Reconstitution of CD40 and CD80 in dendritic cells generated from blasts of patients with acute myeloid leukemia

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Abstract

Acute myeloid leukemia (AML) is a clonal disease of hematopoiesis with poor clinical outcome despite recent improvements in chemotherapy and stem cell transplantation regimens. Immunotherapy with dendritic cells (DCs) eliciting specific T cell responses to leukemia-associated antigens (LAAs) might be a therapeutic option. DCs must express HLA class I/II molecules and the costimulatory molecules CD40, CD80 and CD86 to effectively activate T cells for the subsequent lysis of leukemic blasts. The expression of these antigens on DCs generated from 15 AML patients (AML-DCs) and on DCs generated from 15 healthy volunteers (HV-DCs) was analyzed by FACS. All DCs displayed the typical morphology and tested negative for B, T and NK cell markers. The sustained mRNA expression of LAAs such as PRAME, RHAMM or WT-1 proved that the AML-DCs originated from AML blasts. Compared with AML blasts, the expression of CD40, CD80, CD86 and HLA-DR was upregulated during DC culture to a median of 80-98% on AML-DCs. HLA-ABC was preserved on AML-DCs (median 95%). Expression of CD40, CD80 and CD83 remained lower on AML-DCs than on HV-DCs. AML-DCs express at least one LAA and strongly express HLA and costimulatory molecules, the prerequisites for eliciting T cell responses. AML-DCs may play a role in vaccine-based immunotherapies for AML patients.

Introduction

Acute myeloid leukemia is a hematological disease characterized by the clonal proliferation of undifferentiated myeloid progenitor cells. Most of the patients with AML achieve a complete hematological remission by chemotherapeutical regimes. However, the long-term prognosis for all AML patients is rather poor with a 5-year overall survival of only 20-25% depending on the individual risk profile and the treatment option chosen (1). This clinical outcome suggests that the majority of the patients in complete hematological remission have minimal residual disease, subsequently leading to relapse (2). Obviously the leukemia-bearing host is immunologically
tolerant to the remaining leukemia cells and therefore fails to eradicate the disease. As we demonstrated in a recent study (3), one reason for the insufficient recognition might be the low expression of CD40 and the costimulatory molecule CD80 on leukemic blasts. Both molecules have been repeatedly shown to be key elements in the initiation of primary immune responses (4, 5). The interaction of CD40 on dendritic cells with CD40 ligand (CD40L, i.e. CD154) on naive T cells is critical for DC maturation and the generation of antigen-specific T cell responses (4, 6, 7, 8). Dendritic cells are professional antigen presenting cells with the unique capacity to prime naive T cells (9). Hence, DCs are also considered to be important elements in the induction of specific anti-tumor immune responses (6, 7, 9). Several groups have shown that DCs can be generated from the leukemic blasts of patients with AML or CML (10, 11). Choudhury et al. (12, 13, 14) demonstrated that autologous AML-DCs can stimulate anti-leukemic T cell responses. Up to now, myeloid leukemia is the only malignancy in which DCs can be generated directly from the cancer cells. Therefore AML-DCs might constitutively express leukemia-associated antigens such as PRAME, the antigen preferentially expressed in melanoma (15), the Wilms tumor gene WT-1 (16), or the receptor for hyaluronic acid mediated motility, RHAMM (17).

In the present study, the origin of the AML-DCs was proven by the maintenance of the above-mentioned LAAs. For the efficient presentation of such LAAs, the expression of HLA and the costimulatory molecules CD40, CD80 and CD86 would be essential. In this paper, we show the preservation of HLA molecules and the reconstitution of the expression of costimulatory molecules on AML-DCs. Vaccination with such AML-DCs might be a therapeutic option for AML patients with minimal residual disease or a maintenance therapy.

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**Results**

**Morphology of AML- and HV-DCs**

After 8 days of culture as described in the Material and Methods section, floating and semi-adherent cells generated from AML blasts (AML-DCs) and monocytes from healthy volunteers (HV-DCs) showed a typical DC morphology with characteristic veils, a kidney-shaped nucleus and a diameter of 15-20 µm. AML-DCs visualized by both light and scanning electron microscopy are shown in Figures 1 and 2. The scanning electron microphotograph of an AML-DC (Figure 2) demonstrates that AML-DCs can interact with lymphocytes. The viability of the AML- and HV-DC preparations was >95% as determined by Trypan blue staining.

![Figure 1. Dendritic cells generated from AML blasts visualized by light microscopy (400x).](http://www.cancerimmunity.org/v3p8/030607.htm)
RT-PCR for leukemia-associated antigens

PBMCs from AML patients were subjected to RT-PCR for the three LAAs PRAME, RHAMM and WT-1. All 15 AML patients expressed at least one of these LAAs. Figure 3 shows representative results of an agarose gel for two patients and one healthy volunteer. After DC culture, the generated DC samples were also subjected to RT-PCR. Expression of at least one of the three LAAs tested was detected in all 15 AML-DC preparations. In the majority of the cases PCR signals for LAAs were stronger in AML-DCs than in AML blasts.

Immunophenotyping of PBMCs from healthy donors and of HV-DCs

Immunophenotyping of PBMCs from 15 healthy donors on day 0 and of 15 HV-DCs on day 8 was performed. Figure 4a shows representative flow cytometry results for the expression of HLA class I/II and costimulatory
molecules on freshly prepared PBMCs from healthy volunteers in the monocyte gate. HLA-ABC and -DR were expressed at the highest level. CD86 expression was high, but that of CD40 was very low. We failed to detect CD80 and CD83. Figure 4b demonstrates that a higher level of expression of CD40, CD80, CD83 and CD86 could be observed on day 8 for HV-DCs. HLA-ABC and -DR expression remained unchanged and at high levels. Maturation of DCs was particularly substantiated by the expression of CD83 ranging from 37-89% (median 79%) on DCs in contrast to 0.5-4% (median 1%) before culture. The FACS data for PBMCs before DC culture (day 0) from 15 healthy volunteers are displayed as box plots in Figure 5a. The FACS analysis (Figure 5b) after 8 days of HV-DC culture demonstrates a higher level of CD40, CD80, CD83 and CD86 on all HV-DC preparations as compared to PBMC preparations ($P<0.0001$).

![Figure 4](http://www.cancerimmunity.org/v3p8/030607.htm)

**Figure 4.** Comparison of the expression of HLA and costimulatory molecules on monocytes and DCs. Phenotype of PBMCs from healthy volunteers in the monocyte gate before culture on day 0 (panel a) and of dendritic cells generated from monocytes of healthy donors after 8 days of culture (panel b) as analyzed by flow cytometry.

![Figure 5](http://www.cancerimmunity.org/v3p8/030607.htm)

**Figure 5.** FACS analysis of PBMCs and HV-DCs from healthy donors. The phenotype of PBMCs from 15 healthy donors analyzed by FACS in the monocyte gate on day 0 (panel a) and of DCs after 8 days of culture (panel b) is shown. The data are presented in the box plot format, with the vertical line indicating the range of the data with the minimum and maximum values as endpoints. The upper and lower lines of the box define the 75th and 25th percentile, while the bar within the box corresponds to the median.
Immunophenotyping of AML blasts and AML-DCs

Figure 6a shows representative flow cytometry results for HLA class I/II and costimulatory molecules on AML blasts characterized by the expression of CD34, a marker for hematopoietic progenitor cells. HLA-ABC and -DR are present on most of the cells. The expression level of the CD86 co-stimulatory molecule is high, but that of CD40, CD80 and CD83 is very low. Figure 6b demonstrates that HLA-ABC expression remained unchanged on AML-DCs (at approximately 100%). The FACS analysis of CD34+ blasts from 15 AML patients is displayed in Figure 7a, and that of AML-DCs obtained after 8 days of culture in Figure 7b. The data demonstrate the upregulation of CD40, CD80, CD83, CD86 and HLA-DR on all AML-DC preparations as compared to AML blasts (P<0.001 for HLA-DR and P<0.0001 for all other markers).

Figure 6. Comparison of the expression of HLA and costimulatory molecules on AML blasts and AML-DCs from patient # 9. Shown is the phenotype of leukemic blasts from AML patient # 9 in the monocyte gate before culture on day 0 (panel a) and of DCs generated from blasts of the same AML patient after 8 days of culture (panel b). A monoclonal antibody against CD34 was used to dissect the subpopulations of leukocytes from normal hematopoiesis versus leukemic blasts.
Figure 7. Comparison of leukemic blasts and DCs from AML patients. Shown is the phenotype of leukemic blasts from 15 AML patients on day 0 (panel a) and of DCs from 15 AML patients on day 8 (panel b) as determined by FACS in the monocyte gate. The data are presented in the box plot format, with the vertical line indicating the range of the data with the minimum and maximum values as endpoints. The upper and lower lines of the box define the 75th and 25th percentile, while the bar within the box corresponds to the median.

Comparison of the immunophenotype of PBMCs from healthy volunteers and that of primary AML blasts

To assess the difference between HV-DCs and AML-DCs, we compared the phenotype of PBMCs from 15 healthy volunteers in the monocyte gate with blasts from 15 AML patients before culture. Figure 5a shows the immunophenotype of PBMCs from 15 healthy volunteers in the monocyte gate and Figure 7a the immunophenotype of leukemic blasts from 15 AML patients. Expression of HLA-ABC on blasts was similar to that on monocytes from healthy volunteers, whereas HLA-DR and CD86 were expressed at a lower level. On monocytes from healthy volunteers, CD80 was absent and the median CD40 expression was 19%. No expression of CD40 and CD80 were detected in AML blasts. Expression of CD40, CD86 and HLA-DR was significantly higher on monocytes from healthy volunteers than on AML blasts ($P<0.0001$). No significant difference in HLA-ABC expression was observed between PBMCs and AML blasts ($P>0.05$). Both AML blasts and monocytes from healthy volunteers tested negative for the expression of CD3, CD16, CD19 and CD56 (data not shown).

Comparison of the immunophenotype of DCs from healthy volunteers and AML patients

After 8 days of culture, both HV-DC and AML-DC preparations showed the phenotypic characteristics of monocyte-derived DCs, i.e. no or weak CD14 expression (data not shown) and high levels of major histocompatibility complex (MHC) class II (HLA-DR) molecules. The FACS data obtained for samples from 15 healthy volunteers are shown in Figure 5b and those from 15 AML patients in Figure 7b. No significant differences between HV-DCs and AML-DCs were observed as regards the expression of HLA-ABC (median 98% in HV-DCs versus 95% in AML-DCs), HLA-DR (median 100% in healthy volunteers versus 99% in AML patients) and CD80 (median 74% in HV-DCs versus 80% in AML-DCs) with $P$ values of 0.98 and 0.07 respectively. However, significant differences were observed in the expression of CD40, CD83, CD86, with $P$ values <0.05. The presence of CD83 expression substantiates the maturation of the DCs.
Discussion

As the most professional antigen presenting cells, DCs play a major role in the immune system (6, 8). Clinical trials using autologous DCs loaded with tumor cell lysate, antigen peptides or total RNA extracted from tumor cells are ongoing for patients with melanoma, renal cell cancer or prostate carcinoma (18). New hope for immunotherapies with DCs emerged from phase I/II trials in which immunological and even clinical responses could be observed (19, 20). AML-DCs might express constitutive LAAs such as PRAME (15), RHAMM (17) or WT-1 (16, 20, 21, 22) which have been demonstrated to elicit specific T cell responses subsequently resulting in the lysis of leukemic blasts. Therefore autologous AML-DC preparations might be used as a cancer vaccine for leukemia patients either for maintenance therapy or in the case of relapse for the *ex vivo* generation of specific donor lymphocyte infusions.

Choudhury and co-workers showed earlier (14) that DCs originated from AML blasts by FISH analysis of cytogenetic aberrations. However, FISH analysis does not constitute an appropriate method to demonstrate the origin of AML-DCs in clinical practice. Firstly, only about 50% of AML patients (not only those included in the present study) show chromosomal aberrations (23). Moreover, at least $10^7$ DCs must be generated for FISH analysis, which requires up to $10^9$ PBMCs from AML patients only for this pre-diagnostic procedure, a number which excludes clinical feasibility. Immunophenotyping of AML blasts and AML-DCs is not appropriate to prove the origin of AML-DCs either. For instance, the stem cell marker CD34 is expressed only in 10-20% of AML patients and vanishes as a result of maturation during the eight days of DC culture.

In previous studies (17, 24, 25), we detected the exquisite expression of the LAAs PRAME, RHAMM and WT-1 on AML blasts in contrast to PBMCs from healthy volunteers. In our present RT-PCR assays, the expression at least one of these three LAAs was maintained in each of the 15 AML-DC preparations. In the majority of cases, a stronger PCR signal was observed after eight days of DC culture. This result might be explained by a stronger expression of the LAAs by the AML-DCs as compared to the AML blasts, or rather by a higher percentage of the blast-derived DCs in the whole AML-DC preparation as compared to the percentage of blasts in the original AML sample.

In this study we compared the expression of HLA class I and II molecules and the costimulatory molecules CD40, CD80, CD86 on leukemic blasts and DCs from AML patients with PBMCs and DCs from healthy volunteers. Our findings in this and an earlier study (3) demonstrate a deficient expression of costimulatory molecules in leukemic blasts that might hamper stimulation of T cell responses. The expression of CD40 is considered to be crucial for T cell activation and expansion (5), so the absence of CD40 on blasts might be especially responsible for the insufficient recognition of blasts by the immune system of the AML patient. Moreover, expression of CD80 on blasts was almost absent. Enhancing the expression of CD80 on leukemia blasts increases their costimulatory activity for autologous T cells (26). For both HLA and all costimulatory molecules on AML-DCs a strong upregulation was detected after 8 days of DC culture, therefore providing the prerequisites for eliciting T cell responses.

The comparison of AML-DCs with HV-DCs demonstrated a similar expression of HLA-ABC, -DR and CD80, but a lower expression of CD40 and CD86 on AML-DCs. This indicates that AML-DCs are more potent than AML blasts to elicit T cell responses. However, HV-DCs might be even more efficient through the higher expression of costimulatory molecules. On the other hand, HV-DCs lack the expression of LAAs, making procedures such as pulsing with LAA peptide or transfection with LAA RNA necessary for immunotherapy. The upregulation of HLA and costimulatory molecules on AML-DCs makes them valuable professional antigen presenting cells for the stimulation of specific CTL responses to LAAs presented on the surface of leukemic blasts. Therefore, we initiated a phase I/II clinical trial at our institution using autologous DCs applied intradermally for the treatment of elderly patients with AML in the palliative setting. The first patient showed a 100% increase in CTLs recognizing...
leukemic blasts after three vaccinations with autologous DCs, but did not show a clinical response (27). Future patients and consecutive trials might teach us the most effective route for DC vaccines and the best way of DC preparation for AML patients.

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**Abbreviations**

AML-DC, DC generated from an AML patient; HV-DC, DC generated from a healthy volunteer; LAA, leukemia-associated antigen

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**References**


Materials and methods

Cell samples

Peripheral blood samples from 8 untreated patients with newly diagnosed AML and 7 patients at the time of relapse were taken when the patients were screened for an experimental therapy with autologous DCs. Informed consent as to the use of their blood for scientific purposes was obtained from AML patients who were treated at our institution according to clinical study protocols approved by the local ethics committee. Control samples were prepared from 15 healthy blood donors at our institution after their informed consent was obtained. Table 1 displays the characteristics of the AML patients.

Table 1. Patient characteristics.

<table>
<thead>
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<th>Patient</th>
<th>AML (FAB)</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>WBC (x10^9)</th>
<th>Blasts (%)</th>
<th>Karyotype</th>
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<td>70</td>
<td>M</td>
<td>2.6</td>
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<td>F</td>
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<tr>
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<td>M</td>
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<td>86</td>
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*aAbbreviation: Sec. AML, secondary AML.*
Generation of dendritic cells

DCs were generated from peripheral blood cells as described previously (9, 28, 29, 30). In brief, peripheral blood mononuclear cells were isolated using Ficoll/Paque (Biochrom, Berlin, Germany) density gradient centrifugation of EDTA (Delta-Pharma, Pfullingen, Germany) anticoagulated blood buffy coat preparations from healthy volunteers or from AML patients. PBMCs were seeded (1x10^6 cells/cm^2) into culture flasks (Nunc, Roslild, Denmark) in RPMI-1640 medium (Biochrom AG, Berlin, Germany) supplemented with 2% human AB serum (German Red Cross Blood Center, Ulm, Germany), 2 mM L-glutamine, 100 units/ml penicillin and 100 units/ml streptomycin. After 2 h of incubation at 37°C, non-adherent cells were removed and the adherent CD14+ blood monocytes (purity >80% by FACS analysis) were cultured in RPMI1640 medium supplemented with 100 ng/ml human GM-CSF (Leukomax, Novartis, Basel, Switzerland), 1,000 IU/ml IL-4 (Strathmann, Hannover, Germany) and 10% human AB serum. For the maturation of the cells, the medium was changed on day 6. The new medium contained GM-CSF and IL-4 in the concentrations given above, as well as 50 ng/ml TNF-alpha (Strathmann, Hannover, Germany). The DC cultures were fed with fresh medium and cytokines every three days and cell differentiation was monitored using inverse light microscopy. The expression of cell surface molecules on the DCs was analyzed using flow cytometry after 8 days of culture.

Cell viability and morphologic studies

The viability of the DCs harvested after 8 days of culture was assessed by Trypan blue staining using a Neubauer counting chamber, and the morphology of the cells was analyzed by light and scanning electron microscopy. For light microscopy, DC suspensions were spun and stained with May-Grünwald solution (Merck, Darmstadt, Germany). For scanning electron microscopy, standard protocols were followed. In brief, samples were fixed 1:1 with 5% glutaraldehyde in 0.2 M phosphate buffer and 2% sucrose. 200 µl of the fixed cell suspension containing 10^5 cells were transferred to poly-lysine coated slides, sedimented for 1 hr in a humidified chamber, washed with PBS and slides were cut to the appropriate size. Dehydration by dipping the slides in serial solutions of increasing propanol concentration (30%, 50%, 70%, 90%, 100%) was followed by critical point drying. Samples were coated with gold/palladium particles 20 nm in diameter. Visualization was performed using a scanning electron microscope DSM 962 (Zeiss, Oberkochen, Germany) at 15 kV.

Reverse transcriptase polymerase chain reaction for leukemia-associated antigens

Based on our previous studies on LAAs (17, 24, 25) we tested both AML blasts and AML-DCs for their mRNA expression of the three LAAs PRAME, RHAMM and WT-1 by RT-PCR as described. Briefly, poly(A)+ mRNA was isolated directly from 1x10^7 PBMCs using the µMACS™ mRNA Isolation Kit (Miltenyi, Bergisch Gladbach, Germany). 200 ng of each sample was subjected to cDNA synthesis (1st strand cDNA synthesis kit for RT-PCR, Roche). The primers for RT-PCR were as follows: TBP (RefSeq Accession No. NM_003194.2) 5'-TTAACTTCGTTCCGCTGGC-3' (forward) and 5'-GAAACCCTTGCGCTGGAACT-3' (reverse), PRAME (Accession No. NM_006115) 5'-GTTCCTTAGGCCAGCTAAGT-3' (forward) and 5'-GGAGAGGAGGAGTCTACGCA-3' (reverse), RHAMM (Accession No. NM_012484.1) 5'-CAGGTCACCAAAGAGCTCTCG-3' (forward) and 5'-CAAAGGCTACCCAGTGTTTG-3' (reverse), WT-1 (Accession No. NM_00378.2) 5'-ATGAGGAGATCCCATGGGCCAGCA-3' (forward) and 5'-CCTGGGACACTGAACGGTCCCCGA-3' (reverse). Denaturation, annealing and elongation temperatures were 94°C, 64°C, 72°C for PRAME and WT-1 and 94/95°C, 60°C, 72°C for RHAMM and TBP. All PCR reactions were performed for 35 cycles and with 1.5 mM MgCl2.

Flow cytometry analysis

Harvested cells were washed in FACS medium (PBS containing 1% BSA) and stained at 4°C for 20 min by antibodies directly conjugated with FITC or PE. Thereafter cells were washed three times with PBS and analyzed.
by FACScan (Becton Dickinson, Heidelberg, Germany) using the CellQuest software (Becton Dickinson). The following antibodies were used: FITC-labeled anti-mouse IgG, anti-human HLA-DR, CD33, CD40, CD45 and CD83, as well as PE-labeled anti-mouse IgG, anti-human HLA-ABC, CD1a, CD11c, CD34, CD54, CD80 and CD86 (Becton Dickinson). Double staining was performed using pairs of PE- and FITC-labeled antibodies.

**Statistical analysis**

The statistical evaluation was performed using the statistical software "SAS-Analyst" - a tool of SAS-System V.8 (SAS-Institute, Cary, NC, USA). The expression of HLA and costimulatory molecules in healthy volunteers and in patients with AML was compared using the two-sided Wilcoxon test for parallel groups. The significance level for all comparisons was set at the common standard of 0.05. In the case that the calculated error probability was below 0.05, the zero hypothesis "There are no significant differences in the expression of CD antigens and HLA-molecules between the two groups" was rejected.

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