We have previously reported that leukemic dendritic cells (DC) can be generated ex vivo from myelomonocytic precursors in chronic myelogenous leukemia. In this study we report the generation of DC from acute myelogenous leukemia (AML) cells and their potent ability to stimulate leukemia-specific cytolytic activity in autologous lymphocytes. DC were generated in vitro using granulocyte-macrophage colony-stimulating factor + interleukin-4 in combination with either tumor necrosis factor-α or CD40 ligand (CD40L). Cells from 19 AML patients with a variety of chromosomal abnormalities were studied for their ability to generate DC. In all but 1 case, cells with the morphology, phenotypic characteristics, and T-cell stimulatory properties of DC could be generated. These cells expressed high levels of major histocompatibility complex class I and class II antigens as well as the costimulatory molecules B7-2 and ICAM-1. In three cases these cells were determined to be of leukemic origin by fluorescence in situ hybridization for chromosomal abnormalities or Western blotting for the inv(16) fusion gene product. Autologous lymphocytes cocultured with AML-derived DC (DC-AL) were able to lyse autologous leukemia targets, whereas little cytotoxicity was noted against autologous, normal cells obtained from the patients during remission. We conclude that leukemia-derived DC may be useful for immunotherapy of many AML patients.

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**Materials and Methods**

**Patient samples.** Blood and bone marrow samples for this study were collected from patients with informed consent at the time of sample collection for diagnostic tests under guidelines and procedures approved by the institutional review board of the M.D. Anderson Cancer Center. Relevant clinical and diagnostic laboratory data for the 19 cases are shown in Table 1. Remission samples were collected during morphological remission (<5% marrow blasts) after therapy for the disease. Patients 1 and 11 were the only ones with any history of antecedent hematological disorder.

**Generation of DC from AML cells.** Recombinant human IL-4 was kindly provided by Schering-Plough, Kenilworth, N J. Recombinant human TNF-α was kindly provided by The National Institutes of Health, Bethesda, MD. Recombinant trimeric human CD40L was kindly provided by Immunex Corp, Seattle, WA. This is a trimeric, soluble molecule capable of binding to and activating the CD40 receptor. Peripheral blood mononuclear cells (PBMC) were separated from AML patients and normal donors using a Histopaque-1077 density gradient (Sigma, St Louis, MO) and cultured immediately or cryopreserved. To generate DC, mononuclear cells at 2 to 4 × 10^6 cells/mL were cultured in 75-mm^2 tissue culture flasks (Corning Inc, Corning, NY) in AIM-V serum-free medium (GIBCO Laboratories, Grand Island, NY) with various combinations of human cytokines. Recombinant GM-CSF (Immunex) and IL-4 were added at a final concentration of 1,000 U/mL. Recombinant TNF-α was used at a final concentration of 500 U/mL. Recombinant CD40L was added at a concentration of 1 μg/mL. The medium was changed every 3 to 4 days, and the cells were cultured for 6 to 7 days. The results of this study were reported in accordance with the Code of Federal Regulations, Title 21, Parts 50 and 540.

**Generation of DC.** Mononuclear cells from patients with AML were cultured in 75-mm^2 tissue culture flasks (Corning Inc, Corning, NY) at 2 to 4 × 10^6 cells/mL in AIM-V serum-free medium (GIBCO Laboratories, Grand Island, NY) with various combinations of human cytokines. Recombinant GM-CSF (Immunex) and IL-4 were added at a final concentration of 1,000 U/mL. Recombinant TNF-α was used at a final concentration of 500 U/mL. Recombinant CD40L was added at a concentration of 1 μg/mL. The medium was changed every 3 to 4 days, and the cells were cultured for 6 to 7 days. The results of this study were reported in accordance with the Code of Federal Regulations, Title 21, Parts 50 and 540.

**Purification of DC.** Mononuclear cells from patients with AML were cultured in 75-mm^2 tissue culture flasks (Corning Inc, Corning, NY) at 2 to 4 × 10^6 cells/mL in AIM-V serum-free medium (GIBCO Laboratories, Grand Island, NY) with various combinations of human cytokines. Recombinant GM-CSF (Immunex) and IL-4 were added at a final concentration of 1,000 U/mL. Recombinant TNF-α was used at a final concentration of 500 U/mL. Recombinant CD40L was added at a concentration of 1 μg/mL. The medium was changed every 3 to 4 days, and the cells were cultured for 6 to 7 days. The results of this study were reported in accordance with the Code of Federal Regulations, Title 21, Parts 50 and 540.
unfractionated AML cell cultures were used for phenotypic and functional studies.

**Phenotypic analysis of cells by flow cytometry.** Fluorescein- or phycoerythrin-conjugated mouse monoclonal antibodies against CD3, CD14, CD19, as well as fluorochrome-labeled isotype controls were purchased commercially (Becton Dickinson, Duarte, CA). Fluorescein- or phycoerythrin-labeled antibodies against HLA-D, HLA-A, HLA-B, HLA-C, CD1a, CD40, CD54, CD80, and CD86 were purchased from Pharmingen (San Diego, CA). Freshly isolated or day-14 cells were dispersed to single-cell suspension by gentle trituration through a

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<th>Patient</th>
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<th>Sample</th>
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Numbers in the square brackets represent the number of metaphases counted, showing a particular chromosomal abnormality. The sum of these numbers represent the total number of metaphases analyzed. Percentage blasts were determined by histological examination of a bone marrow aspirate at the time of sample collection.

Abbreviation: BM, bone marrow.
pipette. They were then incubated with various combinations of antibodies, washed with phosphate-buffered saline (PBS)-hIgG and analyzed using a FACScan II flow cytometer and the LYSIS software (Becton Dickinson). Forward scatter versus side scatter gates were set up during analysis to eliminate debris and clumps, and the remaining cell populations were analyzed for expression of phenotypic markers.

Genetic analysis of cells by fluorescence in situ hybridization (FISH). To determine if the DC generated in culture were derived from the leukemic clone or the normal population of the patients’ peripheral blood cells, we selected 2 patients whose leukemic cells were known to exhibit a specific chromosomal abnormality, ie, trisomy of chromosome 8, that could be easily identified in the interphase nuclei by FISH. Freshly isolated PBMNC, as well as dendritic cells generated in the culture following 14 days of incubation with GM-CSF, IL-4, and TNF-α or CD40 ligand were analyzed. Fluorescein-labeled chromosome 8–specific alpha satellite DNA probe (Oncor, Gaithesburg, MD) was used according to the manufacturer’s instructions. At least 400 interphase nuclei were examined in each sample to quantitate the percentage of cells with three hybridization signals for chromosome 8.

Western blotting for CBFB-MYH11. This was performed as previously described9 using polyclonal antipeptide antibodies. Antisera included C2 and an antiserum to an MYH11 peptide developed as described.9

Allogeneic mixed leukocyte response. PBMNC from normal volunteers were used as responder cells. Freshly isolated or 10- to 14-day cultured AML cells irradiated with 30 Gy were used as stimulator cells. A total of 2 × 10⁶ allogeneic PBMNC were added to varying numbers of accessory cells. Lymphocyte proliferation was measured after 5 days of culture with addition of ³H-thymidine for 18 hours.

Expansion of T cells from AML patients and stimulation with autologous DC. PBMNC of AML patients were suspended at a concentration of 5 × 10⁶ cells/mL and cultured in Aim-V containing 1 µg/mL anti-CD3 antibody (Ortho Biotech, Raritan, NJ) and 100 U/mL of IL-2. Nonadherent cells were resuspended in fresh medium containing 100 U/mL IL-2 every 3 days. On day 7 the cells were washed and cocultured at a ratio of 3:1 with washed autologous AML-DC or freshly thawed washed autologous leukemia cells in Aim-V Medium without additives for 3 days. The dendritic cell activated lymphocytes (DC-AL) or fresh leukemia-stimulated lymphocytes were expanded for an additional 4 to 7 days in IL-2 containing medium before being assayed for antileukemic activity.

T-cell cytotoxicity assay. T-cell cytotoxicity was measured in vitro using the lactate dehydrogenase-release assay.10 Cryopreserved, thawed target cells were cocultured with T cells at effector target ratios of 1:1, 1:10, and 1:25 ratios for 6 hours in 96-well round-bottomed plates in phenol red-free RPMI 10.1% human albumin (Miles Inc, Elkhart, IN). Spontaneous release of effector and target cells was controlled by separate incubation of the respective populations. Maximal LDH enzyme release was measured after lysis of the target cells with 0.5% triton X-100 (Sigma). Cell-free supernatants were incubated in a separate 96-well plate with lactate dehydrogenase (LDH) substrate10 for 10 minutes before measuring absorbance using a microplate reader (Dynatech MR5000) at 490 nm with a 650-nm reference. Percentage cytotoxicity was calculated according to the formula:

% Age Cytotoxicity = [{(E – St – Se)/M – St}] × 100
where E is the LDH release by effector-target coculture, St is the

Fig 2. AML cells cultured in the triple cytokine combinations upregulate expression of costimulatory molecules. PBMNC from AML patients were cultured in the triple cytokine cocktail for 14 days. Cells were labeled with fluorescent monoclonal antibodies and analyzed by flow cytometry. (A) Results obtained with the cells of patient 18 cultured for 14 days in Aim-V alone. (B) Cells cultured in GM-CSF, IL-4, and TNF-α. (C) Cells cultured in GM-CSF, IL-4, and CD40L. Cells from patients 7 and 10 through 13 were also studied in similar experiments and comparable increases in CD1a and costimulatory molecule expression were observed.
spontaneous release by target cells, Se is the spontaneous release by effector cells, and M is the maximal release by target cells.

RESULTS

Characteristics of AML PBMNC cultured in various cytokine combinations. PBMNC from AML patients of a variety of FAB types, clinical histories, and cytogenetics were used to generate DC. Clinical details of the AML patients whose cells were used are listed in Table 1. As may be seen, patients had a variety of FAB types and cytogenetic changes. Two patients (patients 1 and 11) had histories of antecedent hematological disorders (anemia or myelodysplastic syndromes) before AML diagnosis. Nearly all cultures for this work were initiated from samples cryopreserved at a previous date. Because of the limited material available from any given patient, not all studies shown could be done for every patient’s leukemia.

Cultures of AML cells were replenished with fresh medium and cytokines every 3 to 4 days. Cells cultured in various cytokine combinations were assessed for total number of viable cells and cells with dendritic morphology after various culture periods. Approximately 25% to 45% of the initial cell population was recovered after 9 days of culture. No significant change in numbers of cells was observed between day 9 and day 20 of culture (not shown). Similar yields of viable cells were noted for AML cell cultures from other patients. Freshly isolated AML PBMNC displayed a spherical morphology without discernable dendritic processes (Fig 1A). Between days 10 and 14, cultures with GM-CSF, IL-4, and TNF-α (Fig 1B) or GM-CSF, IL-4, and CD40L (Fig 1C) consistently displayed an increase in cell size and showed cell clusters with dendritic morphology in all but one AML patient (patient 16).

Phenotype of cultured AML cells. Flow cytometry has been performed on various cases of AML cultured for 11 to 14 days in GM-CSF, IL-4, and TNF-α or GM-CSF, IL-4, and CD40L. In each of these cases the flow cytometric phenotype of the cytokine-cultured cells has been compared with autologous leukemia, either freshly thawed cells or cells cultured for equivalent periods in medium without cytokines. Figure 2 shows the phenotype of AML cells of one representative patient (patient 18), cultured in medium alone (Fig 2A); GM-CSF, IL-4, and TNF-α (Fig 2B); or GM-CSF, IL-4, and CD40L (Fig 2C). Consistent with the increase in cells of dendritic morphology, the triple cytokine cultures showed an upregulation of the dendritic cell lineage marker CD1a as well as the costimulatory molecules B7-1 (CD80), B7-2 (CD86), CD40, and ICAM-1 (CD54). Cells from patients 7 and 10 through 13 were also cultured and analyzed by flow cytometry. In all of these other five cases examined, culture with either cytokine combination generated increases in expression of CD1a, CD80, CD86, and ICAM-1. The cultured cells were negative for CD3 and CD19 (data not shown). Morphological and phenotypic changes of cultured AML cells similar to those described in the current study were also noted with other AML patients in a previous report from our laboratory.11
Demonstration of leukemic origin of cultured DC. To confirm that the DC arising in culture are in fact derived from the leukemic cells we selected cases with identifiable clonal markers. Cells from patient 7, with trisomy 8, were cultured in the triple cytokine cocktails and analyzed by interphase FISH for \( ^{+}8 \) genotype. The results obtained with cells cultured in GM-CSF + IL-4 + TNF-\( \alpha \) are shown in Fig 3. Freshly isolated PBMC from patient 7 displayed trisomy 8 in 93% of the cells. After 14 days of culture in GM-CSF + IL-4 + TNF-\( \alpha \), 92% of the cells showed trisomy 8. Eighty-eight percent of the cells cultured in GM-CSF + IL-4 + CD40L were positive for \( ^{+}8 \).

Given that 44% to 87% of the cells in these cultures expressed the morphology and immunophenotype of DC in these cultures (data not shown), the leukemic clones would have to include the dendritic cells in these cultures. Cells from patient 19 cultured in a similar manner with GM + IL-4 + TNF-\( \alpha \) showed trisomy 8 in 88% of the cells.

Patient 13, whose leukemic karyotype showed inv(16), yielded cells that on culture were 63% DC. These cells were submitted for Western blotting using antisera directed at the two constituent moieties of the CBFB-MYH11 fusion protein. Results are shown in Fig 4. Signals for both CBFB and MYH11 corresponding to the 70-kD signal expected for the type A variant of this protein\(^ {12} \) were seen in both the cultured DC and the thawed native leukemic cells. These bands are similar in intensity, indicating that the cultured cell population continued to express the leukemia associated protein.

Allostimulatory activity of the cultured DC. Freshly isolated and cultured AML cells were examined for their ability to stimulate allogeneic T cells in a mixed leukocyte response (MLR) assay. Allogeneic MLR assays were performed as described in Materials and Methods. Freshly isolated (□), GM + IL-4 + TNF-\( \alpha \)-cultured (●), and GM + IL-4 + CD40L-cultured (▲) AML cells from five patients were compared for their ability to stimulate PBMC from a normal allogeneic donor. Results represent the mean ± SD of triplicate cultures.

Fig 6. Stimulation of autologous, antileukemic cytotoxicity by fresh AML cells, AML-DC cultures, and IL-2 alone. Autologous T cells were cocultured with fresh AML PBMC, AML-DC-rich cultures, or IL-2 alone as described in Materials and Methods. They were then tested for cytotoxicity against autologous leukemia targets by LDH-release assay. Results represent the mean cytotoxicity of triplicate cultures. (□) AML-DC-stimulated T cells, (●) fresh AML PBMC stimulated T cells, (▲) T cells cultured in IL-2 alone. Cytotoxicity of DC-AL compared with IL-2-stimulated T cells at 25:1 ratio is significant (\( P < .005, \) Student’s t-test) for each patient. Cytotoxicity of fresh AML cell-stimulated T cells compared with IL-2-stimulated T cells at 25:1 ratio is significant (\( P < .010, \) Student’s t-test) for patient 13.
(MLR) assay. Figure 5 compares the allostimulatory ability of fresh TNF-α combination and CD40L combination cultured PBMC from five AML patients. In each case GM-CSF + IL-4 together with TNF-α or CD40L showed a higher allostimulatory potential than freshly isolated AML cells.

Stimulation of autologous antileukemic activity by AML derived DC. Autologous T cells were cocultured with freshly isolated or cultured DC-rich AML cells and tested for cytotoxicity against autologous leukemic or autologous PBMC obtained in remission. Figure 6 represents the autologous antileukemic cytotoxicity of T cells stimulated with AML-DC, fresh AML cells, or IL-2 alone. In each of these patients, stimulation of autologous lymphocytes with AML-DC markedly increased their cytotoxicity against autologous leukemia targets. Fresh AML cells could induce a minor level of cytotoxicity in 1 of 4 patients. IL-2-expanded lymphocytes failed to show antileukemic cytotoxicity at the target:effector ratio tested. Figure 7A through C represents the cytotoxicity of DC-AL against autologous leukemia and remission cells. In Fig 7A, GM-CSF + IL-4 + TNF-α was used to generate the DC from five additional patients, whereas GM-CSF + IL-4 + CD40L was used to generate DC in Fig 7B. Figure 7C represents the reactivity of DC-AL used in Fig 7B, against autologous remission cells. Results depicted in Fig 7B and C comprise a single experiment with the same patient samples, whereas those depicted in Fig 7A represent a different experiment.

DISCUSSION

These findings show that cells of DC phenotype and function can be derived from the majority of human AML samples. Given the known biological heterogeneity of AML, it is surprising that the results are variable and that occasional AML cell samples would fail to show differentiation toward DC. The leukemias show highly variable degrees of maturation arrest in vivo and highly variable degrees of monocytoid differentiation. The ontogeny of DC is thought to proceed directly through CD34 progenitors or through the monocyte lineage, and in fact cells of dendritic cell phenotype may be derived from normal monocytes.6 The ability of a given leukemia to differentiate toward DC may be tied to the level of maturation block in the monocytoid lineage.

Santiago-Schwarz8 and colleagues described the generation of DC-like cells from the PBMC of a single AML patient. In their studies, the terminal differentiation of an adherent DC-like population was achieved using a combination of GM-CSF, TNF-α, and IL-6. In the present study IL-6 failed to show any significant effect in generating DC-like cells from AML PBMC or increasing allostimulatory ability of cultured AML-PBMC.
cells. The result of our study suggests that the addition of CD40 on the APC by anti-CD40 antibody or exogenous CD40L interactions. Nature 393:480, 1998

The potential therapeutic implications of this work are substantial. Leukemic DC can potentially be used as a cellular leukemia vaccine in vivo or to generate antileukemic T cells in vitro for adoptive immunotherapy. Their use in immunotherapy may eliminate minimal residual disease and convert transient responses into durable remissions in this normally fatal disease.

**REFERENCES**


Dendritic Cells Derived In Vitro From Acute Myelogenous Leukemia Cells Stimulate Autologous, Antileukemic T-Cell Responses