Induction of Leukemia-specific Cytotoxic Response by Cross-Presentation of Late-Apoptotic Leukemic Blasts by Autologous Dendritic Cells of Nonleukemic Origin

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ABSTRACT

Acute myeloid leukemias (AMLs) are monoclonal proliferations of undifferentiated myeloid progenitors in blood and bone marrow. Long-term remissions are achieved in <50% of patients. There is hope that activation of specific antileukemic immune responses could efficiently eliminate minimal residual disease at the end of chemotherapy and decrease the frequency of relapses. It was demonstrated that AML leukemic blasts can acquire the morphology and phenotype of dendritic cells (DCs), i.e., differentiate into leukemic DCs. However, this method has limitations as a potential immunotherapeutic tool. The alternative approach for the induction of leukemia-specific cytotoxicity we explored in this study consisted of using DCs of nonleukemic origin, pulsed with autologous apoptotic leukemic blasts. We show that mature pulsed nonleukemic DCs were successfully generated from remission samples of all tested patients with minimal interindividual differences. Mature pulsed DCs were used as antigen-presenting cells for leukemia-specific CTL induction. Specific cytotoxic activity against autologous AML blasts was demonstrated. Tumor lysis was autologous blast specific, with no killing activity against allogeneic leukemic cells or autologous mature unpulsed DCs and was MHC class I and class II restricted. In one patient, autologous CTLs stimulated by leukemic DCs or pulsed nonleukemic DCs showed similar significant cytotoxic activity against autologous AML cells. These findings demonstrate the induction of leukemia-specific cytotoxic response by nonleukemic mature DCs cross-presenting apoptotic leukemic blasts and offer a complementary approach to the use of leukemic DCs. We believe that this strategy permits the generation of DC vaccines for the majority of patients with hematological malignancies.

INTRODUCTION

AMLs are monoclonal disorders characterized by the proliferation of undifferentiated myeloid progenitors. The majority of adults with AML achieves complete remission with current chemotherapy protocols. However, long-term disease-free survival occurs in less than half of the cases, with an overall survival of only 15% at 5 years (1). It is well recognized that virtually all patients in complete remission after induction therapy have residual disease that may lead to relapse (2). A number of approaches have been explored to prevent relapse and improve overall survival, including maintenance therapy, intensive consolidation, and myeloablative conditioning regimens with either allogeneic or autologous bone marrow or stem cell transplantation. Several mechanisms are known to be used by leukemic cells to escape to the immune reaction: (a) the low expression of costimulatory or adhesion molecules (3, 4); (b) Fas ligand expression responsible for activated T-lymphocyte apoptosis (5); and (c) secretion of cytokines inhibiting the development of efficient immune responses (6). Thus, manipulations of the immune system could represent a potential additional treatment because recent observations indicate the primordial antitumor role of T lymphocytes in tumor diseases (7). DCs are currently promising adjuvants for cancer immunotherapy because they are the most efficient APCs, and they induce both primary and secondary immune responses (8). DCs are located at the interface of potential pathogen entry sites as immature DCs and take up antigens. After antigen uptake, they move into secondary lymphoid organs, mature, and activate both helper and cytotoxic T cells (9). The first pilot clinical trials showed the feasibility of DC administration in vivo and the objective clinical responses induced by tumor antigen-loaded DCs (10–13). Several systems of tumor antigen delivery to DCs have been used, including defined peptides of known sequences (14), retroviral or adenoviral vectors (15, 16), tumor cell-derived RNA (17), and fusion of DCs with tumor cells (12). Apoptotic tumor cells have been reported as a source of tumor antigen to DCs (18–20). This method does not require the identification of tumor-associated antigens, and it is likely to yield both MHC class I- and MHC class II-restricted epitopes, which represents a major advantage in acute leukemia where only a limited number of well-defined tumor antigens exist, such as AML subtype-restricted fusion proteins or overexpressed normal proteins such as proteinase 3 or WT1 (21–26).

Recently, it has been demonstrated that, under specific culture conditions, leukemic cells can acquire the morphology and phenotype of DCs and differentiate into leukemic DCs (27–31). Thus, leukemic cells that directly present potential tumor antigens on their surface can acquire costimulatory and adhesion molecules and subsequently induce stimulation of leukemia-specific CTLs. However, this method entails several disadvantages for clinical applications. The generation of leukemic DCs is limited to 60% of AML patients with undetermined reproducibility, with respect to the quantity and leukemic origin of cells produced (32). The largest published series demonstrated the induction of antileukemic CTLs in vitro in only 20% of enrolled patients (33). To overcome these constraints, we investigated an alternative approach enabling the generation of a potent antileukemic cytotoxic response, using DCs of nonleukemic origin pulsed with autologous late-apoptotic leukemic blasts. We demonstrate the successful generation of functional monocye-derived DCs in all tested AML patients who achieved complete morphological remission after induction chemotherapy. Moreover, mature DCs efficiently cross-presented apoptotic autologous leukemic blasts and induced specific in vitro CTL responses against the original leukemic cells. It is important to note that the remission samples were obtained from patients who
underwent induction chemotherapy protocols and, thus, were severely immunocompromised. This fact further underlines the great T-cell-priming potential of mature DC preparations.

PATIENTS AND METHODS

Blood Samples. Samples from 10 patients (median age, 65 years; range, 25–71 years) with various FAB subtypes [according to the FAB classification criteria (34)] of de novo AML were used for the experiments performed in this study. Table 1 shows relevant clinical and diagnostic laboratory data for these patients. Samples were taken with informed consent at the time of diagnostic tests. A highly purified population of leukemic blasts was isolated by Ficoll-Paque gradient-density centrifugation. Isolated cells were used immediately or cryopreserved in RPMI 1640 + 10% FCS + 10% DMSO (Sigma, St. Quentin Fallavier, France).

Remission samples were collected between 2 and 5 weeks after the end of aplasia, following induction chemotherapy (1-β-d-arabinofuranosylcytosine and idarubicin for all patients), from patients in complete morphological remission (defined as <5% of blasts in bone marrow). PBMCs were separated by Ficoll-Paque gradient-density centrifugation and used for generation of DCs of nonleukemic origin and as responder cells for the induction of specific CTL responses.

Generation of Leukemic DCs from Leukemic Blasts. Complete CM used for cell cultures consisted of RPMI 1640 (Life Technologies, Inc., Paisley, Scotland) supplemented with 10% heat-inactivated FCS (Eurobio, Les Ulis, France), 1% l-glutamine (Life Technologies, Inc.), and 1% penicillin/streptomycin (Life Technologies, Inc.). Fresh or thawed leukemic blasts were cultured for 7 days at 1 × 10^6 cells/ml in 3 ml of CM/well in six-well plates (Falcon, Le Pont de Claix, France) in the presence of IL-4 (Biosource, Montrouge, France), GM-CSF (Leumocax; Novartis, Rueil-Malmaison, France), and TNF-α (R&D System, Abingdon, United Kingdom). GM-CSF was used at 500 units/ml, IL-4 at 15 ng/ml, and TNF-α at 10 ng/ml. Cytokines were replenished on day 3. Cultured leukemic cells were immunophenotyped by flow cytometry on day 7 and evaluated for their viability by trypan blue (Sigma) exclusion.

Generation of DCs from PBMCs of Patients in Complete Morphological Remission. Immature monocyte-derived DCs were generated from the adherent fraction of PBMCs collected between 2 and 5 weeks after the end of aplasia, following the induction chemotherapy. Briefly, 10 × 10^6 PBMCs were suspended in 3 ml of CM and allowed to adhere to six-well plates. After 2 h of incubation at 37°C, the nonadherent lymphocytes were removed and cryo-preserved in RPMI 1640 + 10% FCS + 10% DMSO. Adherent monocytes were cultured for 5 days in CM with GM-CSF (500 units/ml) and IL-4 (15 ng/ml). Fresh cytokines were added on day 3, and immature DCs were evaluated for their viability by trypan blue exclusion on day 5 and used for additional tests.

Immunophenotype of Fresh AML Blasts, Leukemic DCs, and DCs of Nonleukemic Origin. Phenotypic analyses were performed on 10^6 cells using FITC-conjugated mAbs against CD58, CD83, HLA-DR (Immunotech, Villeneuve-lès-Avignon, France), and phycoerythrin-conjugated anti-CD4 mAb (PharMingen). Cells were incubated with mAbs for 30 min at 4°C, washed twice in PBS, and resuspended in a small volume of PBS for analysis with a FACS Calibur flow cytometer (Becton Dickinson, Grenoble, France). Forward scatter and side scatter gates were established to eliminate cell debris, and dead cells were excluded on the basis of PI staining before the analysis for expression of each phenotypic marker. Appropriate isotype-matched control mAbs were always included.

FISH. To determine the leukemic origin of generated leukemic DCs, we selected patient 10 whose leukemic cells exhibited the amplification of the MLL gene at diagnosis. Leukemic cells were analyzed by FISH before and after culture. Chromosome 11 abnormality was detected using a MLL-specific probe (Vysis, Voisins-le-Bretonneux, France).

Induction of Leukemic Blasts Apoptosis. Apoptosis was induced by UVB irradiation for 60 s, followed by overnight incubation in RPMI 1640. After 24 h, cell death was assessed by trypan blue exclusion, externalization of phosphatidylserine using FITC-labeled annexin V (PharMingen), and staining with PI.

Phagocytosis of Apoptotic Leukemic Cells. Leukemic blasts were labeled with PKH-26 GL membrane dye (Sigma) according to the manufacturer’s instructions, and apoptosis was induced by UVB irradiation. The labeled leukemic blasts were cocultured with immature DCs of nonleukemic origin at a 5:1 ratio. After 24 h, the cells were washed, and the internalization of PKH-26-labeled blasts was visualized by confocal microscopy as described previously (35). Cells were allowed to adhere on Aclian blue-coated slides for 2 h, fixed with 4% paraformaldehyde for 30 min, washed in PBS, and stained with FITC-anti-HLA-DR mAb for 30 min. Labeled slides were mounted with Moviol (Calbiochem, Meudon, France). Confocal microscopy was performed using a confocal laser scanning microscope system (Leica) equipped with a krypton/argon laser. Simultaneous double fluorescence acquisitions were performed using the appropriate laser lines to excite FITC and PKH-26 dyes. Alternatively, the phagocytic activity of immature DCs was evaluated by the use of FITC-labeled latex beads (PharMingen). Immature DCs and latex beads were cocultured for 8 h, and their internalization was evaluated by flow cytometry.

Antigen Pulsing and Maturation of DCs of Nonleukemic Origin. Day 5 immature DCs were mixed with apoptotic autologous leukemic blasts at a 1:5 ratio and incubated for 24 h at 37°C to allow phagocytosis. Pulsed DCs were subsequently matured by the combination of 5 ng/ml TNF-α + 25 μg/ml poly(I:C) (Sigma; Ref. 35) for an additional 24 h and used as APCs. Maturation was confirmed by flow cytometry.

Allogeneic MLR. Responder cells for allogeneic MLR were the nonadherent lymphocytes obtained from the peripheral blood of unrelated healthy donors by gradient-density centrifugation and 2 h of adhesion. Responder cells were plated at 1 × 10^6/well in CM in U-bottomed 96-well plates. Stimulators were added in the final volume of 200 μl at the ratios 1:10, 1:20, 1:40, and 1:80. The proliferation of allogeneic lymphocytes was determined by the uptake of [3H]thymidine (NEN, Boston, MA) added for the last 18 h of a 4-day culture. Simultaneously, the proliferation of donor or remission T cells was assessed by comparison of the responses to the PHA (1 μg/ml; Sigma) stimulation.

Stimulation of Autologous Leukemic Blast-specific T Lymphocytes. Autologous responder cells were derived from thawed or fresh nonadherent fraction of remission blood samples. Autologous leukemic DCs or mature DCs loaded with killed autologous leukemic blasts were used as the stimulatory

<table>
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<th>Sample origin</th>
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a Percentage of positive cells compared with an appropriate isotype control.

b PB, peripheral blood; BM, bone marrow; Biphen, biphenotypic leukemia.

c Del 11q 23, MLL.

d Amp 11q 23, MLL.
cells. Cultures were prepared in 48-well plates by plating stimulatory cells at 5 × 10⁴ with 5 × 10⁵ responders in a final volume of 0.5 ml of CM. CM was supplemented with IL-6 (10 ng/ml; R&D System) for the first week and with IL-2 (50 units/ml; R&D System) + IL-7 (5 ng/ml; R&D System) during the second and third weeks. Responder cells were restimulated on day 7 with the same stimulators that were used at the beginning of culture and on day 14 with CD3/CD28 Dynal T-cell expander beads (Dynal, Compagnie, France) at 1.25 μl/ml. Six days after the last stimulation, cells were harvested, and their cytotoxic activity was tested.

**51Cr Release Cytotoxicity Assay.** Cytotoxicity was measured in a standard 6-h 51Cr release cytotoxicity assay. AML blasts or other target cells were labeled for 2 h with 51Cr and washed three times with CM. Autologous-activated lymphocytes were cultured with 2 × 10⁵ target cells at E:T ratios of 12.5:1, 25:1, 50:1, and 100:1 in 200 μl of CM in U-bottomed 96-well plates. After 6 h, 50 μl of supernatant were collected, and the percentage of specific lysis was calculated using the following formula: [(cpm experimental release − cpm minimal release)/(cpm maximal release − cpm minimal release) × 100]. Minimum 51Cr release was determined from wells containing target cells and medium only. Maximum 51Cr release was determined from wells containing target cells with 100 μl of 5% Triton X-100 (Sigma). Spontaneous release was <15% of the maximal release. To determine the specificity of MHC class I and class II restriction, the target cells were preincubated with MHC class I or class II blocking antibodies (Becton Dickinson).

**Statistical Analysis.** Student t test was used to evaluate the differences between two groups. P < 0.05 was considered to be statistically significant.

**RESULTS**

**Generation and Immunophenotyping of Leukemic DCs.** The mean percentage of blasts in the initial samples was 83.5 ± 10.2%, and leukemic blasts expressed no or very low levels of CD80, CD83, and CD86 at the time of diagnosis. To evaluate the potential of leukemic blasts to develop into leukemic DCs, the blasts were cultured with GM-CSF, IL-4, and TNF-α. After 7 days, the presence of leukemic DCs was assessed morphologically (data not shown) and phenotypically (Fig. IA). Samples that combined typical morphological characteristics of DCs, high-level MHC class II expression, and significant expression of at least one CD80, CD83, or CD86 (25% positive cells at minimum) with negativity of CD14 were defined as leukemic DCs. According to these criteria, leukemic DCs were generated in 5 patients (50%). The blasts from the remaining patients either died rapidly within the first 2 days of culture (4 patients), or they did not correspond to our leukemic DC definition (1 patient). Three independent experiments with the same cytokine combination were done for patients 1, 2, and 3, and one test was performed for the rest of the patients. For patient 2, leukemic DCs were successfully generated from freshly isolated blast cells, although no differentiation occurred when the cryopreserved cells were used. Data concerning the proportion of cells expressing CD80, CD83, and CD86 before and after culture are summarized in Table 1. Note that the mature DC-specific surface molecule CD83 was strongly expressed on the leukemic DCs in patient 1 only. In patients with successful leukemic DC generation, only patient 10 presented a specific chromosomal rearrangement (i.e., the amplification of the MLL gene). FISH analysis demonstrated 100% of leukemic DCs carrying the same chromosomal rearrangement as original leukemic blasts (Fig. IB). Furthermore, in patient 1, the leukemic origin of DCs was suggested by the persistence of strong CD4 expression after 60 s of UVB irradiation, followed by cell death monitored by annexin V/PI staining. At the time of immature DC pulsing, the killed leukemic blast population consisted mainly of annexin V/PI double-positive cells (95 ± 10%), indicating the presence of late apoptosis (Fig. 2A).

**Generation, Pulsing, and Maturation of DCs from Remission Samples.** Remission samples were obtained for only 8 patients because patient 8 died on day 4 of the induction chemotherapy, and patient 5 was lost during follow up. Remission samples were collected between 2 and 5 weeks after the end of aplasia, following induction chemotherapy, while all patients were in complete morphological remission. Immature DCs were generated from peripheral blood adherent monocytes in the presence of GM-CSF and IL-4. On day 5, loosely adherent cells with typical DC morphology were harvested and used for additional assays. The immunophenotype of these cells revealed a homogeneous cell population with immature DC characteristics, i.e., the complete negativity of CD4 and CD83 together
with moderate expression of CD80, CD86, and HLA-DR (Fig. 3A; Table 2). We next determined the ability of immature DCs to capture the surrounding antigens. For this purpose, the immature DCs were mixed with FITC-labeled latex beads, and FACS analysis performed 8 h later revealed rapid phagocytosis of the free beads (Fig. 2A). Finally, the capacity of immature DCs to internalize apoptotic autologous leukemic blasts was confirmed by confocal microscopy using PKH-26-labeled apoptotic leukemic blasts (Fig. 2C). We determined previously that 24-h coculture of five apoptotic cells for one immature DC was optimal for the complete pulsing of the immature DC population (35). Because leukemic blast phagocytosis did not trigger DC maturation (data not shown), the immature DCs were treated for 24 h with a combination of TNF-α + poly(I:C) to ensure their complete activation. The use of these cytokines resulted in a uniform population of phenotypically mature DCs, expressing high levels of the costimulatory molecules (CD80 and CD86), the mature DC-specific marker CD83, and HLA-DR (Fig. 3B). In contrast to the leukemic DCs, mature DCs generated from the remission blood samples were obtained for all patients included in this study with minimal interindividual variations in terms of immature and mature DC phenotype, kinetics of phagocytosis, and response to the maturation-inducing cytokine combination (Table 2). Leukemic DCs and mature DCs loaded with autologous apoptotic leukemic blasts were used as APCs for stimulation of autologous T lymphocytes from the remission blood samples.

Functional Assays: Allogeneic MLR. An allogeneic MLR was used as a first approach to evaluate the immunostimulatory capacity of either leukemic or mature nonleukemic DCs. Complete data for three patients are illustrated in Fig. 4A. For each of these patients, T lymphocytes from an unrelated healthy donor showed significantly higher proliferation in response to leukemic DCs and

![Image](image_url)
In all 3 tested patients (Fig. 6D). The same effect, although to a lesser extent, was observed for an anti-MHC class II blocking antibody. Direct comparison of cytotoxic activity induced by leukemic DCs or killed leukemic blast-pulsed mature DCs was possible for patient 1. As for patients 2 and 4, CTLs harvested after three stimulation cycles efficiently lysed autologous leukemic blasts but not allogeneic targets, and CTLs induced by leukemic DCs or mature DCs pulsed with killed leukemic cells showed comparable leukemia specific cytotoxicity at all E:T ratios tested without statistically significant differences (Fig. 6C).

**DISCUSSION**

As cure rates in AML patients approach their plateau, additional therapeutic modalities are being explored for their ability to prevent disease relapse, the cause of the unfavorable prognosis of AML patients. There is hope that efficient immunotherapy could elicit CTL responses capable of eliminating the residual tumor burden at the end of chemotherapy (36). The importance of the T-cell-mediated immune response for the eradication of low tumor burden is documented by considerably lower relapse rates in patients who undergo allogeneic bone marrow transplantation (37). Its efficacy results not only from high-dose chemotherapy but also from an allogeneic immune mechanism known as graft versus leukemia effect. This effect is currently explored in the treatment of AML relapse because complete remissions could be obtained after injection of initial donor lymphocytes (38). The fact that relapses are more frequent after T-cell graft depletion further underscores the essential role of the immune system in controlling tumor burden (39). Although the graft versus leukemia effect can be attributed to the direct recognition of intact MHC molecules by allogeneic donor T lymphocytes (direct T-cell alloreaction; Ref. 40), a recent report has correlated the detection of autologous nonleukemic DCs than in response to the uncultured leukemic blasts or immature nonleukemic DC stimulation ($P < 0.01$).

In 2 of 3 patients, mature nonleukemic DCs were significantly more potent stimulators than DCs of leukemic origin ($P < 0.05$). The same profile was observed at all DC:T-cell ratios tested. We further compared PHA responses of lymphocytes from healthy volunteers to the lymphocytes from remission blood samples used as responders for CTL induction. Fig. 4B demonstrates the ability of remission T cells to proliferate vigorously in response to PHA ($128,000 \pm 13,000$ cpm; mean $\pm$ SD). However, the PHA response of healthy volunteers’ lymphocytes was always significantly higher for the five donors tested ($408,000 \pm 28,000$; $P < 0.01$).

**Functional Assays: Induction of Leukemia-specific CTL Responses.** For patients 1 and 2, different stimulators were tested in preliminary experiments for their ability to induce leukemia-specific cytotoxic T-cell responses. After three stimulation cycles, the presence of leukemia-specific CTLs was detected only in the bulk cultures primed by leukemic DCs or mature DCs pulsed with killed leukemic blasts (Fig. 5). Fig. 6, A–C, show detailed data for patients 2, 4, and 1, respectively, where sufficient amounts of autologous remission blood samples/cryopreserved leukemic blast pairs were available. In Fig. 6, A and B, mature DCs pulsed with killed autologous leukemia blasts successfully primed leukemia-specific CTLs with a mean percentage of cytotoxicity of $18 \pm 2.9\%$ at the E:T ratio 50:1. Induced CTLs failed to lyse both leukemia blasts from unrelated patients and autologous mature unpulsed DCs (mean cytotoxicity, $1.6 \pm 1.3\%$ of lysed target cells at an E:T ratio of 50:1). Blocking of MHC class I molecules on target cells significantly abrogated leukemic blast lysis in all 3 tested patients (Fig. 6D). The same effect, although to a lesser extent, was observed for an anti-MHC class II blocking antibody. Direct comparison of cytotoxic activity induced by leukemic DCs or killed leukemic blast-pulsed mature DCs was possible for patient 1. As for patients 2 and 4, CTLs harvested after three stimulation cycles efficiently lysed autologous leukemic blasts but not allogeneic targets, and CTLs induced by leukemic DCs or mature DCs pulsed with killed leukemic cells showed comparable leukemia specific cytotoxicity at all E:T ratios tested without statistically significant differences (Fig. 6C).
Efficient T-cell activation not only depends on the interaction between specific T-cell receptor and tumor antigen-MHC complex but also on various accessory molecules present on the surface of DCs that enhance adhesion and signaling (costimulatory molecules) and interact with their corresponding receptors on T cells. Although leukemic blasts often express MHC class II molecules, they usually lack costimulatory molecules, which make them poor APCs (3). It has been proposed that AML blasts could differentiate into cells possessing the morphological, phenotypical, and functional properties of DCs, thus coexpressing leukemic antigens and costimulatory signals and acting as APCs (27). Indeed, several groups have reported recently the feasibility of this approach and the induction of autologous antileukemic cytotoxicity (29–31, 42). However, it is important to keep in mind that this system has considerable limitations. In our experiments, we successfully generated leukemic DCs in 5 of 10 cases (50%) across different FAB subgroups of AML. This percentage is similar, although somewhat lower, to that reported by other groups (30, 33, 42). This discrepancy could be explained by the strict definition of leukemic DCs we used and above all by the fact that the same culture conditions were used for all patients tested, whereas various cytokines combinations were evaluated in previous reports to eliminate individual differences (33). Nevertheless, a certain standardization of culture conditions is necessary if this approach is to be used in immunotherapy clinical trials. Successfully generated leukemic DCs also exhibited considerable heterogeneity in terms of cell yield, purity, viability, and phenotypic characteristics (mean fluorescence intensity and percentage of cells expressing tested surface markers). Because AML encompasses biologically heterogeneous diseases, it is not surprising that response to the growth factors differs substantially among patients, e.g., by the stage of leukemic blast differentiation. Additional studies are necessary to optimize culture conditions and to determine the characteristics of blasts suitable for leukemic DC generation. The addition of FLT3 ligand to the cytokine mixture used for blast culture was reported to enhance leukemic DC yield (43), and expression of the mannose receptor on AML cells was reported to positively correlate with their capacity to differentiate into DCs (44). We demonstrated the leukemic origin of in vitro generated leukemic DCs in 1 patient whose cells exhibited amplification of the MLL gene. For a second patient, the malignant origin of leukemic DCs was indirectly documented by sustained strong surface positivity of the CD4 lymphoid marker aberrantly expressed on initial AML blasts. Human DC precursors initially express CD4, but its expression is gradually lost with maturation (45). However, for the remainder of the patients, leukemic origin could not be verified because of the lack of specific chromosomal rearrangements or aberrant lymphoid marker expression. If leukemic origin is not confirmed, DCs generated at diagnosis could be DCs differentiated from nonmalignant peripheral blood monocytes and lacking tumor antigens. This could explain the unsuccessful activation of CTLs in vitro in the majority of patients (33).

The alternative approach for the induction of CTL responses against autologous leukemic blasts that we explored in this study consisted of the use of DCs loaded with autologous late-apoptotic leukemic blasts as a source of tumor antigens. Fuji et al. (46) reported induction of autologous CTLs against AML cells using clusters of nonleukemic DCs generated from CD34 hematopoietic stem cells of patients in complete remission that had phagocytosed irradiated AML cells. Nevertheless, DC clusters presented in their study were poorly phenotypically characterized and did not exhibit mature DC characteristics. Here, we generated immature nonleukemic DCs from peripheral blood monocytes of AML patients in complete remission 2 weeks after the end of aplasia, following induction chemotherapy. Monocytes are continuously replenished throughout life via repetitive

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**Fig. 6. Cytotoxicity assays for patients 1, 2, and 4.** Leukemia-specific CTLs were activated by patient 2 (A) and patient 4 (B) mature nonleukemic DCs pulsed with autologous killed leukemic blasts and tested for their cytotoxic activity. Primed CTLs efficiently lysed autologous leukemic blasts (●). No significant killing was observed for unrelated leukemic blasts (■) or autologous unpulsed mature DCs (▲). Data points are the means for each ratio; bars, SD. For patient 1 (C), both leukemic DCs (———) and mature nonleukemic pulsed DCs (—) induced CTLs with comparable levels of cytotoxicity against autologous leukemic cells and no unrelated AML blast killing (*, P < 0.05). CTL-mediated lysis was prevented by preincubating the tumor cells with either anti-MHC class I or anti-MHC class II antibodies (D). Presented data show representative results of three independent experiments; bars, SD.
cycles of hematopoietic stem cell differentiation. Complete repopulation generally occurs within 14–21 days (47). Immature DCs generated in culture possessed the typical morphology of DCs, and the phenotypic analysis was in accordance with the immature stage of their life cycle. We show that immature DCs captured autologous leukemic blasts at late-stage apoptosis as characterized by annexin V/PI staining. The phagocytosis of leukemic blasts did not trigger any phenotypical changes indicating DC maturation. Because inhibition of the effector T-cell functions after injection of immature DCs has been described recently (48), incomplete DC maturation might have a substantial effect on induced immune responses. Thus, complete DC activation was achieved by the combination of TNF-α + poly(I:C) that reproducibly led to the homogeneously up-regulation of costimulatory and maturation-associated molecules (35). Mature tumor antigen-pulsed DCs were successfully produced for all patients enrolled in this study with minimal individual variations throughout the key steps of their generation, i.e., phenotype, kinetics of phagocytosis, and response to maturing agents. Notably, immature DC allostimulatory capacity was similar to that of uncultured leukemic blasts, whereas leukemic DCs and mature nonleukemic DCs activated by TNF-α + poly(I:C) stimulated significantly higher proliferation of allogeneic T cells. Moreover, in 2 of 3 patients, mature nonleukemic DCs were significantly more potent stimulators than the DCs of leukemic origin. These data further emphasize the importance of DC maturation for efficient T-cell stimulation and confirm that the allostimulatory capacity corresponds with the level of costimulatory molecule expression (30, 49).

We show that late-apoptotic, leukemic blast-pulsed mature DCs and leukemics DC are the only APCs successfully activating autologous leukemic blast-specific CTLs in vitro. We detected significant and comparable levels of autologous AML blast killing by CTLs induced by killed leukemic blast-pulsed mature DCs for all tested patients. No significant killing was observed against either unrelated leukemic blasts or unpulsed autologous nonleukemic mature DCs. Both anti-MHC class I and anti-MHC class II blocking antibodies substantially abrogated CTL-mediated lysis. Together these results confirm that DCs cross-present phagocytosed apoptotic leukemic blasts and strongly suggest the presence of autologous leukemia-specific CTLs directed against MHC class I- and class II-restricted tumor antigens rather than non-MHC-restricted nonspecific lysis or immune response against self-antigens. Sufficient numbers of leukemic DC's, autologous apoptotic leukemic blast-pulsed mature DCs, and autologous T lymphocytes were available for 1 patient, and we demonstrate comparable autologous leukemic blast cytotoxicity levels induced by both DC preparations. Other authors have already reported higher levels of specific cytotoxicity in AML patients. Beside the fact that different methods were used for CTL detection, this phenomenon might be explained by different culture conditions used for CTL induction. In our system, autologous T lymphocytes were neither polyclonally activated with PHA (4) or anti-CD3 nor nonspecifically stimulated by IL-2 before or during the first cycle of DC stimulation (29). The unique ability of DCs to cross-present dead cells to both CD4 and CD8 T cells allows the exploration of the effect of immunotherapy in diseases like AML, where tumor rejection antigens have not been formally identified to date. It further provides both MHC class I and class II epitopes that could theoretically activate a broad polyclonal repertoire of antigen-specific CD4 and CD8 T cells and induce a diversified immune response. Furthermore, it is applicable regardless of MHC type (50). The additional feature of AML is the fact that leukemic blasts often retain their differentiating potential, and they can be induced to become leukemic DCs. These two complementary systems permit the induction of detectable antileukemic cytotoxic responses in the majority of patients. The use of whole cells as a source of antigen has the potential disadvantage that it might induce pathological autoimmune reactivity to normal tissue antigens (51, 52). Nevertheless, our study evaluating induction of specific CTLs against autologous leukemic blasts does not demonstrate responder T-cell cytotoxicity against autologous nonleukemic DCs.

Perhaps the most intriguing aspect of the use of active immunotherapy is the intensity of chemotherapeutic regimens inducing T-cell depletion. Intensive chemotherapy depletes all lymphocyte subsets, and although the number of CD8 T cells returns to the normal levels within 3 months after therapy, major alterations have been described at the level of restricted repertoire diversity. CD4 T subset recovery is much slower, with CD4 T cells remaining depleted at 6 months after completion of chemotherapy (53). These T-cell subset anomalies could impede the efficacy of DC-based immunotherapies. Effort has to be made to develop the strategies facilitating rapid recovery of the immune system, thus allowing the incorporation of immunotherapy for the treatment of minimal residual disease (47). However, despite the lymphopenia after chemotherapy treatments, DC-based clinical trials have detected specific CTL induction, and objective responses have been observed shortly after the end of chemotherapy regimens (10, 54–56). Considering the complexity of these models, it is obvious that additional comparative studies have to be undertaken to optimize the choice of DC preparations, precise culture conditions, dose, route of injection, and time schedule of eventual vaccination protocols.

Taken together, in this report we clearly show that nonleukemic DCs pulsed with autologous late-apoptotic leukemic blasts can be generated from monocytes of AML patients in complete remission shortly after induction chemotherapy. Despite the immunosuppression after dose-intensive chemotherapy, pulsed mature DCs efficiently cross-presented engulfed leukemic cells and induced CTL responses directed specifically against the original autologous leukemic blasts for all patients tested. Finally, for 1 patient, both leukemic DCs and nonleukemic-pulsed mature DCs were available, and the induced CTLs demonstrated comparable levels of cytotoxicity. Our findings have potential therapeutic implications for the activation of immune responses in vivo using nonleukemic DCs in AML patients. We believe that the approach we offer in this report permits the generation of DC vaccines for use as adjuvant therapy for the majority of patients with hematological malignancies, although its feasibility and efficiency in the elimination of minimal residual disease remains to be addressed in additional experiments.

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Cross-Presentation of Leukemic Blasts by Pulsed DCS


Induction of Leukemia-specific Cytotoxic Response by Cross-Presentation of Late-Apoptotic Leukemic Blasts by Autologous Dendritic Cells of Nonleukemic Origin

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