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ORIGINAL PAPER

The Efficient Generation of Immunocompetent Dendritic Cells from Leukemic Blasts in Acute Myeloid Leukemia: A Local Experience

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Abstract Dendritic cells (DCs) are the most important antigen presenting cells with potentially useful applications in cancer immunotherapy. Leukemic cells of patients with acute myeloid leukemia (AML) could be differentiated to DC-like cells possessing the ability of stimulating anti-leukemic immune response. Despite obvious progress in DC-based immunotherapy, some discrepancies were reported in differentiation potential of AML blasts from all patients toward DC like cells. The present study, as a local experience, was set up to generate DCs from AML blasts of various subtypes. Leukemic Blasts from 16 Iranian AML patients were differentiated into functional DCs by culturing in the presence of rhGM-CSF, rhIL-4 and TNF-alpha for 8 days. The morphology, expression of key surface molecules and allostimulatory activity of resultant DCs were compared with primary blasts and cultured but cytokine untreated control groups. The pattern of angiotensin-converting enzyme (ACE) expression was used to approve the leukemic origin of generated DCs. Neo-expression or upregulation of DC-associated markers were occurred during culturing period in cytokine treated cells compared with primary blasts and cultured but cytokine untreated control groups: CD1a (63.22% vs. 3.22% and 11.79%), CD83 (41.27% vs. 0.11% and 0.70%), CD40 (15.17% vs. 0.00% and 0.04%), CD80 (49.96 vs. 0.02% and 0.32%), CD86 (56.49% vs. 0.50% and 5.71%) and HLA-DR (52.52% vs. 14.32% and 2.49%) respectively. The potency of generated DCs to induce allogeneic T cell proliferation increased significantly compared to pre and post culture control groups (27,533.4±2,548.3, 8,820.4± 1,639.4 and 3,200.35±976 respectively). The expression pattern of ACE in AML-DCs, blast cells and DCs derived from normal monocytes (7.93%, 1.28% and 74.97% respectively) confirmed the leukemic origin of DCs. Our data confirmed the generation of sufficient AML-derived cells with the properties of DCs in all cases. This potency of AML blasts, offers a useful route for active immunotherapy of AML patients.

Keywords Dendritic cell · Differentiation · Immunotherapy · Iranian AML patients · Leukemia

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Introduction

Acute myeloid leukemia represents a group of biologically heterogeneous clonal proliferation of undifferentiated myeloid precursor cells with diverse clinical and biological features [1]. Despite recent advances in therapeutic approaches such as intensive chemotherapy as well as stem cell transplantation, which have dramatically improved the treatment outcome for some patients [1, 2], the overall

survival rate is still low and only 40% to 50% of AML patients can be cured at best and many do not achieve long-term remission [3–5]. The presence of drug resistant residual leukemic cells after intensive chemotherapy is likely to contribute to relapse and therapeutic failure in AML patients. Indeed complete remissions of AML can be induced in most of patients, but relapses occur frequently [5–9]. Allogeneic bone marrow transplantation (allo-BMT) from an HLA matched sibling is now a well established treatment option [10, 11]. The efficacy of allogeneic transplantation may result partly from an allogeneic immune mechanism, known as the graft-versus-leukemia (GVL) effect. Indeed a higher rate of relapse occurs after syngeneic BMT compared to allo-BMT [12]. T cell depletion of the graft significantly increases the rate of leukemic relapse and donor lymphocyte infusion can re-induce a stable hematologic remission in one fourth of AML patients with relapsed disease [13]. Unfortunately, GVL reactivity is frequently accompanied by graft-versus-host disease (GVHD) which results from the lack of specificity of these unselected T cells for the leukemic cells. Therefore, it has been proposed that using cytotoxic T lymphocytes (CTL) with a relative specificity for the leukemic cells may have the advantage of maximizing the anti-leukemia effect whilst minimizing the probability of GVHD. This encourages the controlling or eradicating leukemic residual cells to prevent the relapse of disease [13–16]. However, leukemic cells evade host immune surveillance through several immunosuppressive mechanisms thus a promising approach is to manipulate the immune system by tumor antigen bearing antigen-presenting cells (APCs) that efficiently present tumor antigens to T cells to generate autologous anti-leukemic responses [17–20]. Native AML blasts, although present antigen in the context of class I and even class II MHC, but generally are deficient in the expression of critical co-stimulatory molecules, hence their ability to induce an effective immune response is impaired [21–23].

Dendritic cells are the most important APCs of the immune system that can stimulate quiescent, naive and memory T cells and play pivotal roles in the induction of both cell-mediated and humoral immune responses in vivo [24]. Unlike most tumors, AML blast cells can be induced to differentiate into DCs as APCs possessing the necessary properties to induce anti-leukemic cytotoxic activity. Immunotherapy of AML with DCs in this manner avoids many difficulties inherent in strategies involving DC therapy of cancers. Differentiation of blasts to DCs, obviate the need for the identification of a leukemia-specific antigen or the extra work to prepare that antigen for pulsing of DCs. In fact a good common tumor antigen without any limitation for immunotherapeutic approaches has not been identified in AML yet [25].

However, most of the works on DCs in leukemia have been in patients with CML [26–30], more recently, the generation of DCs from AML blasts has also been proven successful by some investigators [31–35]. Although because of some limitations, this approach is not feasible in all AML cases and generation of leukemic DCs is reported to be successful only in about 50–70% of patients [31, 36–40]. Another limitation is low expression of several key molecules that are related to adhesion, MHC, and the co-stimulatory pathway by the leukemic DCs. Recent reports also suggest that leukemic DCs display several functional defects. The prolonged culture period required for generation of these DCs also results in their reduced viabilities [21, 23, 41–43]. Thus, it is necessary to define an optimal condition that can be used for the generation of DCs from leukemic blasts of AML patients with a variety of FAB subtypes, peripheral blood blast counts and clinical histories, which may be adopted in widespread future clinical applications. In this study, as a local experience, we tried to set up an optimized condition for in vitro generation of leukemic DCs from AML cells of various subtypes.

Materials and Methods

Cell Culture Medium, Cytokines and Antibodies

Liquid RPMI-1640 medium (Gibco, BRL Life Technologies, Paisley, UK) was supplemented with 10% heat-inactivated FBS (Gibco, BRL Life Technologies, Paisley, UK), 100 U/ml penicillin (Sigma, St. Louis, MO, USA), 100 μg/ml of streptomycin (Sigma, St. Louis, MO, USA), 2 mM L-glutamine (Sigma, St. Louis, MO, USA), 50 μM 2-mercaptoethanol (Merck KGaA, Frankfurter, Germany), 1% NEAA (non-essential amino acid solution) (Sigma, St. Louis, MO, USA), and 1 mM sodium pyruvate (Sigma, St. Louis, MO, USA) and used as cell culture medium (this medium afterwards referred as complete RPMI medium).

Human recombinant GM-CSF (hrGM-CSF), human recombinant IL-4 (hrIl-4) and human recombinant TNF-α (hrTNF-α) were obtained from R&D Systems, Abingdon, UK.

The following conjugated mouse anti-human monoclonal antibodies were prepared from BD PharMigen, CA, USA: FITC-conjugated antibody specific for CD83 (IgG1, Clone HB15e), APC conjugated anti-CD14 (IgG2a, Clone M5E2), FITC-conjugated anti-CD1a (IgG1, Clone HI149), RPE-conjugated anti-CD40 (IgG1, Clone 5C3), FITC-conjugated anti-CD80 (IgG1, Clone L307.4), RPE-conjugated anti-CD86 (IgG1, Clone 2331, FUN-1), APC-conjugated anti-HLA-DR (IgG2a, Clone G46-46, L243), FITC-conjugated anti-CD3 (IgG1, Clone UCHT1), and their respective isotype controls. FITC-conjugated antibody specific for

CD143 (IgG1, Clone i2H5) was prepared from Serotec, Oxford, UK.

Blood Samples and Blast Preparation

Heparinized peripheral blood samples were collected from 16 AML patients (10 male and 6 females) referred to oncology centers in Tehran following informed consent. The patients' characteristics are shown in Table 1. The subtypes of AML were determined according to the FAB classification criteria [44, 45]. Three additional blood samples were also obtained from healthy volunteers for generation of normal DCs. The blood cells were separated on a Ficoll-Hypaque (Lymphoprep, Axis-Shield Oslo, Norway) density gradient and the mononuclear cell (MNC) fraction containing the malignant cells was harvested. This fraction was washed twice (400 and 250 g) to minimize platelets contamination. To increase the blast enrichment, only patients with blast counts more than 50% of blood nucleated cells were included in this study. The separated cells were then resuspended in complete RPMI medium and seeded in six-well culture plates (Nunc, Roskilde, Denmark) ($12–16\times10^6$ cells/well). We found that whole PBMC containing blasts was preferred to plastic adherent cells, as starting cell population for leukemic DC generation, through preliminary experiments using both kinds of cells.

Generation of Mature AML-DCs

AML derived dendritic cells were generated from blasts as described elsewhere with some modifications [31–35, 38]. Briefly, complete RPMI medium supplemented with 1,000 IU/ml recombinant human IL-4 and 1,000 IU/ml recombinant human GM-CSF was added to enriched blasts and the cells were incubated at 37°C and 5% CO_2. The cytokines were refreshed every 3 days. In case of an overgrown culture, cell culture wells were split and fresh medium and cytokines were added. On the day 6 of the culture, the appeared aggregates of immature AML-DCs were stimulated with 500 IU/ml TNF-α for two other days. The optimized doses of GM-CSF, IL-4 and TNF-α were determined through culturing of the cells in mediums supplemented with graded concentrations of cytokines. Matured cells were harvested at day 8 for subsequent experiments. In a cytokine untreated culture setting, enriched blasts from each patient were cultured in the absence of any cytokine. These cultures served as controls for cytokine-treated cells.

Immunophenotyping by Flow Cytometry

To assess the differentiation and maturation of AML-DCs, the expression of cell surface markers were analyzed using conjugated monoclonal antibodies specific for CD83, CD14, CD1a, HLA-DR, CD40, CD80 and CD86 and their respective isotype controls. At the end of culturing period, the cells were harvested by replacement of culture medium with 4 ml phosphate-buffered saline (PBS) containing 0.5 mM EDTA. The cells were collected by flushing after 10 min incubation at 37°C and 5% CO_2 followed by centrifugation and washing by ice cold FACS buffer (PBS containing 0.1% NaN3, 5 mM EDTA and 2% FCS). The cells were then aliquoted at 10^5 cells per FACS tube (Falcon 2054, Becton Dickinson, NJ, USA). Normal mouse serum at a final concentration of 1% was added to each tube and the tubes were incubated for 30 min on ice. One microliter of each conjugated mAb was added to the cell's

Table 1 AML patients' characteristics

Patient no.	Sex	Age (years)	FAB subtype	Percent blasts	Clinical status
1	M	26	M2	78	2nd relapse per BMT
2	M	52	M2	75	Relapse (secondary)
3	M	34	M2	90	Refractory pre BMT
4	F	23	M1	73	Newly diagnosed (de novo)
5	M	39	M2	68	Relapse (secondary)
6	F	42	M4	60	Newly diagnosed (de novo)
7	F	40	M3	55	Newly diagnosed (de novo)
8	M	27	M3	64	Newly diagnosed (de novo)
9	F	4	M1	94	Newly diagnosed (de novo)
10	M	34	M3	71	Newly diagnosed (de novo)
11	F	11	M3	59	Newly diagnosed (de novo)
12	M	56	M5	65	2nd relapse
13	M	45	M5	70	Refractory
14	F	31	M4	54	Newly diagnosed (de novo)
15	M	64	M2	56	Refractory
16	M	63	M3	53	Newly diagnosed (de novo)

pellet after washing and the tubes were incubated on ice in the dark for 45 min. Cells were then washed and resuspended in 300–500 μl of FACS buffer and analyzed immediately or fixed by the addition of 1.0% paraformaldehyde. Data acquisition was carried out on a FACS Calibur flow cytometer (Becton-Dickinson, CA, USA) and analyzed using CellQueste software. Data were expressed as percentage of positive cells in comparison to the cells stained with isotype controls. Purity of T cells was identified using anti-CD3 monoclonal antibody. We also used the pattern of angiotensin-converting enzyme (CD143) expression to approve the leukemic origin of generated DCs. To really control the differentiation of primary cell population toward dendritic cell, the phenotypic marker expression were compared on cytokine treated blasts with primary blasts and control groups that were cultured in the absence of supplemented cytokines (cytokine untreated culture setting).

Morphologic Studies

The morphology of cultured cells was analyzed under an inverted microscope during the culture period on days 0, 3, 6 and 8. Cytospin slides were also prepared from some cultures and following fixation with methanol, the cells were stained with Giemsa and analyzed by light microscopy.

Proliferation Assay

Allostimulatory capacity of cells in test (day 8 AML-DCs) and control groups (uncultured fresh AML blasts and cytokine untreated cultured cells) was compared through allogeneic mixed leukocyte reaction (MLR). Responder T cells were prepared from one unrelated healthy and fixed donor. For this purpose lymphocytes were enriched from PBMC fraction as non-adherent (floating) cell population by incubation of cells in six-well culture plates at 37°C and 5% CO_2 for 2 h followed by gentle swirl and elutriation with warm RPMI-1640. T cells were more enriched from non-adherent cells using a sterile nylon wool column. Enriched T lymphocytes were seeded into 96-well round bottomed microplates at 1×10^5 cells/well as responder cells. Irradiated (3,000 rad) fresh AML blasts (pre culture control group), cytokine treated cells (AML-DCs) and cytokine untreated cultured cells (post culture control group) were added in graded numbers (Stimulator/Responder ratios: 1:20, 1:10 and 1:5) and co-cultured for 5 days at 37°C and 5% CO_2. T cells or stimulator cells alone were used as background controls. Maximal stimulation of the T-cell population was also determined by culturing of T cells with PHA (5 μg/ml); 1 μCi of ^{3}H-thymidine (Amersham Pharmacia Biotech, Buckinghamshire, UK) was added to each well for the last 18 h of culture and cell proliferation was measured through ^{3}H-thymidine incorporation by a liquid scintillation β-counter (Wallac, Turku, Finland). All tests were done in triplicate and the results were expressed as the mean counts per minute (cpm) of triplicate cultures.

Statistical Analysis

All experiments were repeated with at least six separate AML samples. The normal distribution of data was confirmed by the Kolmogorov–Smirnov test, then the Paired-Samples T test was used for statistical analysis of the differences between experimental groups and the differences at p values <0.05 were regarded statistically significant. Our results are presented here as $\overline{X} \pm$ SE of the obtained data from separate experiments.

Results

Cell Counts and Viabilities

Table 2 shows total harvested cell counts after 8 days culture in the presence or absence of cytokines compared to the absolute number of cells used to initiate the culture. Although cell counts and viabilities varied between samples, proliferation or increase in cell numbers in cytokine treated cells in comparison to the cells cultured in the absence of cytokine or number of cells in the beginning of culture was found only in patients 10, 14–16. Except for case 11, the viability of cytokine-treated cells was maintained over 84% in all conditions (90.4±1%) but the average of viability in cytokine untreated culture setting was 78.93± 2.83% (Table 2).

Phenotypic Analysis

AML blasts were differentiated into leukemic DCs by culturing in a medium supplemented with hrGM-CSF, hrIL-4 and hrTNF-alpha. To confirm the differentiation of primary blasts, expression of key surface molecules on resultant DCs were compared with primary fresh blasts and cultured but cytokine untreated control groups. For this purpose, cells from cytokine supplemented group and pre and post culture control groups were stained with the same panel of conjugated monoclonal antibodies and the percentage of positive cells for each molecule was assessed using flow cytometric analysis (Figs. 1 and 2). Our results showed that cytokine treated cells express significantly higher levels of DC specific markers compared to pre and post culture control groups ($p<0.03$ in all cases). Although the initiating populations of AML peripheral blood cells

Table 2 Details for cell counts and viabilities related to each sample

Patient no.	D0 WBC count per micro liter[a]	D0 prepared PBMC count[b]	D8 cultured count (w ck)[c]	D8 cultured count (wo ck)[d]	D0 viability[e]	D8 viability (w ck)[f]	D8 viability (wo ck)[g]
1	54×10^3	16×10^6	11.7×10^6	10.5×10^6	93	90	89
2	45×10^3	15×10^6	10×10^6	8×10^6	95	90	78
3	43×10^3	15×10^6	11.8×10^6	12×10^6	96	96	90
4	12.1×10^3	16×10^6	10.9×10^6	11.1×10^6	91	86	84
5	3.54×10^3	15×10^6	10.2×10^6	9×10^6	97	84	66
6	10.8×10^3	15×10^6	11.8×10^6	12×10^6	96	88	86
7	5.1×10^3	15×10^6	12.8×10^6	13.3×10^6	98	96	96
8	3.5×10^3	15×10^6	10×10^6	9.4×10^6	95	93	90
9	162.29×10^3	15×10^6	11×10^6	9×10^6	99	90	84
10	2.9×10^3	4.5×10^6	5.2×10^6	4.4×10^6	99	90	82
11	4×10^3	5×10^6	2.4×10^6	2.5×10^6	98	73	61
12	5.9×10^3	5×10^6	3.8×10^6	35×10^6	95	86	79
13	13.1×10^3	4.5×10^6	4×10^6	2.3×10^6	98	90	72
14	23.2×10^3	4.5×10^6	6.8×10^6	3.2×10^6	96	92	64
15	6.5×10^3	4.5×10^6	6.3×10^6	4.1×10^6	95	97	83
16	2.3×10^3	4.5×10^6	5.8×10^6	3.6×10^6	92	88	59

[a] Absolute number of patient's WBCs from whom AML cells were collected
[b] Absolute number of PBMCs used to initialize the culture
[c] Total harvested cell counts after 8 days culture with cytokines
[d] Total harvested cell counts after 8 days culture without cytokines
[e] The viability of fresh patient's PBMCs at day of sample collection
[f] The viability of cytokine supplemented cells after harvesting on day 8
[g] The viability of untreated cultured cells after harvesting on day 8

containing the blasts were heterogeneous but a mature DC phenotype could be elicited from all samples.

Allostimulatory Capacity of the Cultured Cells (Proliferation Assay)

To investigate the T cell immunostimulatory capacity of leukemic DCs, their ability to stimulate allogeneic T cells in MLR assay was compared with that of the original blasts and cytokine untreated cultured cells. Firstly the purified T cells were co-cultured with graded numbers of irradiated AML blasts or leukemic DCs as stimulator cells to obtain the best ratio of stimulator to responder cells. Our results showed that DC like cytokine treated cells are more potent stimulator of T cells compared to their primary blasts in all tested ratios of stimulator to responder cells. But the difference was more evident when a higher ratio of stimulator cells was used (Fig. 3). Using the 1:5 ratio of

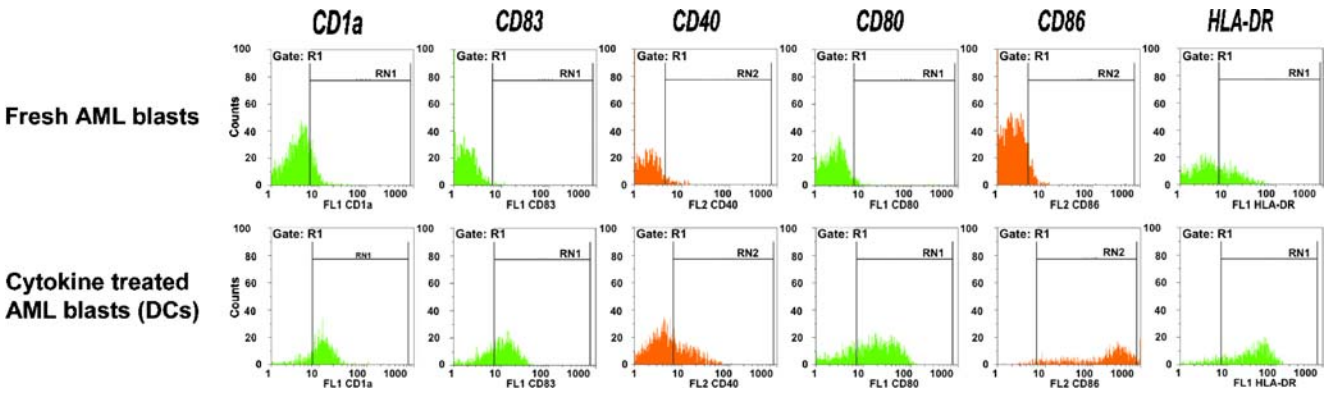

Fig. 1 Phenotypic expression of DC markers. To confirm the differentiation of primary blasts to DC like cells, the expression of key surface molecules was compared on fresh AML blasts and leukemic DCs derived from AML blasts by culturing in a medium supplemented with hrGM-CSF, hrIL-4 and hrTNF-alpha. The results shows flow cytometric analysis of sample 13 as a representative of all samples. Isotype matched monoclonal antibodies were used as isotype controls for each cell population separately

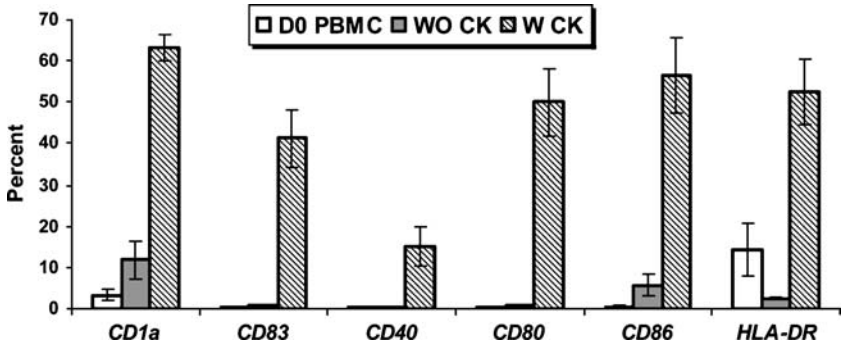

Fig. 2 The phenotypic analysis of cell surface markers. The AML blasts were treated with a cocktail of cytokines as mentioned in the text. The day 8 cells (W CK) were harvested and expression of key surface molecules (percentage of positive cells) were compared with primary blasts (D0 PBMC) and cultured but cytokine untreated cells (WO CK). The data show the mean±SE of 16 separate experiments

stimulator to responder, the potency of T cell stimulation was tested in all cases. For this purpose the irradiated primary AML blasts, leukemic DC like cells and cultured but cytokine untreated blasts were co-cultured with allogeneic T cells in MLR assay. T cells alone, stimulator cells alone and PHA stimulated T cells were also used as controls. Proliferation of the cells was measured following 5 days incubation, through ^{3}H-thymidine incorporation. Our results showed that leukemic DCs were potent stimulators of allogeneic T cells whereas freshly uncultured AML blasts as pre cultured control group and cytokine untreated cultured cells as post cultured control group had a much poorer capacity to stimulate the T cells (p=0.01 and 0.02 respectively) (Fig. 4). Nonetheless, T cells were stimulated to proliferate in response to fresh AML blasts in a greater extent than in response to cytokine untreated cultured cells.

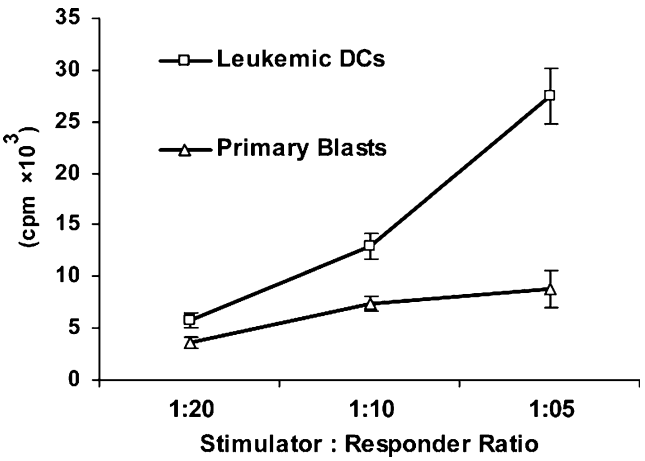

Fig. 3 The effect of stimulator to responder ratio in MLR assay. To obtain the best ratio of stimulator to responder cells graded numbers of irradiated primary blasts from AML patients and their respective leukemic DCs were cultured with fixed number of allogeneic T cells and T cell proliferation was measured through ^{3}H-thymidine incorporation. The data show the mean±SE of counts per minutes (cpm) of six separate samples

Cytokine Treatments Induced Morphologic Changes in AML Blasts

Morphological studies showed that cytokine treatment induced morphologic changes toward typical DC morphology. On the day 4 of cytokine supplemented culture, a large number of cells had ruffle-like membrane structures and a small number of cells developed dendrite-like projections on their surface. On the day 8, more cells demonstrated a dendritic-like appearance with irregular morphology, large amounts of cytoplasm, increased number of cytoplasmic projections and many long and fine dendrites (Fig. 5)

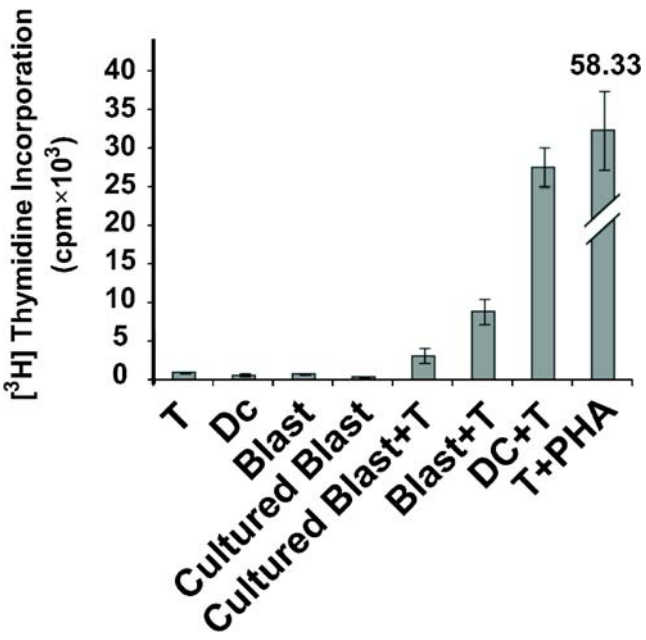

Fig. 4 T cell proliferation assay. The AML derived DCs were cultured with allogeneic T cells (DC+T) at ratio of 1:5 and their capacity to induce T cell responses was compared with primary blasts (Blast+T) and cultured but cytokine untreated blasts (cultured blast+T). T cells alone (T), all kinds of stimulator cells alone (DC, Blast, cultured Blast) and PHA stimulated T cells (T+PHA) were used as controls. The results show the mean±SE of counts per minutes (cpm) of cell populations (n=16)

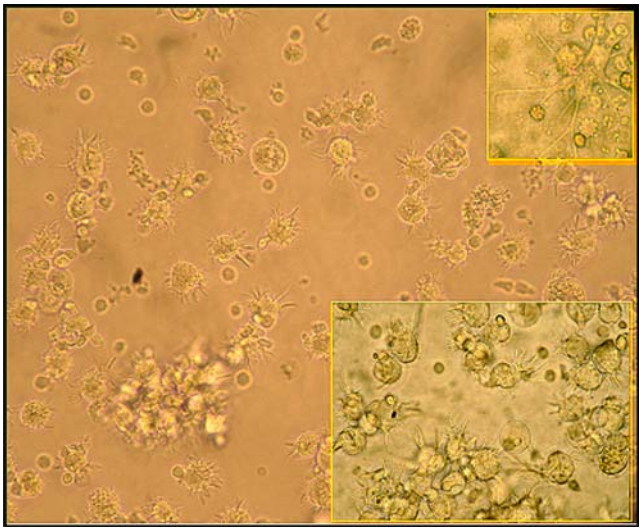

Fig. 5 Morphological characteristics of DCs derived from AML blasts. Cytokine treatment of AML blast induced morphologic changes toward typical DC morphology. The figure shows the cells of three separate samples following 8 days of culturing in the presence of cytokines with dendritic-like appearance, irregular shapes and many long and fine dendrites

whereas, freshly isolated blasts displayed a distinct morphology typical of blast cells without any dendrite. After 8 days of culture in the absence of cytokines, the cells still maintained their blast-like appearance. Interestingly, in some cases, AML blasts exhibited proliferating cell clusters of various sizes following cytokine treatments that were mostly semi-adherent to the plastic surface.

Quantitation of DCs Yield for Evaluation of Culturing Protocol

The yield and number of leukemic DCs generated in each culture was difficult to calculate because of several factors, such as absence of a precise phenotypic marker to define the DC in human, various morphological changes during cultures of different leukemic samples, divers allostimulatory capacity of resultant cells and finally inconsistency of the obtained results from morphologic, immunophenotypic and functional properties in some cases. Regarding the morphologic yield of cultures, although this feature is insufficient to define a DC, we named cells DC initially because of their distinctive morphology and counted the percentage of them in each culture. Although a typical dendritic morphology often could be seen throughout the culture period, even as early as 48 h after cytokine treatment, but morphologic analysis at the end of culture, showed in all cases a clear population with a dendritic-like appearance varying from 50% (case 11) to 95% (case 13) (mean=75.3%) of the cultured cells.

Immunophenotypically, cells that combined high-level expression of MHC class II after culture and expression of at least one of CD86, CD1a and CD83 without expression of CD14 were defined as leukemic DCs. Thereby based on this immunophenotypic profile, our yield in leukemic DC generation was 52.5% (range 33–79%). Although there was no correlation between morphologic and phenotypic yield in some cases, but with respect to leukemic nature of primary cells and biologically heterogeneous blast cells in different AML patients, our yield as we anticipated, will be more than 63%.

Finally based on T cell stimulating capacity, in all cases we succeeded to obtain DC like cells with relatively higher T cell stimulatory potential than primary leukemic blasts.

Derivation of DCs from the Leukemic Blasts

In order to determine whether the cultured DCs were originated from the malignant clone (blasts), a new phenotypic analysis based on ACE expression proposed by Sergei M. Danilov et al. [46] was performed. The mean percentage of positive cells for ACE molecule in six separate experiments on fresh and cytokine supplemented cultured blasts were 1.28±0.56% and 7.93±2.52% respectively (Fig. 6). We also compared ACE expression in DCs derived from patient's blasts and DCs derived from normal monocytes of healthy volunteers. In contrast to AML blast derived DCs, DCs obtained from monocytes of healthy donors produced high levels of ACE (74.97±5.47%, n=3). Thus, the negligible expression of ACE on AML-DCs proved their leukemic origin.

Discussion

Whilst great advances have been made in the treatment of AML patients, significant challenges still remain, including low overall survival rate, drug resistance feature of residual leukemic cells and the toxicity resulting from commonly used chemotherapeutic regimens. Although chemotherapy is capable of inducing disease remission in 60–85% of AML patients, long-term survival is achieved by less than 40% of individuals and relapses occur frequently. Hence, there is a great need for the development of novel therapeutic approaches such as immunotherapy which, ideally, would target the leukemic cells specifically and circumvent the present limitations [4]. However, generation of an efficient autologous immune response against leukemic cells appears much difficult to build. From a more general point of view, the major problem of tumor immunotherapy is the induction of tolerance by cancer cells. This tolerance appears to be partly mediated via a defect in antigen presentation. Indeed one of the major reasons for non-efficient recognition of AML blasts by T lymphocytes in vivo is that 70% of the blasts do not express the costimulatory molecules which are necessary for

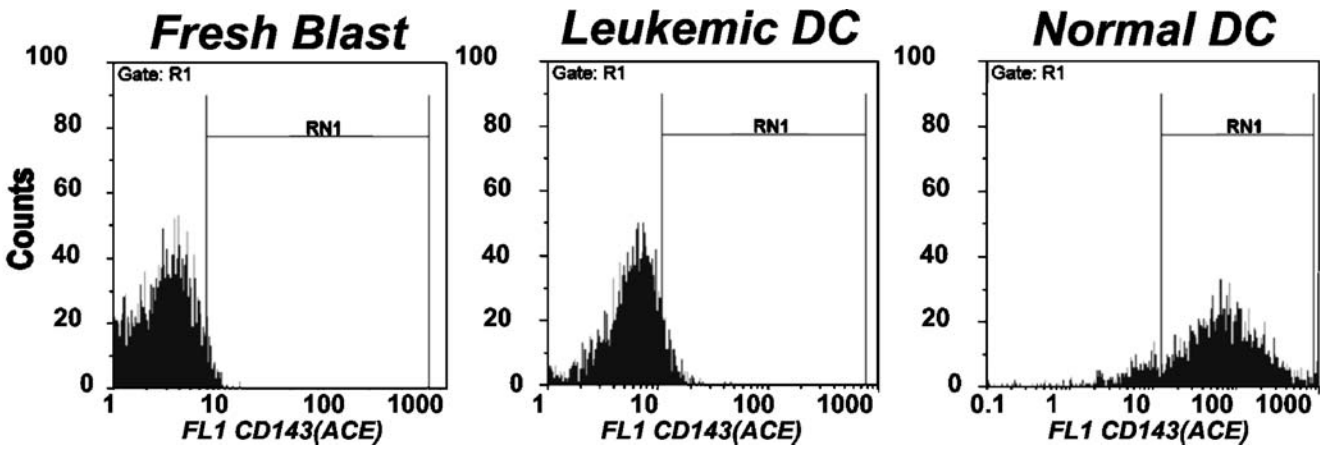

Fig. 6 Flow cytometric analysis of surface expression of ACE (CD143). In order to determine the origin of cultured DCs, the expression of ACE on primary blasts, leukemic DCs and DCs originated from normal monocytes were compared using flow cytometric analysis. Each cell sample had its own isotype control. The data are representative of six separate experiments. Figure shows negligible expression of ACE on leukemic DCs. However normal monocytes derived DCs express high level of ACE

efficient T cell activation [17, 18, 21, 40, 41]. In AML, blast cells can be differentiated into DCs or DC like cells with upregulated expression of co-stimulatory molecules and increased immunogenicity. The major advantage of such an approach is the fact that all of the putative leukemia-specific antigens would be presented along with co-stimulatory signals by the modified AML cells and therefore the probability of generating specific CTLs would be maximized. However this strategy is also hampered by several limitations, including some defects in their functional capacities, low expression of molecules related to adhesion, MHC, and the co-stimulatory pathway and excessive mortality of these leukemic DCs. Another limitation lies in the difficulties associated with the generation of leukemic DCs in some patients. Therefore, it is necessary to define the optimized conditions of cultures in terms of successful leukemic DC generation which may be used in future clinical applications. In this study, as a local experience, we tried to optimize the in vitro generation of DCs from leukemic cells of Iranian AML patients from various subtypes.

Indeed, we investigated the ability of leukemic blasts to obtain DC-like characteristics upon culture in the presence of the most commonly used cytokine combination (GM-CSF + IL-4 + TNF-α). Our results showed that the cells from all AML cases can be converted to leukemic DCs in some extent. In fact in the majority of the AML patients with a variety of FAB subtypes, peripheral blood blast counts and clinical histories, we succeeded in generating a substantial proportion of leukemic cells with typical morphology, phenotype, and immunostimulatory capacity of normal DCs. Our findings are consistent with some previous studies [31–35]. However, based on some other in vitro studies, it has been reported that only in about 50–70% of AML patients, the blasts are successfully capable to differentiate toward dendritic cells [36, 38, 39].

We identified the optimal conditions for the induction of potent leukemic DCs from AML blast cells. Comparison between two different culturing periods (8 and 14 days) according to the phenotypic assay revealed that although in some experiments leukemic DCs like cells were apparent as early as day 2 of culture, the optimal culture duration for their generation was 8 days (data not shown). We also found that the least combination of cytokine is GM-CSF, IL-4 and TNF-alpha that were effective with most patients. Although in some experiments we used IL-3 and SCF together with the above combination, but there was no significant difference in expression of phenotypic markers between two groups (data are not shown). Also addition of cytokines in a fractionated schedule was preferable compared to their addition in the beginning of culture by measurement of respective cells recovery and viability. Therefore, because of relatively limited half-life of cytokines, dividing of their total dose into two smaller, equal doses delivered over the period of culturing is recommended.

It is well established that IL-4, in combination with GM-CSF, promotes the differentiation of DCs from their precursors, especially from those of myeloid lineages. The role of IL-4 is more critical when used to generate DCs from human peripheral blood monocytes, where most of the cells do not differentiate into mature DC with GM-CSF alone. Although suppression of myeloid differentiation along the macrophage pathway by IL-4 has been reported, the precise mechanism by which IL-4 promoted the differentiation of DCs is not well understood [47]. Although SCF itself doesn't induce a DC phenotype, preliminary experiments had shown that it is necessary for cell proliferation and viability, especially under serum-free

conditions [48]. IL-3 can also support DC growth in vitro, but has been neglected for some years.

Caux C et al. showed that IL-3 in combination with GM-CSF enhance DC differentiation [49]. Mackensen et al. also reported that CD34+ cells could be used as a major source of DCs for immunotherapy when cultured in cytokine cocktails including SCF, IL-3, IL-6, IL-4, GM-CSF [50]. As the blasts from many AML patients are CD34 positive, and because of the above findings, we postulated that addition of SCF and IL-3 to the conventional cytokines, would have beneficial effects in our differentiation system. However we didn't observe any significant difference following addition of them to the cytokine cocktail.

TNF-α has traditionally been used from the initiation of the culture or very early in culture period to induce DC maturation from progenitor cells [51–53]. In our culture setting, the addition of TNF-α at last 48 h of culture was sufficient to induce maturation of DCs.

The immunostimulatory capacity of cytokine treated cultured blasts was much more potent compared to fresh AML blasts (approximately 2.5 fold) and cytokine untreated cultured blasts (approximately 12.5 fold) especially at higher leukemic DC/T cell ratios. Cytokine treated cultured blasts also expressed higher levels of CD83, CD1a, CD40, CD80, CD86 and HLA-DR compared to fresh AML blasts and cytokine untreated cultured blasts. The phenotypic results presented here are similar to those we previously reported for the development of DCs from normal monocytes [54]. These results together with the morphological results confirmed the competency of AML blasts cultured in a medium supplemented with appropriate cytokines to serve as potent APCs. In our best condition, the yield of leukemic DC generation based on phenotypic and morphologic criteria was 52%, 75% respectively. However, in all cases we succeeded to obtain DC like cells with relatively higher T cell stimulatory potential than primary leukemic blasts.

To show the leukemic origin of dendritic like cells, we analyzed ACE expression profile. ACE (CD143 or kininase II), a zinc-metalloprotease with a broad substrate specificity, is generally known as an important regulator of blood pressure and fluid and electrolyte homeostasis. The differentiation of monocytes to macrophages and DCs is accompanied by a dramatic increase in its expression. ACE activity is 50-fold higher in macrophages and 150-fold higher in DCs than in monocytes. It is reported that normal DCs express the highest level of ACE per cell among the all human cells tested. In contrast, DCs derived from AML blasts fail to up-regulate ACE and do not show an elevated level of ACE during their DC-type differentiation, although they acquired DC markers such as CD80, CD40, and CD86 upon cytokine or calcium ionophore treatment. Interestingly, a lack of ACE up-regulation is noted for cells derived from patients with various AML subtypes, characterized by different genetic abnormalities. Therefore ACE expression can be used as a marker to functionally distinguish DCs generated from normal monocytes and leukemic blast cells [46]. In our investigation the leukemic origin of resultant DCs confirmed based on the negligible expression of ACE on cultured DCs. However, in contrast to leukemic DCs, normal monocytes derived DCs expressed high level of ACE.

In conclusion, the generation of AML-APC by an 8-days culture method resulted in highly potent, mature leukemic DCs with T cell stimulatory capacity. Such an approach could be feasible for vaccination purposes in many AML patients. Immunotherapy with DCs derived in this manner avoids the need for the identification of a leukemia-specific antigen or for the extra work to prepare antigen-pulsed DCs for clinical protocols. Our results show that the cells from AML patients from various subtypes can be converted to some acceptable extent to leukemic DCs that ease the design of future clinical trials.

All together, we demonstrated the feasibility and good yield of AML-DC generation in Iranian leukemic patients that is comparable to and consistent with other studies [31–35, 38] and thereby strongly offer the need of more extensive investigations and initiation of controlled clinical studies about safety, feasibility and benefit of AML-DC vaccine therapy in Iranians patients as a new treatment in the near future.

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References

1. Wang ZY, Chen Z (2008) Acute promyelocytic leukemia: from highly fatal to highly curable. Blood 111:2505–2515
2. Mato AR, Morgans A, Luger SM (2008) Novel strategies for relapsed and refractory acute myeloid leukemia. Curr Opin Hematol 15:108–114
3. Capra M, Vilella L, Pereira WV, Coser VM, Fernandes MS, Schilling MA, Almeida D, Gross M, Leite M, Hellwig T, Natchigal G, Zelmanowicz A, Paskulin G, Neumann J, Silla L (2007) Estimated number of cases, regional distribution and survival of patients diagnosed with acute myeloid leukemia between 1996 and 2000 in Rio Grande do Sul, Brazil. Leuk Lymphoma 48:2381–2386
4. Pulte D, Gondos A, Brenner H (2008) Improvements in survival of adults diagnosed with acute myeloblastic leukemia in the early 21st century. Haematologica 93:594–600
5. Yanada M, Garcia-Manero G, Borthakur G, Ravandi F, Kantarjian H, Estey E (2008) Relapse and death during first remission in acute myeloid leukemia. Haematologica 93:633–634

6. Kim HR, Shin JH, Lee JN, Lee EY (2007) Clinical significance of quantitation of WT1 gene expression for minimal residual disease monitoring of acute myelogenous leukemia. Korean J Lab Med 27:305–312
7. Ho MM, Hogge DE, Ling V (2008) MDR1 and BCRP1 expression in leukemic progenitors correlates with chemotherapy response in acute myeloid leukemia. Exp Hematol 36:433–442
8. Hussein K, Jahagirdar B, Gupta P, Burns L, Larsen K, Weisdorf D (2008) Day 14 bone marrow biopsy in predicting complete remission and survival in acute myeloid leukemia. Am J Hematol 83(6):446–450
9. Ommen HB, Nyvold CG, Braendstrup K, Andersen BL, Ommen IB, Hasle H, Hokland P, Ostergaard M (2008) Relapse prediction in acute myeloid leukaemia patients in complete remission using WT1 as a molecular marker: development of a mathematical model to predict time from molecular to clinical relapse and define optimal sampling intervals. Br J Haematol 141(6):782–791
10. Aversa F (2008) Haploidentical haematopoietic stem cell transplantation for acute leukaemia in adults: experience in Europe and the United States. Bone Marrow Transplant 41:473–481
11. Hamadani M, Awan FT, Copelan EA (2008) Hematopoietic stem cell transplantation in adults with acute myeloid leukemia. Biol Blood Marrow Transplant 14:556–567
12. Fefer A, Sullivan KM, Weiden P, Buckner CD, Schoch G, Storb R, Thomas ED (1987) Graft versus leukemia effect in man: the relapse rate of acute leukemia is lower after allogeneic than after syngeneic marrow transplantation. Prog Clin Biol Res 244:401–408
13. Huang XJ, Wang Y, Liu DH, Xu LP, Chen H, Chen YH, Han W, Shi HX, Liu KY (2008) Modified donor lymphocyte infusion (DLI) for the prophylaxis of leukemia relapse after hematopoietic stem cell transplantation in patients with advanced leukemia—feasibility and safety study. J Clin Immunol 28:390–397
14. Slavin S, Ackerstein A, Naparstek E, Or R, Weiss L (1990) The graft-versus-leukemia (GVL) phenomenon: is GVL separable from GVHD? Bone Marrow Transplant 6:155–161
15. Ge X, Brown J, Sykes M, Boussiotis VA (2008) CD134-allodepletion allows selective elimination of alloreactive human T cells without loss of virus-specific and leukemia-specific effectors. Biol Blood Marrow Transplant 14:518–530
16. Hartwig UF, Nonn M, Khan S, Link I, Huber C, Herr W (2008) Depletion of alloreactive donor T lymphocytes by CD95-mediated activation-induced cell death retains antileukemic, antiviral, and immunoregulatory T cell immunity. Biol Blood Marrow Transplant 14:99–109
17. Buggins AG, Milojkovic D, Arno MJ, Lea NC, Mufti GJ, Thomas NS, Hirst WJ (2001) Microenvironment produced by acute myeloid leukemia cells prevents T cell activation and proliferation by inhibition of NF-kappaB, c-Myc, and pRb pathways. J Immunol 167:6021–6030
18. Narita M, Takahashi M, Liu A, Nikkuni K, Furukawa T, Toba K, Koyama S, Takai K, Sanada M, Aizawa Y (2001) Leukemia blast-induced T-cell anergy demonstrated by leukemia-derived dendritic cells in acute myelogenous leukemia. Exp Hematol 29:709–719
19. Orleans-Lindsay JK, Barber LD, Prentice HG, Lowdell MW (2001) Acute myeloid leukaemia cells secrete a soluble factor that inhibits T and NK cell proliferation but not cytolytic function—implications for the adoptive immunotherapy of leukaemia. Clin Exp Immunol 126:403–411
20. Duluc D, Delneste Y, Tan F, Moles MP, Grimaud L, Lenoir J, Preisser L, Anegon I, Catala L, Ifrah N, Descamps P, Gamelin E, Gascan H, Hebbar M, Jeannin P (2007) Tumor-associated leukemia inhibitory factor and IL-6 skew monocyte differentiation into tumor-associated macrophage-like cells. Blood 110:4319–4330
21. Hirano N, Takahashi T, Ohtake S, Hirashima K, Emi N, Saito K, Hirano M, Shinohara K, Takeuchi M, Taketazu F, Tsunoda S, Ogura M, Omine M, Saito T, Yazaki Y, Ueda R, Hirai H (1996) Expression of costimulatory molecules in human leukemias. Leukemia 10:1168–1176
22. Costello RT, Mallet F, Sainty D, Maraninchi D, Gastaut JA, Olive D (1998) Regulation of CD80/B7-1 and CD86/B7-2 molecule expression in human primary acute myeloid leukemia and their role in allogenic immune recognition. Eur J Immunol 28:90–103
23. Bruserud O (1999) Acute myelogenous leukemia blasts as accessory cells during T lymphocyte activation: possible implications for future therapeutic strategies. Leukemia 13:1175–1187
24. Adams S, O'Neill DW, Bhardwaj N (2005) Recent advances in dendritic cell biology. J Clin Immunol 25:87–98
25. Greiner J, Dohner H, Schmitt M (2006) Cancer vaccines for patients with acute myeloid leukemia—definition of leukemia-associated antigens and current clinical protocols targeting these antigens. Haematologica 91:1653–1661
26. Heinzinger M, Waller CF, von den Berg A, Rosenstiel A, Lange W (1999) Generation of dendritic cells from patients with chronic myelogenous leukemia. Ann Hematol 78:181–186
27. Eisendle K, Lang A, Eibl B, Nachbaur D, Glassl H, Fiegl M, Thaler J, Gastl G (2003) Phenotypic and functional deficiencies of leukaemic dendritic cells from patients with chronic myeloid leukaemia. Br J Haematol 120:63–73
28. Takahashi T, Tanaka Y, Nieda M, Azuma T, Chiba S, Juji T, Shibata Y, Hirai H (2003) Dendritic cell vaccination for patients with chronic myelogenous leukemia. Leuk Res 27:795–802
29. Tong XM, Jin J, Qian WB, Meng HT, Xue YQ (2005) Biological features of dendritic cells derived from chronic myeloid leukemia cells in vitro. Zhejiang Da Xue Xue Bao Yi Xue Ban 34:348–352, 357
30. Zhang YF, Wu CY, Zhang LS, Chai Y (2006) In vitro cytokines-induced differentiation in mononuclear cell derived dendritic cells from chronic myeloid leukemia. Zhongguo Shi Yan Xue Ye Xue Za Zhi 14:137–141
31. Kohler T, Plettig R, Wetzstein W, Schmitz M, Ritter M, Mohr B, Schaekel U, Ehninger G, Bornhauser M (2000) Cytokine-driven differentiation of blasts from patients with acute myelogenous and lymphoblastic leukemia into dendritic cells. Stem Cells 18:139–147
32. Oehler L, Berer A, Kollars M, Keil F, Konig M, Waclavicek M, Haas O, Knapp W, Lechner K, Geissler K (2000) Culture requirements for induction of dendritic cell differentiation in acute myeloid leukemia. Ann Hematol 79:355–362
33. Claxton D, Choudhury A (2001) Potential for therapy with AML-derived dendritic cells. Leukemia 15:668–669
34. Yan KH, You SG, Bian SG, Ma GJ, Ge W, Ma S, Liu SH, Zhao CH (2003) Dendritic cells (DC) induced from acute myeloid leukemia (AML) cells with cytokine cocktails. Zhonghua Xue Ye Xue Za Zhi 24:365–368
35. Houtenbos I, Westers TM, Ossenkoppele GJ, van de Loosdrecht AA (2006) Leukemia-derived dendritic cells: towards clinical vaccination protocols in acute myeloid leukemia. Haematologica 91:348–355
36. Robinson SP, English N, Jaju R, Kearney L, Knight SC, Reid CD (1998) The in-vitro generation of dendritic cells from blast cells in acute leukaemia. Br J Haematol 103:763–771
37. Choudhury BA, Liang JC, Thomas EK, Flores-Romo L, Xie QS, Agusala K, Sutaria S, Sinha I, Champlin RE, Claxton DF (1999) Dendritic cells derived in vitro from acute myelogenous leukemia cells stimulate autologous, antileukemic T-cell responses. Blood 93:780–786
38. Brouwer RE, van der Hoorn M, Kluin-Nelemans HC, van Zelderen-Bhola S, Willemze R, Falkenburg JH (2000) The generation of dendritic-like cells with increased allostimulatory

function from acute myeloid leukemia cells of various FAB subclasses. Hum Immunol 61:565–574
39. Harrison BD, Adams JA, Briggs M, Brereton ML, Yin JA (2001) Stimulation of autologous proliferative and cytotoxic T-cell responses by "leukemic dendritic cells" derived from blast cells in acute myeloid leukemia. Blood 97:2764–2771
40. Roddie PH, Horton Y, Turner ML (2002) Primary acute myeloid leukaemia blasts resistant to cytokine-induced differentiation to dendritic-like leukaemia cells can be forced to differentiate by the addition of bryostatin-1. Leukemia 16:84–93
41. Almand B, Resser JR, Lindman B, Nadaf S, Clark JI, Kwon ED, Carbone DP, Gabrilovich DI (2000) Clinical significance of defective dendritic cell differentiation in cancer. Clin Cancer Res 6:1755–1766
42. Bello-Fernandez C, Stasakova J, Renner A, Carballido-Perrig N, Koening M, Waclavicek M, Madjic O, Oehler L, Haas O, Carballido JM, Buschle M, Knapp W (2003) Retrovirus-mediated IL-7 expression in leukemic dendritic cells generated from primary acute myelogenous leukemias enhances their functional properties. Blood 101:2184–2190
43. Orsini E, Guarini A, Chiaretti S, Mauro FR, Foa R (2003) The circulating dendritic cell compartment in patients with chronic lymphocytic leukemia is severely defective and unable to stimulate an effective T-cell response. Cancer Res 63:4497–4506
44. Van der Reijden HJ, van Rhenen DJ, Lansdorp PM, van't Veer MB, Langenhuijsen MM, Engelfriet CP, von dem Borne AE (1983) A comparison of surface marker analysis and FAB classification in acute myeloid leukemia. Blood 61:443–448
45. Neame PB, Soamboonsrup P, Browman GP, Meyer RM, Benger A, Wilson WE, Walker IR, Saeed N, McBride JA (1986) Classifying acute leukemia by immunophenotyping: a combined FAB-immunologic classification of AML. Blood 68:1355–1362
46. Danilov SM, Sadovnikova E, Scharenborg N, Balyasnikova IV, Svinareva DA, Semikina EL, Parovichnikova EN, Savchenko VG, Adema GJ (2003) Angiotensin-converting enzyme (CD143) is abundantly expressed by dendritic cells and discriminates human monocyte-derived dendritic cells from acute myeloid leukemia-derived dendritic cells. Exp Hematol 31:1301–1309
47. Conti L, Cardone M, Varano B, Puddu P, Belardelli F, Gessani S (2008) Role of the cytokine environment and cytokine receptor expression on the generation of functionally distinct dendritic cells from human monocytes. Eur J Immunol 38:750–762
48. Hassan HT, Zander A (1996) Stem cell factor as a survival and growth factor in human normal and malignant hematopoiesis. Acta Haematol 95:257–262
49. Caux C, Saeland S, Favre C, Duvert V, Mannoni P, Banchereau J (1990) Tumor necrosis factor-alpha strongly potentiates interleukin-3 and granulocyte-macrophage colony-stimulating factor-induced proliferation of human CD34+ hematopoietic progenitor cells. Blood 75:2292–2298
50. Mackensen A, Herbst B, Chen JL, Kohler G, Noppen C, Herr W, Spagnoli GC, Cerundolo V, Lindemann A (2000) Phase I study in melanoma patients of a vaccine with peptide-pulsed dendritic cells generated in vitro from CD34(+) hematopoietic progenitor cells. Int J Cancer 86:385–392
51. Brunner C, Seiderer J, Schlamp A, Bidlingmaier M, Eigler A, Haimerl W, Lehr HA, Krieg AM, Hartmann G, Endres S (2000) Enhanced dendritic cell maturation by TNF-alpha or cytidine-phosphate-guanosine DNA drives T cell activation in vitro and therapeutic anti-tumor immune responses in vivo. J Immunol 165:6278–6286
52. Morel Y, Truneh A, Sweet RW, Olive D, Costello RT (2001) The TNF superfamily members LIGHT and CD154 (CD40 ligand) costimulate induction of dendritic cell maturation and elicit specific CTL activity. J Immunol 167:2479–2486
53. Cignetti A, Bryant E, Allione B, Vitale A, Foa R, Cheever MA (1999) CD34(+) acute myeloid and lymphoid leukemic blasts can be induced to differentiate into dendritic cells. Blood 94:2048–2055
54. Bagheri K, Delirezh N, Moazzeni SM (2008) PPD extract induces the maturation of human monocyte-derived dendritic cells. Immunopharmacol Immunotoxicol 30:91–104