly upregulated in all culture conditions. The most pronounced increase in CD40 and CD54 expression was observed with methods 2a (PMA/TNFα/IL-4) and 3a (A23187/IL-4). The mean fold increase in MFI was 12.4 ± 12.1 (median: 7.1) and 17.7 ± 19.4 (median: 8.6) for CD40, respectively, and 16.4 ± 13.2 (median: 12.1) and 11.5 ± 8.0 (median: 13.2) for CD54 (see Table 4). These increases were significantly higher as compared to those in methods 1 and 4a. The costimulatory molecules CD80 and CD86 were significantly upregulated in method 3a, with a mean fold increase of 12.7 ± 21.8 (median: 5.3) and 6.4 ± 6.5 (median: 4.1), respectively. Except for the cytokine culture with respect to CD80, no significant upregulation of CD80 and CD86 was found for any of the other methods. CD83 was significantly upregulated by method 2a with a mean fold increase of 2.6 ± 2.0 (median: 1.9). No significant increase in the expression of CD1a and HLA-DR was achieved by either method.

Overall, method 3a (A23187 and IL-4) resulted in the generation of more mature APC based on their immunophenotypic profile as compared to the cytokine-based APC culture [CD40 ($P = 0.007$), CD54 ($P = 0.017$), CD80 ($P = 0.078$) and CD86 ($P = 0.028$)].

Although a trend towards an increase in total number of viable cells during culture was seen with cytokine-enriched medium (method 1; 14 days culture), no significance was found compared to all other methods (Fig. 4A). In addition, no significant differences regarding the relative APC yield were found between the different culture methods (Fig. 4B).

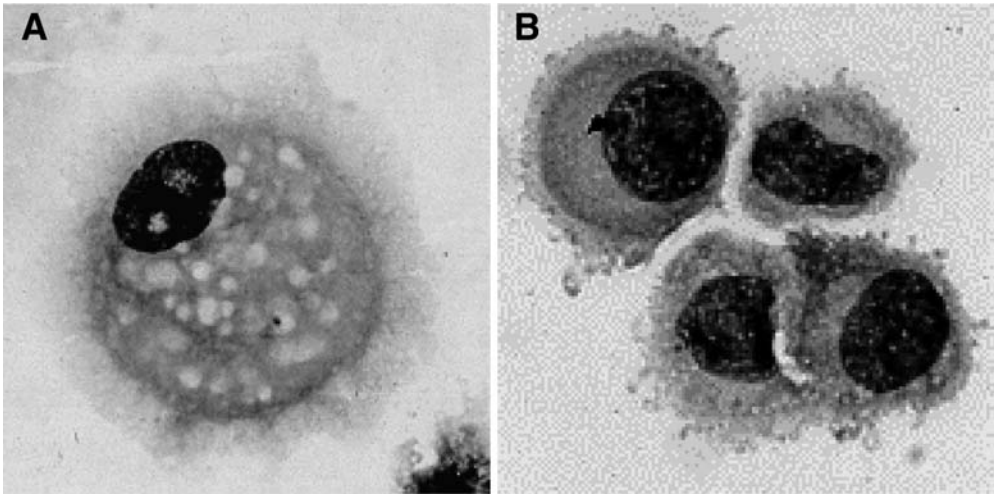

Fig. 2 A, B Morphology of cells after AML APC culture. Cultured cells were spun onto slides and stained with May-Grünwald-Giemsa. A representative example is shown of AML APC cultured from bone marrow AML cells of patient 23 (AML-M5a; see Table 1) in **A** cytokine-enriched medium (method 1, 1000×) as well as of cells cultured with **B** A23187 and IL-4 (method 3a, 1000×)

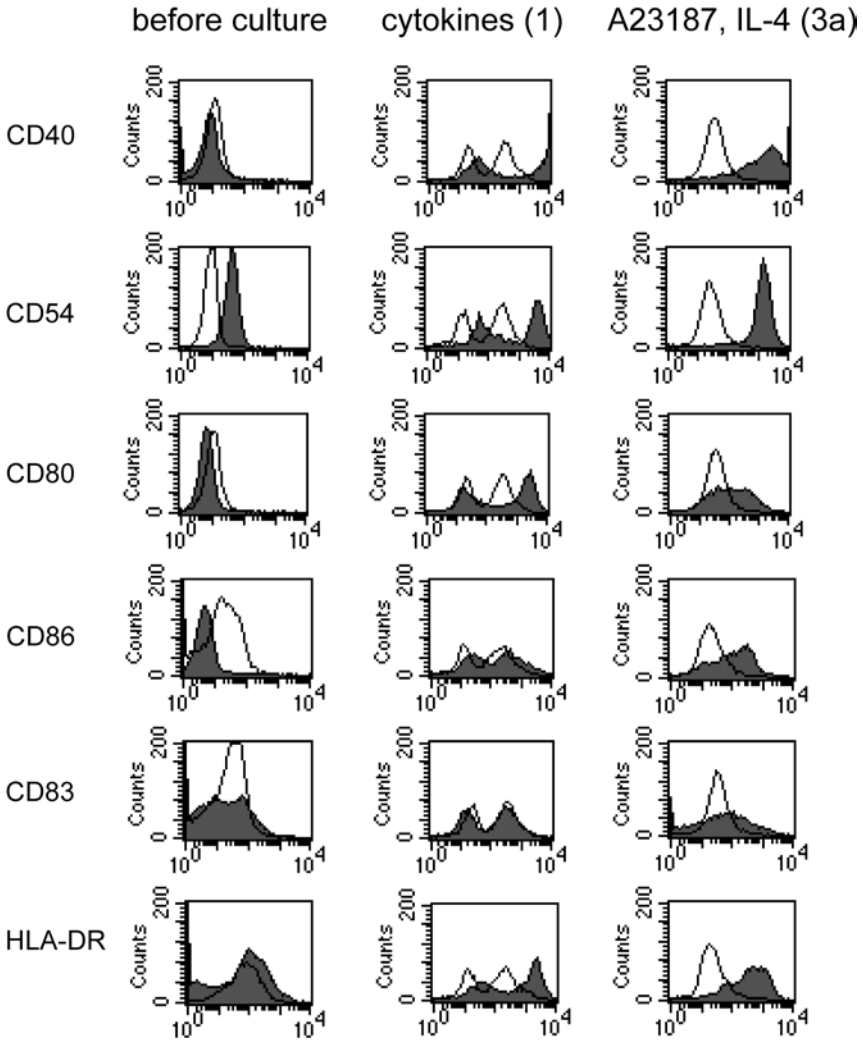

Fig. 3 Immunophenotypic analysis of untreated AML cells and cells after APC culture. APC culture method 1 (cytokines) and method 3a (A23187 plus IL-4) are depicted for one representative patient (patient 25, AML-M5a; see Table 1). The *left column* shows phenotypic analysis of AML blast cells before culture, the *middle column* shows results of APC culture in cytokine-enriched medium, and the *right column* results of APC culture by method 3a. The *open profile* shows the isotype control and the *closed profile* the expression of the antigen depicted on the left side of the figure

Mixed leukocyte reaction

The T cell stimulating capacity of AML-APC cultured by the different methods showed large heterogeneity within a particular AML sample. MRL results for two representative cases are shown in Fig. 5A, B. In almost all cases, AML-APC cultured by method 3a were found to have the strongest T cell stimulating capacity, which was consistent with the immunophenotypical data. Addition of IL-4 to non-cytokine culture methods, tested in 5 patients, resulted in a significant further increase in T cell proliferation ($P = 0.005$; data not shown). Finally, similar results were obtained with responder cells from a second donor tested simultaneously (data not shown).

Autologous T cell mediated cytotoxicity

A sufficient amount of autologous T cells was generated from the de novo AML samples from 2 patients. The AML-APC that were used to stimulate the autologous T cells were generated in culture with A23187 and IL-4 (method 3a). An increase in apoptosis and secondary necrosis of AML blast cells was found after stimulation of the autologous T cells with AML-APC as compared to unstimulated autologous T cells (patients 22 and 27: 19% and 35% increase, respectively; see Fig. 6). A dose-response effect was observed (data not shown). For patient 22, cytokine cultured AML-APC were also used to stimulate the autologous T cells. However, these cytokine cultured APC did not induce T cell mediated cytotoxicity. AML-APC-mediated apoptosis of the autologous T cells was approximately 2% at an E/T ratio of 20:1.

Reproducibility

In 5 patients (patients 5, 10, 18, 23 and 25), the reproducibility of the results by either method was confirmed. Immunophenotypic analysis showed that the results could be reproduced in all patients. There was a significant correlation between the two sets of experiments with respect to CD40-MFI ($r = 0.824$, $P < 0.001$) and CD54-MFI ($r = 0.560$, $P = 0.010$). The MLR assay showed a similar alloreactivity when comparing both sets of experiments (data not shown).

Table 3 Mean fluorescence index of DC defining antigens after immunophenotypic analysis of uncultured AML blasts and cells after APC culture (n.t.: not tested)

Patient	CD54					CD83					HLA-DR				
	Culture method					Culture method					Culture method				
	Day 0	1	2a	3a	4a	Day 0	1	2a	3a	4a	Day 0	1	2a	3a	4a
3	5.1	51.5	125.7	43.9	14.6	0.8	9.6	17.3	4.2	0.9	0.4	1.8	6.6	6.6	7.1
4	6.3	4.6	27.7	15.5	2.6	0.8	0.9	0.9	4.1	0.6	2.2	2.8	2.0	10.3	1.4
5	7.5	14.5	38.1	54.6	45.6	0.6	2.7	1.1	3.2	3.4	0.6	1.7	1.4	4.6	3.1
8	4.8	12.6	30.6	16.9	5.5	0.6	7.8	5.3	8.6	1.3	1.6	21.8	7.5	13.3	1.9
10	1.1	4.5	12.6	4.1	3.7	1.5	1.0	0.4	1.2	1.3	1.0	1.1	0.5	1.9	1.5
12	6.1	22.8	53.0	24.8	10.1	0.7	2.9	1.4	4.1	1.1	1.2	2.0	1.6	4.6	1.6
14	3.2	12.4	31.6	58.5	15.8	1.3	1.8	2.2	5.3	1.4	4.5	3.0	8.6	17.2	4.4
16	1.8	13.6	5.6	12.4	2.7	1.3	1.5	1.2	3.3	0.9	2.6	1.4	3.5	12.4	1.2
18	3.6	8.3	42.2	36.4	29.2	0.6	1.6	2.6	3.3	2.4	1.7	1.3	2.8	4.5	3.2
21	2.1	14.0	13.7	30.3	11.0	1.1	1.2	0.7	3.3	0.7	5.1	2.1	5.5	5.1	1.1
22	1.9	34.5	n.t.	101.3	n.t.	0.8	3.1	n.t.	29.8	n.t.	2.7	2.0	n.t.	33.5	n.t.
23	1.5	18.2	47.3	50.7	39.3	2.2	4.7	2.7	5.0	3.0	1.3	2.2	3.4	5.3	3.1
25	5.0	11.0	35.5	39.4	39.1	1.1	2.1	3.7	3.9	3.7	3.7	2.6	2.7	4.2	4.3
27	1.9	24.0	86.2	77.0	31.0	0.9	7.9	5.8	41.0	5.4	0.6	4.7	5.6	15.0	4.4
30	1.5	57.8	9.9	109.5	35.2	3.3	3.4	0.8	20.1	3.8	7.6	5.1	2.9	58.1	10.4
31	5.0	12.7	16.3	153.4	79.0	0.7	3.6	0.7	56.8	11.4	12.5	1.3	10.4	35.3	31.2
3	5.8	14.2	83.8	17.5	15.8	0.8	1.6	6.3	1.2	1.4	16.4	4.4	7.8	6.9	13.7
4	1.6	3.7	72.8	37.9	2.9	1.1	0.8	1.2	1.7	1.3	11.9	2.3	2.9	6.9	3.8
5	5.6	11.4	34.8	46.2	40.0	0.9	1.1	1.9	1.5	1.6	11.4	4.2	4.8	14.4	16.8
8	1.7	5.1	76.0	31.2	4.3	0.6	0.8	0.7	1.1	0.8	12.9	1.6	5.5	14.6	4.2
10	3.0	4.4	20.5	2.1	2.7	0.5	0.7	0.7	0.6	0.7	38.4	2.7	4.8	2.2	2.3
12	2.4	6.6	22.7	9.3	4.0	1.3	1.2	1.8	1.2	0.9	4.0	4.7	6.5	4.7	7.5
14	1.8	5.3	39.1	19.0	10.8	0.8	0.9	3.3	5.7	1.3	20.4	4.9	37.3	58.3	21.5
16	1.2	4.5	15.2	8.8	1.5	0.8	0.9	1.5	4.5	1.0	1.1	1.2	2.6	1.1	1.1
18	1.6	4.5	27.5	16.9	16.2	0.6	1.1	1.1	1.6	1.1	14.7	2.9	2.8	4.5	3.2
21	1.3	59.8	7.4	6.4	5.9	0.7	0.8	1.7	2.7	1.0	5.1	1.3	1.7	1.2	2.1
22	3.8	15.4	n.t.	63.7	n.t.	1.0	1.1	n.t.	10.4	n.t.	7.0	20.7	n.t.	20.3	n.t.
23	4.2	9.7	46.9	34.6	35.9	1.2	1.0	2.4	3.3	2.1	12.9	4.9	15.1	14.0	16.7
25	3.5	5.3	42.3	36.3	33.5	1.9	0.8	1.3	1.1	1.3	3.3	6.1	21.9	16.4	17.2
27	3.7	6.4	99.5	56.8	16.5	0.7	1.9	3.9	6.6	1.2	8.3	6.5	17.3	31.2	18.3
30	5.1	23.9	29.0	92.9	27.7	0.4	0.9	1.8	10.7	1.2	3.5	11.4	1.0	6.7	18.1
31	2.5	1.9	15.3	72.3	51.4	1.5	1.0	1.8	54.9	35.8	111.4	3.7	21.8	191.9	101.2

Table 4 Fold increase in MFI of DC defining antigens of AML APC for the various culture conditions as compared to uncultured AML blasts

	CD40				CD80				CD86			
	Culture method				Culture method				Culture method			
	1	2a	3a	4a	1	2a	3a	4a	1	2a	3a	4a
Mean	7.4*	12.4*	17.7*	8.3*	4.0*	3.7	12.7*	3.1	2.6	3.1	6.4*	3.4
SD	9.4	12.1	19.4	8.3	4.1	5.5	21.8	4.1	3.7	4.4	6.5	4.7
Median	3.9	7.1	8.6	5.2	2.1	1.7	5.3	1.4	1.1	1.4	4.1	1.5
	CD54				CD83				HLA-DR			
	Culture method				Culture method				Culture method			
	1	2a	3a	4a	1	2a	3a	4a	1	2a	3a	4a
Mean	5.4*	16.4*	11.5*	5.8*	1.3	2.6*	6.9	3.0	0.7	0.9	1.2	1.3
SD	11.3	13.2	8.0	5.1	0.6	2.0	10.5	5.7	0.9	0.8	1.0	1.3
Median	2.5	12.1	13.2	4.5	1.1	1.9	2.7	1.6	0.3	0.4	1.1	1.0

*$P < 0.05$ as compared to uncultured AML blasts. Patient 22 was excluded from analysis since only culture methods 1 and 3a were utilised

Optimisation of APC culture conditions

Cell culture with A23187 and IL-4 (method 3a) resulted in most the mature APC after 2 days of culture. The cytokine culture method resulted in high cell viability and cell numbers after 14 days of culture. Combining these two methods could lead to improved viability and hence APC yield compared to either method used on

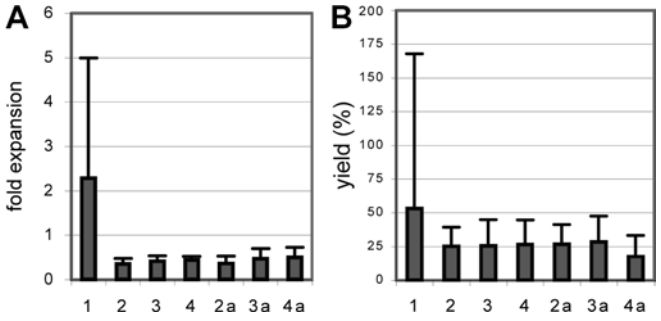

Fig. 4 A, B Fold expansion of total number of viable cells and relative APC yield. **A** Fold expansion of viable cells after culture compared to viable cells before culture is shown. **B** Relative APC yield: the percentage of the $CD40^{bright}/CD54^{bright}$ cell population multiplied by fold expansion is shown. Mean and standard deviation are indicated for every culture method tested

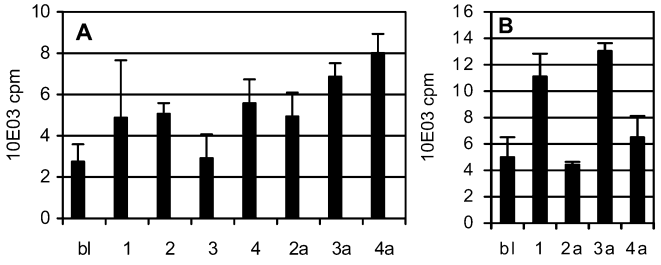

Fig. 5 A, B T cell stimulating capacity of cultured cells from several AML APC culture methods. After AML APC culture, cells were harvested and co-cultured for 5 days with allogeneic PBMC. [^{3}H]thymidine was added for 5 h, and the uptake was analysed. [^{3}H]thymidine incorporation of PBMC stimulated by uncultured blast cells (*bl*) or APC at a ratio of 5:1 is shown for 2 patients and several culture conditions **A** (patient 23, AML-M5a; see Table 1) and **B** (patient 15, AML-M3). Culture methods are depicted along the *X-axis*: cytokine-based culture (method 1), PMA/TNF$\alpha \pm$ IL-4 (methods 2 and 2a), A23187 $\pm$ IL-4 (methods 3 and 3a) and ionomycin $\pm$ IL-4 (methods 4 and 4a). All results are the means of triplicate determinations

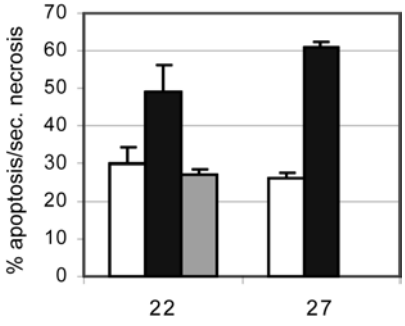

Fig. 6 T cell mediated cytotoxicity against autologous AML blasts. Percentage apoptosis and secondary necrosis are shown for an E/T ratio of 20:1 for patients 22 and 27 (see Table 1). *Open bars* depict results for unstimulated T cells, *black bars* results for T cells stimulated by A23187/IL-4 cultured AML-APC (method 3a) and *grey bars* results for T cells stimulated by cytokine cultured AML-APC. Results are means of duplicate determinations

its own. Therefore, AML cells were incubated with various combinations of cytokines together with or followed by A23187. The results are shown in Fig. 7.

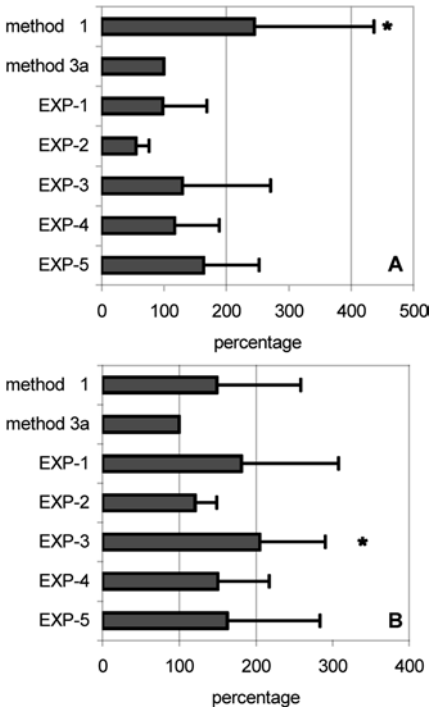

Fig. 7 A, B Influence of cytokine-induced expansion combined with A23187 on APC yield, viability and maturation. Expansion/differentiation methods are described in Table 2. In short: EXP-1: GM-CSF, IL-3, SCF, FLT3-L, A23187 and IL-4 for 2 days ($n = 6$); EXP-2: preincubation with IL-3, SCF and FLT3-L ($n = 3$); EXP-3: preincubation with EXP-2 cytokines plus GM-CSF ($n = 3$); EXP-4: preincubation with EXP-3 cytokines plus TNF-α and IL-4 ($n = 6$); EXP-5: sequential addition of TNF-α, GM-CSF, IL-3, SCF, FLT3-L, IL-4 and IFN-γ ($n = 3$). EXP-2, 3, 4 and 5 were combined with 2 days incubation with A23187 and IL-4. **A** This panel shows cell viability. **B** This panel shows APC yield after preincubation with cytokines compared to incubation with A23187 and IL-4 alone (viability and yield of method 3a cultures were set at 100%; $n = 15$). Mean and standard deviation are indicated. $^*P < 0.05$

The cytokine culture method (method 1) showed a significantly higher cell viability compared to method 3a ($P = 0.011$; Fig. 7A). No significant improvement in cell culture viability was obtained by preincubation with cytokines before A23187 treatment. Preincubation of AML cells with IL-3, SCF, FLT3-L and GM-CSF, followed by A23187 and IL-4 (EXP-3) resulted in significantly more APC compared to culture with A23187 and IL-4 alone ($P = 0.03$; Fig. 7B). In all culture methods tested, preincubation with cytokines followed by A23187 and IL-4 resulted in a significantly lower maturation level of AML-APC than that obtained by A23187 and IL-4 alone. These results paralleled a decrease in MLR compared to culture with A23187 and IL-4 alone (data not shown). If cytokines and A23187 were used from the start of culture (method EXP-1), no significant difference in the level of maturation compared to that obtained by A23187 and IL-4 alone was observed. Finally, no increase in HLA-DR expression could be achieved by adding IFN-γ to the culture medium.

Determination of leukaemic origin

The sorting of APC after culture was performed in 2 cases (patient 10, using culture method 1 and patient 16, using culture method 3a). After flow-cytometric sorting of AML-APC, chromosome 8 trisomy was detected by FISH analysis in the APC from patient 10, thereby proving the leukaemic origin of the cultured APC. The micro-granular staining pattern of the PML/RARα fusion protein was detected by immunostaining in 98 out of 100 sorted cells from patient 16, thereby proving the presence of t(15;17) in these APC and their leukaemic origin.

Fresh versus frozen/thawed material

Table 5 provides a comparison between cultures performed with freshly isolated and frozen/thawed AML cells with respect to the percentage of CD40-positive cells and APC yield. With regard to the percentage of CD40-positive cells and APC yield, no significant difference was found between freshly isolated and frozen/thawed APC cultures. The immunophenotype and the functional quality of the cultured cells were comparable.

Discussion

PMA treatment of $CD34^+$ progenitor cells results in rapid differentiation into functional DC [21]. Likewise, CI treatment of peripheral blood monocytes appears to rapidly result in truly mature and activated DC [10]. In leukaemic cell lines, more restricted characteristics of DC maturation are encountered after calcium mobilisation; moreover, maturation could be enhanced by GM-CSF and IFN-γ treatment [19]. Engels et al. have shown that DC-like cells can be generated from leukaemic cells in CML by culture with the calcium ionophore A23187 [13].

In this study, we have investigated the ability of AML cells to obtain DC-like characteristics upon a rapid culture method based on PMA or CI compared to a conventional culture method based on cytokines. Our data show that the generation of these cells with PMA and CI is also possible in AML. Our results support the data of Waclavicek et al. [34]. In the majority of the AML patients, we succeeded in generating DC-like APC after rapid culture (2 days) with PMA, A23187 or ionomycin and in 2 weeks with a cytokine-based method. The leukaemic origin of the APC was confirmed in 2 patients tested. Culturing with A23187 and IL-4 resulted in the generation of the most mature APC. This was evidenced by a significant increase in the expression of CD40, CD54, CD80 and CD86 as well as by an increase in alloreactivity (MLR) compared to the other methods tested. The upregulation of these molecules was also significantly increased by PMA/TNF-α/IL-4 and ionomycin/IL-4 compared to that observed in cytokine-based methods. In general, IL-4 was shown to further improve the characteristics of AML-APC, especially in the non-cytokine culture methods.

Autologous T cell mediated cytotoxicity towards AML blast cells in vitro was observed in 2 patients tested. In these cases, the AML-APC used were cultured with A23187 and IL-4. This culture method resulted in a significantly more mature APC phenotype and hence autologous T cell mediated cytotoxicity.

In this study, the autologous T cells were generated from de novo AML samples. T cells isolated from patients in remission might be a better option, because these cells can be used without CD3/CD28 manipulation, and moreover they represent the situation during patient vaccination. However, T cells isolated from patients in remission were not available in the selected subjects.

The heterogeneity of AML and the putative intrinsic variations in the differentiative ability of leukaemic cells might reflect the heterogeneous results for different AML samples under varying culture conditions. No

Table 5 Percentage and absolute number of AML APC after culture using fresh and frozen/thawed material

Patient no.	Culture method	CD40 (%)		Abs. no. APC ($*10^6$)[a]	
		Fresh	Frozen/thawed	Fresh	Frozen/thawed
3	1	37	34 (92)	55	6 (11)
5	1	88	29 (33)	9	13 (139)
11	1	9	7 (78)	9	48 (522)
18	1	45	36 (80)	227	50 (22)
25	1	40	18 (45)	155	28 (18)
35	1	54	28 (52)	62	121 (192)
36	1	22	26 (118)	8	2 (25)
3	2a	91	92 (101)	40	25 (62)
7	2a	85	54 (64)	59	8 (14)
3	3a	14	41 (290)	4	13 (320)
25	3a	64	76 (118)	60	30 (50)
27	3a	54	56 (103)	34	29 (85)
7	3a	83	64 (77)	86	43 (50)
Mean ± SD		53 ± 28	43 ± 24	62 ± 64	116 ± 151
Median		54	36	55	50

[a]Data were extrapolated to an input of 100×10^6 viable cells at the start of culture. Shown in brackets are the percentage CD40 and absolute number of cells compared to fresh cultures

correlation between AML FAB classification and culture outcome could be found. The culture of AML-APC might therefore require a patient-oriented approach. An optimal method for generating AML-APC should be established at diagnosis for each individual patient. From our data, we propose that the culture conditions should include a CI and IL-4, e.g. A23187/IL-4, which provided the most mature AML-APC. In addition, the generation of AML-APC from frozen/thawed material was possible. Therefore, leukaemic cells can be cryopreserved until use for large-scale generation of AML-APC, based upon the most optimal method defined at diagnosis for the preparation of an AML-APC vaccine.

A pilot study on active specific immunisation with autologous leukaemic DC in CML, performed at our department, showed that 10×10^6 CML DC could be generated for each of 4 vaccines (manuscript in preparation). This number corresponds fairly well to the number of CML DC used in a vaccination study reported by Westermann et al. [35]. Table 4 shows that in 10 out of 13 cultures, the amount of 10×10^6 AML APC could be obtained if the initial cultures contained at least 100×10^6 viable frozen/thawed AML cells. The recovery of cryopreserved blast cells usually amounted to about 50%; the average APC yield in the cells from 15 patients cultured by the non-cytokine methods was 24%. Assuming that an AML APC vaccination regimen requires 4 vaccines of 10×10^6 cells each, similar to that shown for CML, approximately 4×10^8 viable AML cells are needed at diagnosis. In 25 out of 36 patients (70%), we were able to isolate this amount of cells at diagnosis.

In the majority of AML samples, the expansion of cells during 14-days culture in a cytokine-enriched medium could be shown. FLT3-L and SCF are reported to sustain long-term expansion of normal DC progenitors [1, 9, 28, 38]. Expansion of these progenitor cells did not effect DC differentiation and stimulation of T cells [9]. Woichiechowsky et al. reported that addition of FLT3-L to GM-CSF and TNF-α based AML DC cultures was effective in increasing leukaemic DC yield [37]. In previous experiments IL-3, SCF and FLT3-L in addition to GM-CSF, TNF-α and IL-4 based-cytokine cultures appeared to increase viability and APC yield (data not shown). The use of these cytokines prior to culture in the non-cytokine culture system might result in an increase of cell number and hence the yield of APC. However, our findings do not support a significant improvement in these parameters by the sequential use of cytokines and CI.

In conclusion, the generation of AML-APC by a rapid 2-day culture method with A23187 and IL-4 resulted in mature AML-APC with functionally intact T cell stimulating capacity. Such an approach could be feasible for vaccination purposes in approximately 70% of AML samples. This culture method provides a time-saving and cost-effective approach in preparing anti-leukaemia vaccines for the development of an active specific immunisation programme with AML-APC for patients with minimal residual disease.

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