Circulating blood dendritic cells from myeloid leukemia patients display quantitative and cytogenetic abnormalities as well as functional impairment

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**Dendritic cells (DCs)** are bone marrow–derived leukocytes that are responsible for the initiation of immune responses. Two distinct subsets of blood DCs have been characterized thus far. Myeloid DCs (MDCs) and plasmacytoid monocytes (PDCs) have been shown to be able to promote polarization of naive T cells. This study shows a dramatic quantitative imbalance in both circulating blood DC subsets in 37 patients with acute myeloid leukemias. Eleven patients (30%) displayed a normal quantitative profile (MDC mean, 0.37% ± 0.21%; range, 0.01% to 0.78%; PDC mean, 0.21% ± 0.24%; range, 0.04% to 0.62%), whereas 22 (59%) showed a tremendous expansion of MDCs (9 patients: mean, 16.76% ± 14.03%; range, 1.36% to 41%), PDCs (4 patients: mean, 7.28% ± 6.84%; range, 1% to 14%), or both subsets (9 patients: MDC mean, 10.86% ± 12.36%; range, 4.25% to 37.1%; PDC mean, 4.25% ± 3.78%; range, 1.14% to 13.04%). Finally, in 4 patients (11%), no DC subsets were detectable. Both MDC and PDC subsets exhibited the original leukemic chromosomal abnormality. Ex vivo, leukemic PDCs, but not leukemic MDCs, had impaired capacity for maturation and decreased allostimulatory activity. Also, leukemic PDCs were altered in their ability to secrete interferon-α. These data provide evidence that DC subsets in vivo may be affected by leukemogenesis and may contribute to leukemia escape from immune control.

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**Patients, materials, and methods**

**Patient samples**

AML cell samples were obtained after informed consent from 37 patients at diagnosis and before any chemotherapy. AML peripheral blood mononuclear cells (PBMCs) analyzed in this study were derived from patients with ages ranging from 15 to 66 years treated at the Institut Paoli-Calmettes (Marseille, France) between 1993 and 2000. AMLs were subtyped according to the FAB classification. PBMCs from patients and healthy controls (Regional Transfusion Center, Marseille, France), to which the patients’

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samples have been compared, were isolated by standard density gradient centrifugation with Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) before cryopreservation. At all times, leukemic samples and healthy controls were handled similarly to prevent functional differences that would arise from cryopreservation and cell separation.

Cell lines
Murine L cells transfected with human CD40L were kindly provided by Schering-Plough (Laboratory for Immunological Research, Dardilly, France) and used after 75 Gy irradiation.

Blood DC detection and sorting
Blood DCs were identified by 3-color staining performed on PBMCs using the following monoclonal antibodies: ZM3.8-PC5 (mAb against ILT3, an immunoglobulinlike transcript recently used to isolate DCs from the blood, mouse IgG1),17 BU15-phycoerythrin (PE) (mAb against CD11c), and fluorescein isothiocyanate (FITC)-labeled mAbs against lineage markers CD14, CD16, CD19, and CD56 (Beckman-Coulter, Marseille, France). Cells that did not label with these lineage markers were designated as lin−. The purity of each DC subpopulation after cell sorting was always greater than 98% and was assessed by fluorescence-activated cell sorting (FACS) before subsequent fluorescence in situ hybridization (FISH) and functional experiments. The following mAbs were used for flow cytometry studies: HLA-DR, CD13, CD33, CD45RA, CD45RO, CD4, CD5, CD11c, CD1a, and CD83 (Beckman-Coulter); and CD80, CD86, CD123, and CD40 (Pharmingen, San Diego, CA). Stained cells were analyzed on a FACSCalibur cytometer using Cellquest software (Becton Dickinson, San Jose, CA). DCs were sorted on a FACSVerse cytometer (Becton Dickinson).

FISH analysis
Interphase FISH was performed as described previously on cytopsin preparations with sorted MDCs and PDCs from 4 patients selected for detection of their cytogenetic abnormality.18 For detection of trisomy 8 Interphase FISH was performed as described previously on cytospin preparations. For the detection of some 8 (D8Z2 probe) or chromosome 7 (D7Z1 probe), both purchased from Oncor (Gaithersburg, MD). For the detection of translocations associated with the q23 region of chromosome 11 (UPN109), LSI MLL dual-color DNA probe (Vysis, Downers Grove, IL) was used. At least 200 nuclei were analyzed at diagnosis and before treatment by flow cytometry after cryopreservation and cell separation. At all times, leukemic samples and healthy controls were handled similarly to prevent functional differences that would arise from cryopreservation and cell separation.

Confocal microscopy
Cells were adhered to polylysine-coated glass slides for 30 minutes at room temperature, fixed in 4% paraformaldehyde, and permeabilized in 0.1% Triton in phosphate-buffered saline. Cells were then labeled with different primary mAbs and revealed by species-specific Alexa 488, tetramethylrhodamine isothiocyanate (TRITC; Molecular Probes, Eugene, OR), or cyanin 5–labeled secondary antibodies (Jackson Immunoresearch, West Baltimore Pike, PA). DC-LAMP mAb was kindly provided by Serge Lebecque (Schering-Plough, Laboratory for Immunological Research). Slides were then mounted using fluorescent mounting medium (Dako, Trappes, France). Confocal analysis was performed with a TCS NT microscope equipped with argon and krypton ion lasers and a X100 1.3NA PL fluorite objective (Leica Microsystem, Heidelberg, Germany).

Reverse transcriptase–polymerase chain reaction analysis
Expression of pre-Ta transcript on freshly sorted MDCs and PDCs was analyzed as described previously.20 Briefly, total cellular RNA was extracted from the FACS-sorted cells using TRIzol reagent (Gibco BRL, Cergy-Pontoise, France). First-strand cDNAs were prepared using oligo(dT) primers and murine Moloney leukemia virus reverse transcriptase (RT; Gibco BRL). Polymerase chain reaction (PCR) amplification was performed for 35 cycles (1 minute at 94°C, 1 minute at 55°C, and 2 minutes at 72°C) with Taq DNA polymerase (Gibco BRL). Oligonucleotide primers for pre-Ta were 5′-GAGCGATCTCTGCTGAAGC-3′ and 5′-CGAGTCCCTGGCGTGAAGC-3′ for sense and antisense primers, respectively.

Functional analysis
To evaluate T-cell proliferation capacity, we sorted ILT3+/CD11c− (PDCs) and ILT3−CD11c+ (MDCs) from peripheral blood from healthy volunteers (HV) and patients with acute myeloid leukemia (AML) at diagnosis and before treatment by flow cytometry after cryopreservation and cell separation. At all times, leukemic samples and healthy controls were handled similarly to prevent functional differences that would arise from cryopreservation and cell separation.

Results
Imbalance of blood DC subsets in myeloid leukemia patients
Circulating blood DC subsets from 37 leukemic patients were analyzed at diagnosis and before treatment by flow cytometry after cryopreservation and cell separation. At all times, leukemic samples and healthy controls were handled similarly to prevent functional differences that would arise from cryopreservation and cell separation.

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isolated from blood of patients revealed their typical DC morphology (Figure 3).

**Leukemic origin of circulating blood DC subsets**

The leukemic origin of freshly isolated MDC and PDC populations from patients with AML was investigated by examining the presence of cytogenetic abnormalities by FISH experiments. MDCs and PDCs from 4 patients taken in both groups I and II (UPN79, UPN109, UPN156, and UPN223) displaying cytogenetic abnormalities on their leukemic blasts at diagnosis were analyzed. All freshly sorted MDCs and PDCs from these patients, with or without quantitative expansion of DC subsets, exhibited the initial cytogenetic abnormalities. Examples of MDCs from one AML patient with trisomy 8 at diagnosis (UPN156) are depicted in Figure 4. A red hybridization signal on all of the analyzed nuclei showed the presence of trisomy 8 on sorted MDCs. These results demonstrated the leukemic status of blood circulating DC subsets in patients with myeloid leukemia.

**Figure 2. Immunophenotype of freshly isolated MDCs, PDCs, and leukemic blasts**

![Image](https://www.bloodjournal.org)
Impaired in vitro maturation of PDCs isolated from patients

Freshly sorted MDCs and PDCs isolated either from HVs or from leukemic patients were cultured for 3 days in the presence of GM-CSF and IL-4 for MDCs and IL-3 for PDCs. We examined their capacity to undergo maturation after either simultaneous in vitro culture with appropriate cytokines and CD40L for 3 days or 3 days with cytokines followed by 2 days of stimulation by CD40L. Phenotypic analysis indicated that both subsets isolated from HVs acquired the expression of CD83 and the costimulatory molecules CD80 and CD86. The expression of HLA-DR was up-regulated, especially on the MDC subset (Figure 3A,C). In leukemic patients, the MDC subset could acquire CD83 and the costimulatory molecules CD80 and CD86 (Figure 3B). Acquisition of CD83 and distribution of HLA-DR molecules on leukemic MDCs were further confirmed by confocal microscopy staining (Figure 3B,C). Furthermore, confocal staining of MDCs indicated that they expressed DC-LAMP after maturation in culture (Figure 3D), which is a newly described DC-specific lysosomal marker specifically induced in maturing DCs. In contrast, PDCs from leukemic patients never acquired CD83 or costimulatory molecules, even after prolonged culture or with the addition of tumor necrosis factor-α (TNF-α; Figure 3D and data not shown). These results strikingly indicate that PDCs isolated from leukemic patients display altered capacities for maturation as compared with their healthy counterparts.

Functional properties of MDCs and PDCs from leukemic patients

MDCs and PDCs were assessed for their ability to stimulate naive CD4+ T cells in an allogeneic mixed lymphocyte reaction. Freshly isolated and 3-day–cultured MDCs obtained either from HVs or leukemic patients could efficiently stimulate the proliferation of
naive CD4+ T cells (Figure 6A and data not shown). As described previously,2,26 freshly isolated PDCs from HVs induced weak, if any, proliferation of naive allogeneic T cells (Figure 6B). After culture in the presence of IL-3 and CD40L, there was an increase in the allostimulatory activity of PDCs from HVs (Figure 6B). In marked contrast, even after culture, leukemic PDCs were inefficient to induce proliferative responses by naive T cells (Figure 6A,B).

PDCs proved recently to be the main producers of type I IFN-α, an important cytokine in immune responses, after microbial challenge.4,6,27 To investigate the functional properties of PDCs from leukemic patients, we tested their capacity to produce IFN-α when stimulated with HSV. PDCs from HVs could produce large amounts of IFN-α. In contrast, PDCs from leukemic patients produced small amounts of IFN-α (25-fold less) compared with that produced by PDCs from HVs (Figure 7). Thus, decreased production of IFN-α in response to viral stimuli appears to be another prominent feature of PDCs from leukemic patients.

Discussion

Our results show that the 2 circulating DC subsets can be identified in the blood of patients with AML. No specific marker is currently available for positive identification of circulating DCs. A method based on a lin-1/HLA-DR+ DC phenotype has been used to isolate circulating blood DCs.28,29 This approach is not applicable in patients with AML because leukemic cells can express HLA-DR. Furthermore, expression of HLA-DR on activated cells during illness may also complicate the issue, creating a potential for error when expression of HLA-DR is used to monitor blood DCs.21 In this study, ILT3 was the reference antibody because it has already been proved to be able to identify the 2 circulating DC subsets in fresh blood without any previous culture technique.6,17 However, our definition of leukemic MDCs and PDCs was not exclusively based on the expression of the ILT3 antigen. Leukemic MDCs and PDCs presented many specific features that could clearly distinguish them from leukemic blasts. Leukemic MDCs exhibited after culture a potent allostimulatory activity toward naive T cells. This allostimulatory activity of leukemic MDCs could also be demonstrated when we used freshly isolated normal and leukemic MDCs without any further differentiation or activation, in comparison with monocytes and leukemic blasts from the same patient. The ability to stimulate naive T cells is not a common feature of freshly isolated leukemic blasts. Exhaustive phenotypic analysis of the sorted DC subpopulations was performed using a large panel of cell-surface markers expressed by human DCs.30 These markers could distinguish MDCs and PDCs as populations distinct from the leukemic blasts. In addition, leukemic MDCs expressed DC-LAMP, which is a DC-specific lysosomal marker specifically induced in maturing DCs.18 This marker is not currently known to be expressed by fresh leukemic blasts. Although leukemic cells can express some markers of unknown significance, the high number of leukemic samples we studied (37 different AMLs) argues against an aberrant expression of the ILT3 antigen, which is another specific marker of antigen-presenting cells.6,17,31 On the morphologic level, leukemic MDCs and PDCs had a distinctive morphology. Although this feature is insufficient to define a DC, DCs were named initially based on their distinctive morphology, and leukemic MDCs in our study exhibited in vitro a typical dendritic morphology as soon as 24 hours after culture. This was not the case for leukemic blasts. On the other hand, freshly isolated leukemic PDCs exhibited a lymphoid or plasma cell–like shape, which could clearly distinguish them among blasts with common myeloid morphology. Moreover, these leukemic PDCs were shown to express lymphoid-associated pre-Tc transcript. At present, little is known about the origin of PDCs, although some evidence suggests their lymphoid origin. Recently, it has been shown that PDCs in the thymus20 and human committed DC precursors that are present in adult peripheral blood and thymus21,22 share with T-cell precursors the expression of pre-Tc mRNA.23 We extended these observations
by showing that the PDC subset we isolated from leukemic patients expressed pre-Tα transcript. The finding of a transcript associated with the T-cell differentiation pathway adds support to the putative lymphoid origin of the leukemic PDC subset we have analyzed. The latter finding is a major feature that can distinguish the PDC subset from leukemic blasts that are of myeloid origin in patients with AML. Taken together, the aforementioned features of leukemic MDCs and PDCs can rigorously help to define these cells toward the DC lineage.

Our results among 37 AML patients show an important quantitative imbalance for MDCs and PDCs. The clinical relevance of increased blood DC numbers is not yet clear. Recently, it has been proposed that PDCs might be responsible for maintaining peripheral T-cell tolerance to self-antigens.3 The balance of these cells in vivo influences the character of cell-mediated immune and inflammatory responses and may interfere in the mechanisms regulating antitumor immunity versus tolerance. FISH analysis confirmed that MDCs and PDCs had the same cytogenetic abnormalities expressed by freshly isolated leukemic cells. Therefore, DCs in leukemic patients may be part of the malignant clone, or “dendritopoesis” is affected by the leukemic process. The latter does not exclude that normal MDC and PDC subsets without cytogenetic abnormalities might circulate in the blood of these leukemic patients. Because of the quantitative predominance of leukemic MDC and PDC subsets, such normal subsets could not be detected. Furthermore, at present, the different differentiation pathways of DCs are not clearly established. At this stage, though MDC and PDC development appears to be affected by the leukemic process, we cannot confirm whether circulating DCs in leukemic patients originate exclusively from the leukemic clone or from another stem cell with expression of leukemic cytogenetic markers.

The recognized characteristics of DCs include the ability to present antigens and to stimulate specific T-cell responses. Leukemic MDCs and PDCs might express at least some leukemia-related proteins associated with the cytogenetic abnormality, but this does not necessarily mean that they will be efficiently presented to T cells. The crucial role of costimulatory molecules in the generation of an antileukemic response has been shown in murine leukemia models.32,33 In the current study, we have demonstrated that PDCs from leukemic patients could not acquire CD80 and CD86. The same held true for HLA-DR, which was weakly expressed on leukemic PDCs after culture. On the other hand, MDCs could achieve increased expression of the costimulatory molecules, which correlated with their capacity to induce naive CD4+ T-cell proliferation. This maturation process was associated with the expression of CD1a and CD83. These properties and markers helped to define the maturation pattern of these cells toward the DC lineage.30

Our results established a clear difference in the function of leukemic circulating DCs compared with their normal counterparts. Stimulation through the CD40 pathway failed to enhance the allostimulatory activity of PDCs from leukemic patients, which is compatible with the absence or low expression of HLA-DR and costimulatory molecules.

It has been shown recently that PDCs produce large amounts of type I IFN after microbial challenge.1,4,6,27 The in vivo effects of type I IFN are associated with promoting an antiviral state, including a broad spectrum of cellular targets.34 Type I IFN plays an essential role in antiviral immunity and is widely used to treat viral hepatitis and various types of malignancies, especially chronic myeloid leukemia.35 These effects appear to be due to direct inhibition of viral replication in infected cells and to the pleiotropic immunomodulating activity of type I IFN.35 Type I IFN may act by enhancing the cytotoxic activity of natural killer cells and macrophages,35 by inducing T-cell activation,36 and by maintaining the survival of activated T cells.37 It has also been shown that IFN-α can induce Th1 development in human CD4+ T cells.38,39 and HSV infection results in the development of IFN-γ-secreting CD4+ T cells with cytolytic activity.40 Furthermore, type I IFN can induce the expression of TNF-related apoptosis-inducing ligand on T cells and thereby enhance T-cell cytotoxicity.41 Taken together, all of these findings illustrate the critical role of PDCs for linking innate and adaptive immune responses against tumors. The dramatically decreased secretion of IFN-α by leukemic PDCs would have serious consequences on the induction of antileukemic immune responses.

In summary, in AML patients, both circulating DC subsets exhibit quantitative abnormalities and exhibit leukemia cytogenetic features. The leukemic process, through interference with the DC system, appears to contribute to creating a state of profound immune suppression in AML patients. Although leukemic, MDCs retain the ability to differentiate, whereas PDCs show altered immunogenicity. The latter suggests that PDCs may play a key role in AML and other cancers where the immune system appears to be suppressed or otherwise “tolerized” to the tumors. Further studies are warranted to investigate the clinical relevance of altered circulating blood DC numbers in leukemic patients and the potential role of PDCs in the induction of tolerance to leukemic cells.

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References


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