Stimulation of autologous proliferative and cytotoxic T-cell responses by “leukemic dendritic cells” derived from blast cells in acute myeloid leukemia

Beth D. Harrison, Julie A. Adams, Mark Briggs, Michelle L. Brereton, and John A. Liu Yin

Effective presentation of tumor antigens is fundamental to strategies aimed at enrolling the immune system in eradication of residual disease after conventional treatments. Myeloid malignancies provide a unique opportunity to derive dendritic cells (DCs), functioning antigen-presenting cells, from the malignant cells themselves. These may then co-express leukemic antigens together with appropriate secondary signals and be used to generate a specific, antileukemic immune response. In this study, blasts from 40 patients with acute myeloid leukemia (AML) were cultured with combinations of granulocyte-macrophage colony-stimulating factor, interleukin 4, and tumor necrosis factor α, and development to DCs was assessed. After culture, cells from 24 samples exhibited morphological and immunophenotypic features of DCs, including expression of major histocompatibility complex class II, CD1a, CD83, and CD86, and were potent stimulators in an allogeneic mixed lymphocyte reaction (MLR). Stimulation of autologous T-cell responses was assessed by the proliferative response of autologous T cells to the leukemic DCs and by demonstration of the induction of specific, autologous, antileukemic cytotoxicity. Of 17 samples, 11 were effective stimulators in the autologous MLR, and low, but consistent, antileukemic cytotoxicity was induced in 8 of 11 cases (mean, 27%; range, 17%-37%). This study indicates that cells with enhanced antigen-presenting ability can be generated from AML blasts, that these cells can effectively prime autologous cytotoxic T cells in vitro, and that they may be used as potential vaccines in the immunotherapy of AML.

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Introduction

The major cause of treatment failure in acute myeloid leukemia (AML) is relapse of the disease. Relapse rates are considerably lower in patients who undergo allogeneic bone marrow transplantation (BMT). This is attributed to a graft-versus-leukemia effect mediated by the donor-derived immune system, principally T cells. 

Stimulation of autologous proliferative and cytotoxic T-cell responses by “leukemic dendritic cells” derived from blast cells in acute myeloid leukemia

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precursors and that reversal of the granulocyte maturation pathway can also yield candidate DCs in the presence of GM-CSF, IL-4, and TNF-α. Myeloid malignancies therefore provide a unique opportunity to derive APCs from the malignant cells themselves, which may then combine expression of leukemic antigens with the presence of the necessary costimulatory signals and be used to generate a specific antileukemic immune response. DCs have been generated from AML blasts in vitro, but published data on the ability of these “leukemic DCs” to generate autologous cytotoxic T-cell responses is sparse.

In this study, several combinations of GM-CSF, IL-4, and TNF-α were used in suspension cultures of AML blasts to generate leukemic DCs from a wide range of AML French-American-British (FAB) types. Morphological, immunophenotypic, and functional parameters were used to assess DC production and possible differences in DC potential, and the optimal cytokine conditions for different AML FAB types were studied. We investigated the ability of the generated leukemic DCs to stimulate proliferative and cytotoxic responses by autologous T cells; these data were used as markers of effective antigen presentation and cytotoxic T lymphocyte (CTL) generation by leukemic DCs.

Patients, materials, and methods

Patient samples
PB and bone marrow (BM) samples were from 40 patients with AML at presentation or relapse. Samples were taken with informed consent at the time of diagnostic tests. To obtain homogeneous blast populations, mononuclear cells (MNCs) from consecutive samples with high percentages of blasts were isolated by centrifugation of whole PB or BM over a Ficoll-Paque density gradient (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, United Kingdom). Cells were used immediately or cryopreserved in 90% fetal calf serum (FCS) (Applied Protein Products, West Midlands, United Kingdom), 10% dimethyl sulfoxide (Sigma Aldrich, Poole, United Kingdom). Patient details are given in Table 1.

Generation of leukemic DCs from AML blasts
Cryopreserved MNCs were thawed and washed in serum-free medium; fresh and cryopreserved cells were suspended at 1 x 10^5 cells per milliliter in McCoys 5A medium (Life Technologies, Paisley, Scotland) supplemented with 10% to 15% heat-inactivated serum or plasma and cultured in 12-well plates (Falcon, Oxford, United Kingdom) with various combinations of cytokines. The majority of samples were cultured in 10% human blood group AB serum, and in initial experiments, this was compared with 15% FCS or 10% autologous plasma (with 20 U/mL preservative-free heparin [CP Pharmaceuticals, Wrexham, United Kingdom]). GM-CSF (Schering-Plough, Kenilworth, NJ) was used at 100 ng/mL, IL-4 (Sigma Aldrich) at 20 ng/mL, and TNF-α (Sigma) at 10 ng/mL. Samples were cultured for 7 to 14 days with GM-CSF, with or without IL-4 and with or without TNF-α. TNF-α was added at day 0 or 2 days before the end of the culture period. Samples were also cultured without cytokines and with each cytokine alone. All cultures, mixed lymphocyte reactions (MLRs), and cytotoxicity assays were maintained at 37°C in 5% CO₂ and a humidified atmosphere. Cultured cells were observed by phase-contrast microscopy for evidence of increasing size and formation of clusters. At the end of culture, cell count was measured on an automated cell counter (Sysmex, Milton Keynes, United Kingdom). Cyto centrifuge preparations of unmanipulated and cultured blasts were stained with Giemsa. Review of these cytospin preparations allowed assessment of viability and maturation. Cells that had increased in size, acquired copious gray cytoplasm without cytoplasmic granules or vacuoles, and developed long cytoplasmic processes were defined as mature leukemic DCs morphologically (Figure 1).

Immunophenotype of fresh and cultured AML blasts
Unmanipulated and cultured AML samples were stained with fluorescein isothiocyanate (FITC)-, phycoerythrin-, and peridinin chlorophyll protein (PerCP)-conjugated mouse monoclonal antibodies (mAbs) against CD1a, CD14, CD68 (Pharmingen, Oxford, United Kingdom), HLA-DR (Becton Dickinson, Oxford, United Kingdom), and CD83 (Coulter, Latun, United Kingdom), or with appropriate isotype-matched control mAbs. Cells were incubated with mAb for 30 minutes at 4°C, washed once in phosphate buffered saline (PBS), and resuspended in a small volume of PBS for analysis by means of a FACScan flow cytometer (Becton Dickinson) with CellQuest version 3.1 acquisition and analysis software (Becton Dickinson). Forward- and side-scatter gates were established to exclude cell debris and clumps prior to analysis for expression of each phenotypic marker.

Fluorescence in situ hybridization
To determine the leukemic origin of the DCs generated in culture, cells from patients with leukemias that exhibited trisomy 8 on diagnostic cytogenetic testing were examined by means of fluorescence in situ hybridization (FISH) before and after culture. Cells were treated with colcemid for 2 hours and then fixed in 3:1 methanol to acetic acid. Chromosome 8 was identified in both metaphase and interphase cells by means of an alpha satellite probe for chromosome 8 labeled with Texas red.

Allergenic and autologous MLRs
Responder cells for the autologous MLRs were MNCs obtained by density centrifugation of PB from normal volunteers. Responder cells for the autologous MLRs were MNCs obtained by separation of thawed PB progenitor cell (PBPC) collections or fresh PB taken from patients in continuing morphological remission. Responders were plated at 1 x 10^5 per well in McCoys 5A medium with 10% AB serum in 96-well U-bottomed plates (Falcon). Leukemic DCs were used as stimulus cells after being irradiated (30 Gy). Stimulators were added to responder cells, in triplicate wells, in a total volume of 200 μL. Stimulator-to-responder combinations were plated at as many possible different ratios (1:1024, 1:256, 1:64, 1:16, 1:4, 1:1) as numbers of cultured cells allowed. Controls were uncultured AML blasts, blasts cultured in the absence of cytokines, and, for the autologous MLRs, autologous MNCs or leukemic DCs. All controls were irradiated. Lymphocyte proliferation was measured by means of 3H-thymidine (ICN, Oxfordshire, United Kingdom) incorporation (37 kBq per well). 3H-thymidine was added for the last 18 hours of a 5-day culture. Proliferative responses more than 4-fold greater than controls were considered positive for both autologous and autologous MLRs. As a nonspecific measure of T-cell proliferation potential, some wells contained responders alone stimulated with phytohemagglutinin (PHA) 1%. The proliferative potentials of normal and remission T cells were assessed by comparison of the responses with PHA stimulation. Flow cytometric analysis using an FITC-conjugated mAb against CD3 (Becton Dickinson) was used to measure percentages of T cells in MNCs from normal PB and PBPC collections.

Stimulation of T cells from AML patients by autologous DCs
Autologous responder cells derived from thawed PBPC collections or fresh PB were suspended at 1 x 10^6 cells per milliliter in McCoys 5A medium supplemented with 10% AB serum and IL-2 (Becton Dickinson). 20 U/mL. Washed, irradiated, autologous leukemic DCs derived from DC cultures with the optimum cytokine combination for each sample were added at responder-to-stimulator ratios of between 5:1 and 10:1. Further IL-2 was added every 3 to 4 days, and cultures were refed with media at day 7. Controls were (1) responder cells cultured with IL-2 but not primed with leukemic DCs and (2) normal allogeneic responders primed with leukemic DCs (positive control). Cytotoxicity assays were performed on day 14.

Cytotoxicity assays
Cytotoxicity assays were performed with lactate dehydrogenase (LDH)—release and flow cytometric methods. In the majority of cases, the
for targeted, T-cell–mediated cellular cytotoxicity. 21,22 Briefly, targets were
removed debris and dead cells. Targets were stained with 3,3-
9-dioctadecyloxacridine (DiOC18), a green fluorescent membrane dye (emission
maximum 501 nm, visualized in the FL1 channel by flow cytometry) that is
permanently incorporated into cell membranes, for 20 minutes in the dark at
37°C and then washed twice in Phenol Red free RPMI medium (Life
Technologies) containing 5% FCS. Day-14 stimulated T-cell populations
("effectors") were also centrifuged over a Ficoll-Paque density gradient and
washed. We added 10^4 to 5 × 10^6 effectors to polystyrene tubes (Falcon)
and set up in quadruplicate wells. For personal use only. on October 22, 2017. For personal use only.
The effects of differences in length of culture, sera, source of cytokines (Figure 3, Table 1). Samples that combined high-level expression of MHC class II after culture and expression of at least one of CD86, CD1a, and CD83 with very low-level expression of CD14 (on fewer than 10% of cells) were defined as leukemic DCs immunophenotypically. At the end of the culture period, cells from 13 cases combined expression of MHC class II with expression of one other DC marker; cells from 15 cases expressed MHC class II and at least 2 other phenotypic markers of DCs. None of these expressed CD14 at the end of culture. Among these 28 cases, for blasts cultured with the cytokine combination shown in Table 1 in each case, 71% ± 21% of cells were positive for MHC class II; 24 cases expressed CD86 on 32% ± 16% of cells; 17 cases expressed CD1a on 28% ± 19% of cells; and 9 (of 10 tested) expressed CD83 on 24% ± 14% of cells. After culture, expression of CD14 was low overall and was further reduced in the presence of IL-4 ($P < .00001$). This difference was also significant for FAB types M4 and M5 considered alone; initial expression of CD14 on cells from M4/M5 AMLs was 24% ± 27%; expression of CD14 after culture in the absence of IL-4 was 16% ± 23% and, in the presence of IL-4, 12% ± 24% ($P < .01$). This is in addition to significantly greater morphological maturation of cells from M4/M5 AMLs in GM-CSF, IL-4, and TNF-α than in GM-CSF and TNF-α alone (Table 2).

The percentages of expression of each of the phenotypic markers of DCs studied (MHC class II, CD86, CD1a, and CD83) were individually correlated with functional data from allogeneic and autologous MLRs and cytotoxicity assays.

**Statistical analyses**

Results were analyzed by means of standard equations for the mean, SD, Student paired and unpaired $t$ tests, and correlation coefficients.

**Results**

**Cultures**

The mean percentage of blasts in the initial populations was 93% ± 12% (mean ± SD); percentage viability was 99% ± 2%. After 7 to 14 days' culture, cell counts varied widely; overall, total density of viable cells at the end of culture was $1.19 \pm 0.73 \times 10^6$ cells per milliliter (initial cell density was $1 \times 10^6$ cells per milliliter). For 25 of 40 samples, total cell numbers at the end of culture with at least one cytokine combination had increased, whereas total counts at the end of culture were fewer than 50% of initial cell numbers in 11 cases and fewer than 25% in 5 of these. In 29 cases, mature cell numbers exceeded 25% of the starting cell number; in 17 of these, they exceeded 50% (Figure 1). For each sample, numbers of mature cells after culture are given in Table 1. The effects of differences in length of culture, sera, source of cultured cells (fresh or cryopreserved), FAB type, and cytokine combination on total cell numbers and numbers of DCs are listed in Table 2. Because the trend is that cultures longer than 9 days yield fewer cells and fewer mature cells than shorter cultures (Table 2), the majority of samples in this study were cultured for 7 to 8 days. Overall, the timing of the addition of TNF-α did not consistently affect development of leukemic DCs, although the results indicated that the "best" cytokine combinations for maturation of leukemic DCs differed between samples. The cytokine combinations that best combined evidence of morphological maturation with phenotypic and functional evidence of maturation for each sample are given in Table 1, and the leukemic DCs generated in these combinations were used in subsequent cytotoxicity assays.

**Immunophenotype**

The immunophenotype of the cultured blasts was compared with that of fresh, uncultured blasts or blasts cultured in the absence of cytokines (Figure 3, Table 1). Samples that combined high-level expression of MHC class II after culture and expression of at least one of CD86, CD1a, and CD83 with very low-level expression of CD14 (on fewer than 10% of cells) were defined as leukemic DCs immunophenotypically. At the end of the culture period, cells from 13 cases combined expression of MHC class II with expression of one other DC marker; cells from 15 cases expressed MHC class II and at least 2 other phenotypic markers of DCs. None of these expressed CD14 at the end of culture. Among these 28 cases, for blasts cultured with the cytokine combination shown in Table 1 in each case, 71% ± 21% of cells were positive for MHC class II; 24 cases expressed CD86 on 32% ± 16% of cells; 17 cases expressed CD1a on 28% ± 19% of cells; and 9 (of 10 tested) expressed CD83 on 24% ± 14% of cells. After culture, expression of CD14 was low overall and was further reduced in the presence of IL-4 ($P < .00001$). This difference was also significant for FAB types M4 and M5 considered alone; initial expression of CD14 on cells from M4/M5 AMLs was 24% ± 27%; expression of CD14 after culture in the absence of IL-4 was 16% ± 23% and, in the presence of IL-4, 12% ± 24% ($P < .01$). This is in addition to significantly greater morphological maturation of cells from M4/M5 AMLs in GM-CSF, IL-4, and TNF-α than in GM-CSF and TNF-α alone (Table 2).

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**Functional tests: MLRs**

All allogeneic and autologous MLRs were performed at several stimulator-to-responder ratios; complete data from 2 experiments are illustrated in Figures 4 and 5. Cultured cells that stimulated $^3$H-thymidine uptake by normal allogeneic responders more than 4-fold the $^3$H-thymidine uptake in response to controls (unmanipulated AML blasts or blasts cultured without cytokines, each from the same patient as the cultured cells) at stimulator-to-responder ratios of 1:4 were defined as leukemic DCs functionally. Cultured cells from 31 samples were tested as stimulators in an allogeneic MLR in this study. Cells from 25 samples induced proliferation of

![Figure 1. Cultured Leukemic DCs.](image-url)
versus specific foreign epitopes in association with self-MHC, we
the numbers of T cells responding to foreign MHC molecules
MLRs. Although this difference may simply reflect differences in
H-thymidine uptake, were much lower than those in the allogeneic
responses to controls (mean, 2-fold; range, less than 1-3).

Remission material as a source of autologous responder cells
for the autologous MLR and for cytotoxicity assays was available
for 17 patients. Cultured leukemic DCs from 11 patients induced
stimulation by cultured cells were up to 4-fold greater than
normal, allogeneic T cells at least 4-fold greater than responses to
controls (mean, 62-fold; range, 4-193) (Table 1). For 6 of the 31
samples tested, proliferative responses of normal allogeneic cells
to stimulation by cultured cells were up to 4-fold greater than
response to controls (mean, 2-fold; range, 1-3) and were therefore
considered negative.

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spontaneous target death, which appeared to be partly due to the staining procedure and which prevented assessment of specific antileukemic cytotoxicity in 6 cases in which autologous T-cell/blast pairs were available.

When expression of phenotypic markers was correlated with activity in allogeneic MLRs, $P$ values $<.05$ were found for the following: MHC class II, $r = 0.40$, $P < .05$; CD86, $r = 0.57$, $P < .002$; CD83, $r = 0.72$, $P < .01$. Expression of CD1a was not

Figure 2. FISH showing trisomy 8 in cultured cells from patient 5. For this patient, 79% of cells carried trisomy 8 before culture; 55% carried trisomy 8 after culture.

Figure 3. Immunophenotype of cultured and uncultured AML blasts. Filled and unfilled histograms indicate expression of the labeled mAb and of an appropriate isotype control. Numbers indicate percentage of positive cells after subtraction of controls (mean fluorescence intensity of positive cells). (A) The immunophenotype of day-0 and day-7 cells from patient 3 (cultured with GM-CSF with TNF-$\alpha$ added for the last 2 days). (B) The immunophenotype of day-0 and day-7 cells from patient 14 (cultured with GM-CSF, IL-4, and TNF-$\alpha$ all from day 0).

Figure 4. Allogeneic MLR. Leukemic blasts from patient 36 cultured with various combinations of cytokines and tested as stimulators in an allogeneic MLR. Responders were normal PB MNCs. G = GM-CSF, I = IL-4, T = TNF-$\alpha$, 0 = day 0, 5 = day 5 of culture. Data points plus error bars indicate mean $\pm$ SD for each ratio tested for each cytokine combination.

Figure 5. Autologous MLR. Leukemic blasts from patient 29 cultured with various cytokine combinations and tested as stimulators in an autologous MLR. Responders in this experiment were autologous PBPC MNCs. Data points plus error bars indicate mean $\pm$ SD for each ratio.
Laboratory capacity was poor or not investigated because of an antigen capture and leads to up-regulation of MHC and accessory proteins.23 The addition of TNF-α to DC cultures from normal PB MNCs halts myeloid and macrophage development from myelomonocytic precursors. IL-4 suppresses macrophage cytokine to which myeloid DCs are universally responsive, as are myeloid and macrophage progenitors. Proliferative responses in allogeneic and autologous MLRs were well correlated for the 17 samples for which paired data were available, r = 0.80, P < .001. No significant relationships between functional activities in allogeneic or autologous MLRs and in cytotoxicity assays were detected.

Discussion

In this study, cells possessing the morphological, phenotypic, and allostimulatory properties of DCs have been generated from MNC populations from PB or BM of 24 of 40 patients with AML at presentation or relapse. In addition, stimulation of proliferative responses of autologous T cells was demonstrated in 11 of 17 cases, and induction of autologous antileukemic cytotoxicity in 8 of 11.

AML encompasses a biologically heterogeneous group of clonal disorders of myeloid precursors. To maximize the potential for development of DCs from the leukemic blasts, several cytokine combinations were investigated in this study. GM-CSF is a cytokine to which myeloid DCs are universally responsive, as are myeloid and macrophage progenitors. IL-4 suppresses macrophage and monocyte development from myelomonocytic precursors.23

The addition of TNF-α to DC cultures from normal PB MNCs halts antigen capture and leads to up-regulation of MHC and accessory molecules and T-cell stimulatory capacity.15,24 We have investigated development of DCs from primitive (M0/M1), maturing (M2), and myelomonocytic (M4/M5) leukemias. However, in 16 of 40 samples there was no evidence of generation of leukemic DCs; either the blasts did not survive in culture,7 there was no morphological or immunophenotypic evidence of maturation,8 or allostimulatory capacity was poor1 or not investigated because of an insufficient number of cells after culture.7 The wide variations in our ability to generate leukemic DCs from different samples persisted across FAB types. It was not possible to postulate any characteristic of an AML predictive of generation of cells with evidence of antigen-presenting capability from our data, in part owing to the relatively small numbers of samples studied in each subgroup.

The leukemic origin of the DCs generated is supported by data from the FISH analysis, indicating survival of cells carrying trisomy 8. In other cases, the leukemic origin of cultured cells was not proven formally.

In 19 of the 24 samples producing leukemic DCs, the cytokine combination that gave greatest evidence of morphological maturation to DCs also gave highest expression of relevant DC markers and greatest allostimulatory capacity. We went on to use these combinations to generate leukemic DCs for stimulation of autologous T-cell effectors for cytotoxicity assays.25

In the allogeneic MLR, functional DCs are dramatically better stimulator cells than other APCs, such as monocytes or B cells,25 and should stimulate significant proliferation of allogeneic T cells at low DC–to–T-cell ratios. Leukemic DCs generated from the majority of samples in this study were effective stimulators in the allogeneic MLRs. Proliferative responses in the autologous MLRs indicated presentation of “foreign” antigens to autologous T cells by leukemic DCs. That these antigens represent extraneous proteins introduced during the experiment, or self-, nonleukemic antigens, cannot be formally excluded. However, stimulatory capacities in allogeneic and autologous MLRs were closely correlated (P < .001), and allostimulatory capacity was correlated with expression of 3 specific DC markers: MHC class II, CD86, and CD83 (P < .05 for each comparison).

It is not always possible to predict the clinical efficacy of an immunotherapeutic strategy from data generated from the in vitro assessment of the function of experimentally primed T cells. In particular, data from cytotoxicity assays are difficult to interpret and often poorly reproducible. These experiments are heavily reliant on the initial viability of thawed targets. In our experience, LDH-release and chromium-release assays have particular problems with high background release, which swamps experimentally induced cell death. Nonetheless, despite practical difficulties, we

![Figure 6. Cytotoxicity of unprimed and primed effectors against unmanipulated autologous AML blasts.](image)

![Figure 7. Cytotoxicity assay, patient 33.](image)
have demonstrated low but consistent stimulation of autologous killing of leukemic blasts by cytotoxic T cells through priming with leukemic DCs in 8 of 11 cases (mean cytotoxicity, 27%; range, 17%-37%), using a flow cytometric cytotoxicity assay. Other groups have demonstrated higher levels of specific cytotoxicity in fewer patients.17,19 However, their T cells did not undergo polyclonal stimulation with anti-CD3, and our targets were unmanipulated. In addition, positive and reliable cytotoxicity data do not exclude escape from cytotoxicity of the leukemic stem cell; an in vivo cytotoxic response to these cells would be fundamental to the eradication of minimal residual disease through immunotherapy. Here, data from autologous T-cell–proliferation assays have been used to support cytotoxicity data, and together these indicate that stimulation with autologous DCs derived from AML blasts can generate antileukemic T-cell responses. Useful future studies will include assays of inhibition of leukemic colony formation and, ultimately, clinical trials.

We have attempted to harness the power of professional APCs to take up, process, and present antigens to potential CTLs through the generation of functional DCs co-expressing leukemic antigens and costimulatory signals. This is of importance in targeting the generation of functional DCs co-expressing leukemic antigens to take up, process, and present antigens to potential CTLs through cytotoxic T cells than DCs derived from normal precursors and primed with exogenous leukemic antigens.

In conclusion, our study confirms and extends evidence of the potential for induction of antigen-presenting function on AML blasts through culture with appropriate cytokines in a larger group of patients encompassing a wide range of FAB types. Furthermore, we show evidence of induction of specific, autologous T-cell stimulatory capacity through data from both proliferative and cytotoxicity assays. We believe that these data support the feasibility of using cultured AML blasts to present antigens to autologous T cells and their possible use in clinical trials.

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