Human Acute Myeloid Leukemia CD34⁺/CD38⁻ Progenitor Cells Have Decreased Sensitivity to Chemotherapy and Fas-induced Apoptosis, Reduced Immunogenicity, and Impaired Dendritic Cell Transformation Capacities

Régis T. Costello, Françoise Mallet, Béatrice Gaugler, Danielle Sainty, Christine Arnoulet, Jean-Albert Gastaut, and Daniel Olive


ABSTRACT

The destruction of cells capable of initiating and maintaining leukemia challenges the treatment of human acute myeloid leukemia. Recently, CD34⁺/CD38⁻ leukemia progenitors have been defined as new leukemia-initiating cells less mature than colony-forming cells. Here we show that CD34⁺/CD38⁻ leukemia precursors have reduced in vitro sensitivity to daunorubicin, a major drug used in leukemia treatment, in comparison with the CD34⁺/CD38⁻ counterpart, and increased expression of multidrug resistance genes (mrp/frp). These precursors show lower expression of Fas/Fas-L and Fas-induced apoptosis than CD34⁺/CD38⁻ blasts. Moreover, the CD34⁺/CD38⁻ leukemic subpopulation induces a weaker mixed leukocyte reaction of responding T-lymphocytes than the CD34⁺/CD38⁻ leukemic counterpart, either in a MHC-unmatched or MHC-matched settings. This weaker immunogenicity could be linked to lower expression on CD34⁺/CD38⁻ leukemia precursors of major immune response molecules (MHC-DR, LFA-3, B7-1, or B7-2) than CD34⁺/CD38⁻ leukemic cells. Nonetheless, the susceptibility of the immature CD34⁺/CD38⁻ precursors to cytotoxicity was not different from the sensitivity of the CD38⁻ counterpart. Finally, CD34⁺/CD38⁻ leukemia precursors, in contrast with CD38⁺ precursors, failed, under appropriate conditions, to differentiate into dendritic cells, a central step for antigen recognition. This is to our knowledge the first demonstration that the very immature phenotype of CD34⁺/CD38⁻ leukemic progenitors confers both chemotherapy resistance and decreased capacities to induce an immune response. Because the susceptibility of the immature leukemia cells as cytotoxic targets is maintained, our data underline the importance of improving the initial steps of leukemia recognition, more particularly by defining optimal conditions of dendritic cell transformation of the very immature hematopoietic precursors.

INTRODUCTION

AML relapse occurs after intensive myeloablative chemotherapy and, although less frequently, after allogeneic stem cell transplantation, despite the antitumor immune response called “graft-versus-

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2 To whom requests for reprints should be addressed, at Immunologie des Tumeurs, Institut Paoli-Calmettes, 232 Boulevard Sainte Marguerite, 13009 Marseille, France. Phone: (33)-4-91-75-84-15; Fax: (33)-4-91-22-36-10; E-mail: olive@marseille.inserm.fr.

3 The abbreviations used are: AML, acute myeloid leukemia; SCID, severe combined immunodeficient; DC, dendritic cell; mAb, monoclonal antibody; Fas-L, Fas ligand; IL, interleukin; PBMC, peripheral blood mononuclear cell; DR, daunorubicin; FACS, fluorescence-activated cell sorter; MLR, mixed lymphocyte reaction; LAK, lymphokine-activated killer; GM-CSF, granulocyte/macrophage colony-stimulating factor; RT-PCR, reverse transcription-PCR; LRP, lung resistance-related protein; MRP, multiple resistance-associated protein; MFI, mean fluorescence intensity; ICAM, intercellular adhesion molecule; Pgp, P-glycoprotein.

leukemia" effect (1). These data show that some leukemic cells with self-renewal and proliferative properties escape from both intensive chemotherapy and immune response. Recent data demonstrate that immune recognition of progenitors has a role in leukemia treatment. In chronic myelogenous leukemia, T cells recognizing CD34⁺ progenitors mediate the antileukemic effect of donor lymphocyte infusions (2). Moreover, an antileukemia clone developed against both CD34⁺ early progenitors and leukemia cells suggests that differentiation-dependent alloimmune response plays a role in the antileukemic response (3). The limited proliferative capacities of most human AML cells support the hypothesis that the blast population may be maintained by rare stem cells instead of the whole leukemic CD34⁺ population. Recently, the origin of leukemic precursors has been refined, within the CD34⁺ hematopoietic cells, to the CD38⁻ subpopulation. Human leukemia-initiating cells that engraft SCID mice to produce large numbers of colony-forming progenitors able to differentiate in vivo into leukemic blasts are CD34⁺/CD38⁻ (4, 5). The majority of cells capable of long-term colony-forming units are CD34⁺/CD38⁻ (6). Cytogenetic abnormalities suggest that, in AML, malignant transformation as well as disease progression may occur at the level of CD34⁺/CD38⁻ precursors (7). Finally, these data on leukemia progenitors are in line with those obtained with normal hematopoietic precursors, which demonstrate a central role for the CD34⁺/CD38⁻ precursors in hematological reconstitution (8–10). This prompted us to focus our attention on the chemosensitivity, immune recognition, and susceptibility as cytotoxic targets of CD34⁺/CD38⁻ human leukemia precursors, in comparison with their CD34⁺/CD38⁻ counterpart and nonleukemic CD34⁺ stem cells. Because the potential use of DCs generated from leukemic cells has been suggested for cancer immunotherapy, we also evaluated the differential generation and maturation of DCs from CD34⁺/CD38⁻ versus CD34⁺/CD38⁻ leukemic subpopulations.

Here we demonstrate, for the first time to our knowledge, that the recently identified CD34⁺/CD38⁻ leukemia progenitors, which are responsible for leukemia initiation and development, have reduced drug influx, chemotherapy sensitivity, Fas/Fas-L expression, and Fas-induced apoptosis sensitivity in comparison with their CD38⁻ counterpart. We also show that CD34⁺/CD38⁻ leukemia progenitors elicit a weaker alloimmune response, in line with lower adhesion/costimulatory molecule expression. Nonetheless, the susceptibility to cytotoxicity of immature CD34⁺/CD38⁻ leukemia progenitors is identical to their CD38⁻ counterpart. Finally, we demonstrate that CD34⁺/CD38⁻ leukemia progenitors, but not their CD34⁺/CD38⁻ leukemia counterpart, fail to differentiate, under appropriate conditions, into DCs. These new data on the clonogenic population of leukemia shed light on the mechanism of leukemia cell eradication and may refine therapeutic strategies, more particularly regarding immunotherapy.
MATERIALS AND METHODS

Patient Samples. Sixteen newly diagnosed patients with AMLs (3 AML-M0, 2 AML-M1, 3 AML-M2, 4 AML-M4, and 4 AML-M5) of the French-American-British classification (11) with ≥2% CD34+/CD38+ blasts (to be able to functionally analyze the CD34+/CD38+ leukemic subfractions) were included in this study. The median percentage of CD34+/CD38+ leukemic cells was 4% (range, 2.5–27). Peripheral blood samples, obtained before specific antileukemic therapy and after informed consent by the patient, were part of diagnostic procedures. Slides were independently reviewed by two pathologists (D.S. and C.A.).

mAbs and Cytokines. mAbs and cytokines were used in flow cytometric studies: anti-B7-1 (Becton Dickinson, San Jose, CA), anti-B7-2 (PharMingen, Paris, France), anti-MHC-class II DR asacitic fluids (kind gift of Dr. P. Coulié, Ludwig Institute, Brussels, Belgium), anti-ICAM-1 (Immunotech, Marseille, France), anti-LFA-3 (Immunotech), anti-Fas UB2 clone (Immunotech), and anti-CD40 purified IgG (PharMingen). Apoptosis was detected using the APO2.7 mAb (Immunotech), which recognizes a M6, 38,000 protein localized on the membrane of mitochondria, the expression of which appears to be restricted to cells undergoing programmed cell death. To induce Fas-dependent apoptosis, we used the CH-1 anti-Fas (Immunotech) mAb at 125 ng/ml, a concentration corresponding (manufacturer’s indications) to 90% death of the WR191-12a Fas-transfected cell line. To detect Fas-L expression, anti-Fas-L mAb (CD95-L, PharMingen) was used in addition to the blocker of Fas-L cleavage matrix metalloproteinase inhibitor (12) KB8301 used, as specified by the manufacturer (PharMingen), at 10 μM. For each mAb, an isotype-matched immunoglobulin was used as control in all experiments.

Cytokine Production and Assay. T lymphocytes were incubated with leukemic cells in RPMI 1640 (Bioproducts, MA) with 10% fetal bovine serum, and supernatants were harvested after a 5-day incubation. Cytokines were generated according to the conditions determined in our laboratory, which were suspended in Trizol (Life Technologies, Cergy Ponfoise, France), and supernatants were harvested after a 5-day incubation. Cytokines were tested using immunoenzymatic assays with sensitivity of 5 pg/ml for IL-2, IFN-γ, IL-4, IL-10, and IL-12 (Immunotech).

Cell Separation. PBMCs from healthy donors or leukemic patients were isolated on Ficoll-Hypaque gradients and viably frozen in liquid nitrogen until use (13). The leukemic CD34+/CD38− or CD34+/>CD38− subpopulations were obtained by flow cytometry cell sorting using double staining with anti-CD34 and anti-CD38 mAbs, with an exclusion of at least 20 channels between the CD38+ and CD38− subpopulations. The purity of the preparation (>99% CD34+ blast cells and ≥99% CD38− and CD38+ cells) was assessed by flow cytometry reanalysis of sorted cells. The experiments related to DNR accumulation were performed on purified CD34+ leukemic cells from samples chosen for CD34 positivity on all blasts. These samples were depleted from nonleukemic cells by two rounds of negative selection using magnetic beads coated with anti-CD3, anti-CD19, anti-CD56, and, depending on the leukemia phenotype at diagnosis, anti-CD14 or anti-CD13 mAbs (Immunotech). The nonleukemic CD34+ hematopoietic progenitors were obtained from granulocyte-colony stimulating factor mobilized patients and purified using a MACS column isolation kit (Tebu, Le Perray-en-Yvelines, France). For experiments regarding the CD38− and CD38+ subpopulations, normal hematopoietic precursors were further sorted by flow cytometry under the same conditions than leukemic cells. In all cases, the purity of the preparation (>99%) was verified by flow cytometry reanalysis of separated cells.

Flow Cytometry Studies and Cell Sorting. Cell analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson). The DNR intracellular detection, performed using pure CD34+ leukemic cells, was based on the intrinsic fluorescence of DNR labeling associated, for double staining, with anti-CD38 mAb (Immunotech) labeling. For DNR detection, we used a laser excitation of 488 nm, whereas fluorescence was filtered through a 575-nm filter. Cell sorting was performed on FACS Vintage cell sorter (Becton Dickinson), with an exclusion of at least 20 channels between the CD38− and CD38+ subpopulations; as a consequence, the CD38+ cells correspond to “high-level” expression of CD38 but are referred as CD38− for simplicity. Sorted CD34+/CD38− and CD34+/>CD38− cell purity, evaluated by reanalysis, was in all cases ≥99%.

Primary Mixed Lymphocyte Reaction (MLR). For MHC-unmatched MLR against leukemic cells, responding T lymphocytes were isolated from three unrelated healthy blood donors and purified by sheep erythrocyte rosetting plus overnight adhesion on plastic dishes, as described previously (13). In one case, MHC-matched T lymphocytes from an intrafamilial identical sibling were used in MLR. Because in this case freshly thawed leukemic cells failed to induce MHC-matched T-lymphocyte proliferation (data not shown), sorted CD34+/CD38− and CD34+/CD38+ blasts were separately cultured under DC generation conditions (see below) and added at a 1:10 ratio to MHC-matched T lymphocytes. Culture experiments were performed in RPMI 1640 (Bioproducts) with 10% fetal bovine serum (Bioproducts), 1% l-glutamine (Life Technologies, Gaithersburg, MD), 1% sodium pyruvate (Life Technologies) and 5 × 10−5 β-mercaptoethanol (Sigma Chemical Co, St. Louis, MO). Leukemic cells had γ-irradiation at 50 Gy and were then incubated (from 5 × 106 per well to 2000/well) with T lymphocytes (5 × 105 per well) for 6 days and then pulsed for the 10 last h with [3H]thymidine (Amersham, Buckinghamshire, United Kingdom). Thymidine incorporation was assessed with a direct beta counter (Matrx 9600; Packard Instruments, Rungis, France).

Chemotherapy Sensitivity. Cells (1 × 106/ml) incubated with GM-CSF (Sandoz, Copenhagen, Denmark) at 100 ng/ml and IL-4 (Genzyme Corp., Cambridge, MA) at 10 ng/ml for 6 days. Medium was replenished with cytokines every 2 days. At day 6, 50 G y-irradiated L-cell stable transfectants for CD40L or control CD32 (kindly provided by Dr. J. Banchereau, Schering-Plough, Lyon, France) were added (1 × 106/ml) to leukemic blasts (1 × 106/ml) and coinfected for 48 h to induce DC maturation.

Drug Accumulation. The study was performed as described previously by Feller et al. (17). Briefly, blast cells (1 × 106) were incubated at 37°C in 2 μM DNR (Sigma). The fluorescence was then analyzed at different incubation times, with the influx being stopped by washing the cells with ice-cold medium. Nonspecific binding of DNR was evaluated by adding ice-cold drug-containing medium to the cells and washing them immediately.

Nucleic Acid Preparation, RT-PCR Amplification, and Primers. Briefly, total RNA was isolated from 0.1 to 0.5 × 106 cells for each sample, which were suspended in Trizol (Life Technologies, Cergy Pontoise, France), and extracted with phenol-chloroform, as recommended by the manufacturer. The reverse transcription was performed using standard procedures (19) using Moloney murine leukemia virus Superscript reverse transcriptase and random hexamers according to the manufacturer’s instructions (Life Science). For PCR, 2.5 μl of this cDNA were used as the target in a total volume of 25 μl containing 1.5 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl (pH 8.5), 200 μM each of dNTPs, 1 μM of primers, and 1.25 units of Taq polymerase (Perkin-Elmer). The amplification was performed in a Touchdown Temperature Cycling System thermal cycler (Hybaid, Teddington, United Kingdom): first cycle at 94°C for 3 min and then annealing at 65°C for 30 s, extension at 72°C for 30 s, and then denaturation at 94°C for 30 s (25 cycles...
for β-actin, 35 cycles for LRP and MRP), terminating with 10 min at 72°C. The detection of the housekeeping gene β-actin was used to assess reverse transcription and PCR efficiency, with the following primers; sense 5'-ggc atc gtc gag tcc g-3' and the antisense 5'-gtt gag tgg tgg aca ggc a-3'. The oligonucleotides used for MRP were sense 5'-tct ggg act gga atg tca cgt-3' and antisense 5'-cag gaa tat gcc ctc gcg act-3', and for LRP, sense 5'-gac gtc gcc ttc gtc act-3' and antisense 5'-cca gga agg tgg aca gcg a-3'. Half RT-PCR product was run on a 1.5% agarose gel, and the molecular weights of migrating products were evaluated in comparison with φX 174 RF DNA HaeIII fragments. Evaluation of transcript amounts was performed by gel analysis using the Bio-Imaging Analyzer MacBAS V2.5 (Fuji Photo Film Co., Ltd., Koshin Graphic Systems Inc., Tokyo, Japan).

Statistical Analysis. Statistical analysis was performed using the SPSS software (20). The Kolmogorov-Smirnov test was used to determine whether the data fitted a normal distribution. Because this test rejected the assumption of normality for all variables, comparisons were made using the nonparametric Wilcoxon matched-pairs signed-rank test.

RESULTS

The CD34+/38− Leukemia and Normal Progenitor Cells Have Reduced DNR Accumulation and Chemosensitivity in Comparison with the CD38+ Counterpart, Together with Higher mRNA Expression of Drug Resistance Genes. We analyzed the uptake of DNR in pure CD34+ blast (Fig. 1A) or normal CD34+ hematopoietic progenitors (Fig. 1B) cells. The DNR accumulation was lower (paired sample test, P < 0.05) in CD38+ cells than in the CD38− subpopulation, both in normal and leukemic samples. We then tested blast and nonleukemic cell chemosensitivity to DNR (Fig. 1C). The CD38− and CD38+ subpopulations were sorted by flow cytometry and cultured with GM-CSF, and their proliferation in the presence or absence of DNR was tested. A significantly higher (42% ± 14 versus 18 ± 7; P < 0.05) residual proliferation was observed in the CD38− subpopu-

Fig. 1. Differential DNR influx and chemosensitivity of CD34+/CD38− progenitors versus CD34+/CD38+ cells. Purified CD34+ leukemic cells (A) or nonleukemic CD34+ hematopoietic progenitors (B) were incubated for different periods of time with DNR, an anthracycline widely used in acute leukemia chemotherapy. Because of its inherent fluorescence, DNR influx was directly studied by flow cytometry in the CD38− and CD38+ blast subpopulation using FITC-labeled anti-CD38 mAb. Each point represents duplicate evaluation of MFI from seven different samples; bars, SD. In (C), we measured sorted CD38− (■) and CD38+ (□) cell chemosensitivity by evaluating the residual proliferation of GM-CSF-incubated blasts or nonleukemic progenitors in presence or absence of DNR using a [3H]thymidine incorporation assay. Results are expressed as the percentage of residual proliferation corresponding to the ratio (cells + DNR/cells), corresponding to triplicate data from four different samples; bars, SD. D, we evaluated DNR-induced apoptosis in AML blasts using the APO2.7 mAb. Results are expressed as the percentage of positive cells (left columns) or MFI (right columns), after subtraction of the isotypic control background, in the CD38− (■) and CD38+ (□) blast cell populations (data from duplicate analysis of four different samples; bars, SD).
Proliferation in the CD38$$^2$$ (Fig. 3A) a decreased susceptibility to Fas-mediated apoptosis and Fas-L expression in one representative patient. We also observed a comparable result was observed with nonleukemic CD34$$^1$$ blasts (30, 35, and 40). Data in Fig. 2 illustrate one representative experiment of four performed. Higher expression of LRP mRNA (Fig. 2, first row) is observed in the CD34$$^1$$/CD38$$^2$$ subpopulation because a specific band is detected in this subpopulation at the lowest PCR cycle number (30), whereas no signal is detectable at the same cycle number in the CD34$$^1$$/CD38$$^2$$ subset. In the same way, a specific band for MRP mRNA (Fig. 2, second row) was detectable at 35 cycles in the CD34$$^1$$/CD38$$^2$$ subpopulation but not in the CD34$$^1$$/CD38$$^2$$ counterpart. As a control, comparable actin signal was detected in the two subsets (last row).

The CD34$$^+$$/CD38$$^-$ Leukemia Progenitors Express Fas and Fas-L at a Lower Level than CD34$$^1$$/CD38$$^2$$ Blasts and Have Decreased Susceptibility to Fas-induced Apoptosis. The Fas/Fas-L system has been involved in the apoptotic activity of drugs used in leukemia chemotherapy (25) and in the T cell-mediated apoptosis in AML (26), although these data are still debated (27, 28). That prompted us to determine differential Fas/Fas-L expression and function in the leukemic progenitor CD34$$^1$$/CD38$$^2$$ and CD34$$^1$$/CD38$$^-$ subpopulations.

The percentage of Fas-L-positive leukemic cells (Fig. 3A, left) was higher ($P < 0.05$) in the CD38$$^-$ subpopulation (21% ± 11%) than in the CD38$$^+$ counterpart (5% ± 3.5). Regarding the MFI, a comparable significant difference was observed between the CD38$$^-$ blasts (53 ± 23) and the CD38$$^+$ leukemia cells (3 ± 1). In line with data from literature (29), we observed (Fig. 3A, right) a higher ($P < 0.05$) Fas expression in the leukemic CD34$$^1$$/CD38$$^2$$ subpopulation (% positive cells, 53 ± 26; MFI, 14 ± 5) than in the CD38$$^-$ cells (% positive cells, 11 ± 9; MFI, 5 ± 1). Fig. 3B shows FACS data of Fas and Fas-L expression in one representative patient. We also observed (Fig. 3C) a decreased susceptibility to Fas-mediated apoptosis ($P < 0.05$), as evaluated by APO2.7 mAb staining, of the CD38$$^-$ subset (% of positive cells, 18 ± 7; MFI, 19 ± 15) in comparison with the CD38$$^+$ counterpart (% of positive cells, 35 ± 9; MFI, 77 ± 21).

The Human Leukemia CD34$$^+$$/CD38$$^-$ Hematopoietic Precursors Have Reduced Allogeneic (MHC-unmatched and MHC-matched) Immunogenicity. The CD34$$^+$$/CD38$$^-$ and CD34$$^+$$/CD38$$^-$ leukemic progenitors from five different patients were sorted by flow cytometry, and the response of allogeneic PBMCs was evaluated regarding proliferation and cytokine secretion (Table 1). As a comparison with normal physiology, MLR against CD38$$^-$ and CD38$$^+$ cells obtained from granulocyte-CSF-mobilized CD34$$^+$ cells from three different donors was performed under the same conditions as AML cells. Titration of leukemia cells:responding lymphocytes ratio was performed (from 2:1 to 1:50), and the 1:1 ratio was chosen because it provided significant and reproducible T-lymphocyte proliferation (data not shown).

When compared with MLR elicited against CD38$$^-$ leukemia cells, lymphocyte proliferation against the CD38$$^+$ population was decreased, in parallel with IL-2 secretion and IFN-$$\gamma$$ secretion. To get data more relevant to clinical conditions, we performed comparable experiments in an allogeneic but MHC-matched context with leukemic cells from a patient who underwent allogeneic transplant from a MHC-matched intrafamilial sibling. When compared with MLR elicited against CD38$$^-$ leukemia cells, lymphocyte proliferation against the CD38$$^+$ population was decreased, in parallel with IL-2 secretion and IFN-$$\gamma$$ secretion. Regarding nonleukemic progenitors, the results were quite comparable because the CD38$$^-$ subpopulation elicited a higher T-lymphocyte proliferation, IL-2 and IFN-$$\gamma$$ secretion than the CD38$$^+$ counterpart. In all cases, we failed to observed significant differences regarding IL-10 secretion. In addition, no IL-4 secretion was detected in all of the conditions tested (data not shown).

As a comparison, under our experimental conditions, we observed in standard MLR (i.e. normal mononucleated cells against mononucleated cells) a mean proliferation of 4461 cpm ± 1200, mean IL-2 secretion of 402 pg/ml ± 220, a mean IFN-$$\gamma$$ secretion of 1500 pg/ml ± 100, and a mean IL-10 secretion of 71 pg/ml ± 60.

The CD34$$^+$$/CD38$$^-$ Leukemic Cells Have Identical Susceptibility as Cytotoxic Targets as Their CD34$$^+$$/CD38$$^+$ Counterpart. Because we have shown previously that immature leukemic CD34$$^+$$/CD38$$^-$ progenitors elicited a decreased alloimmune response, we also wanted to test their susceptibility as cytotoxic targets. We first tested their susceptibility to LAK cytotoxicity; as seen in Fig. 4A, the CD34$$^+$$/CD38$$^-$ and CD34$$^+$$/CD38$$^+$ leukemic subpopulation had comparable susceptibility (respective maximum lysis of 18% ± 2 and 17% ± 3) to LAK cytotoxicity, which was lower than lysis of the control target cell line Daudi (maximum lysis, 64% ± 3). We then tested for CTL generation by AML cells; as seen in Fig. 4B, the CD34$$^+$$/CD38$$^-$ and CD34$$^+$$/CD38$$^+$ leukemic subpopulations had comparable susceptibility (respective maximum lysis of 26% ± 2 and 25% ± 3), which was lower than the lysis of the control target EBV cell line (maximum lysis, 66% ± 2).

The CD34$$^+$$/CD38$$^-$ Cells Have Lower Expression of MHC-DR, LFA-3, B7-1, and B7-2 Molecules When Compared with Their More Differentiated CD34$$^+$$/CD38$$^+$ Counterpart. To get more insight into the differential alloimmune recognition of CD38$$^+$ versus CD38$$^-$ leukemia precursors, we compared their expression of adhesion/costimulatory molecules. With comparable percentages of positive cells (data not shown), significant differences were observed regarding MFI (Fig. 5A). The expression of MHC-DR, LFA-3, and B7-2 was higher in the CD38$$^+$ cells than in the CD38$$^-$ cells, although no significant statistical difference was observed regarding ICAM-1, B7-1, or CD40.

As comparison, we tested the differential expression of the same
molecules in nonleukemic CD34⁺ hematopoietic precursors (Fig. 5B). We observed a statistically significant higher expression of B7-1 and B7-2 in the CD38⁺ subpopulation regarding the CD38⁻ cells. The expression of the other tested markers (MHC-DR, ICAM-1, LFA-3, and CD40) was not statistically different between CD38⁺ and CD38⁻ cells.

The CD34⁺/CD38⁻ Leukemic Precursors Have Limited DC Differentiation Capacities in Comparison with CD38⁺ Leukemic Cells. Purified leukemic cell CD34⁺/CD38⁻ and CD38⁺ subpopulations were incubated 6 days with GM-CSF and IL-4 and then cocultured with CD32-expressing fibroblasts to allow DC generation and maturation from AML cells (14–16).

We tested the phenotypic differentiation of the two leukemic cell subpopulations in response to these DC differentiation culture conditions (Fig. 6A). CD40L coinoculation induced higher B7-1 expression in the CD38⁻ cells (18.5% ± 3 positive cells/25 MFI ± 3) than in the CD38⁺ counterpart (9% positive cells ± 1/15 MFI ± 1). A more drastic induction differential was observed with B7-2 expression, favoring the CD38⁻ cells (32% positive cells ± 6/299 MFI ± 30) in comparison with CD38⁺ precursors (7% positive cells ± 2/11.5 MFI ± 1). The MHC class II DR molecule was more efficiently induced in the CD38⁻ cells (24% positive cells ± 5/110 MFI ± 18) than in the CD38⁺ cells (14% positive cells ± 3/13 MFI ± 2). Finally, the induction of CD83 was restricted to the CD38⁻ cells (38% positive cells ± 6/105 MFI ± 26) because no positive cells were detected after culture of leukemic CD38⁺ precursors. The control CD32-positive fibroblasts had very weak effects on both CD34⁺/CD38⁻ and CD34⁺/CD38⁺ subpopulations with low expression of B7-1 (2% positive cells ± 1, MFI 15 ± 2 versus 8% positive cells ± 1, MFI 7 ± 1), B7-2 (11.5% positive cells ± 2, MFI 25 ± 4).

Table 1 Comparison of the alloimmune stimulating capacities of CD34⁺/CD38⁻ versus CD34⁺/CD38⁺ leukemic cells or granulocyte-CSF mobilized hematopoietic precursors

The proliferative response and cytokine secretion of T lymphocytes to CD38⁻ and CD38⁺ subpopulations were tested in MLR. The results correspond to the median and range of experiments using three different allogenic lymphocyte unrelated donors or, for the case of UPN41, with a MHC-matched donor in triplicate experiments. As comparative control, we used nonleukemic granulocyte-CSF-mobilized CD34⁺ cells obtained from three different patients with nonhematological malignancies, each tested against lymphocytes from three unrelated donors.


**CHEMOSENSITIVITY AND IMMUNOGENICITY OF LEUKEMIA PRECURSORS**

**DISCUSSION**

Leukemia treatment must target not only terminally differentiated blasts but also the most primitive leukemic progenitors with high proliferative and self-renewal capacities, which are responsible for human leukemia initiation and development. Recent data have determined that AML is hierarchically organized and originates from a primitive hematopoietic cell for which CD34⁺/CD38⁻ precursors constitute one of the most immature stage (4, 5, 7, 8, 32). This prompted us to focus our in vitro study on this specific subset regarding the sensitivity of these immature precursors to anticancer drug and allogeneic immunogenicity.

Drug resistance in leukemia is related to many different mechanisms (33). The MDR is the best characterized and can be explained, in some cases, by enhanced expression of certain genes: MDR1 and its product P-gp, MRP or LRP, high expression of which generally indicates a poor response to therapy in AML (34, 35). We failed to detect by flow cytometry analysis increased expression of P-gp in the immature progenitor CD34⁺/CD38⁻ subpopulation (data not shown). In contrast, we detected in the CD34⁺/CD38⁻ leukemic cells an increased expression (RT-PCR) and function (decreased DNR influx, increased chemoresistance/lower apoptosis) of MDR family proteins LRP and MRP, the latter being more particularly related to DNR pumping (17, 36). In adult AML, CD34 positivity is an adverse prognostic factor associated with in vitro DNR resistance (18). It will be of interest to determine, in large studies, whether these data correlate only to CD34 expression or more precisely to the relative importance of the most immature CD34⁺/CD38⁻ subset. Of note, the same differential influx of DNR and sensitivity to DNR-induced inhibition of proliferation was observed between the CD34⁺/CD38⁻ and CD34⁺/CD38⁺ nonleukemic subpopulation. This suggests that leukemic cell progenitor-reduced drug sensitivity could proceed from a physiological mechanism involved in hematopoietic stem cell pro-

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**DISCUSSION**

Leukemia treatment must target not only terminally differentiated blasts but also the most primitive leukemic progenitors with high proliferative and self-renewal capacities, which are responsible for human leukemia initiation and development. Recent data have determined that AML is hierarchically organized and originates from a primitive hematopoietic cell for which CD34⁺/CD38⁻ precursors constitute one of the most immature stage (4, 5, 7, 8, 32). This prompted us to focus our in vitro study on this specific subset regarding the sensitivity of these immature precursors to anticancer drug and allogeneic immunogenicity.

Drug resistance in leukemia is related to many different mechanisms (33). The MDR is the best characterized and can be explained, in some cases, by enhanced expression of certain genes: MDR1 and its product P-gp, MRP or LRP, high expression of which generally indicates a poor response to therapy in AML (34, 35). We failed to detect by flow cytometry analysis increased expression of P-gp in the immature progenitor CD34⁺/CD38⁻ subpopulation (data not shown). In contrast, we detected in the CD34⁺/CD38⁻ leukemic cells an increased expression (RT-PCR) and function (decreased DNR influx, increased chemoresistance/lower apoptosis) of MDR family proteins LRP and MRP, the latter being more particularly related to DNR pumping (17, 36). In adult AML, CD34 positivity is an adverse prognostic factor associated with in vitro DNR resistance (18). It will be of interest to determine, in large studies, whether these data correlate only to CD34 expression or more precisely to the relative importance of the most immature CD34⁺/CD38⁻ subset. Of note, the same differential influx of DNR and sensitivity to DNR-induced inhibition of proliferation was observed between the CD34⁺/CD38⁻ and CD34⁺/CD38⁺ nonleukemic subpopulation. This suggests that leukemic cell progenitor-reduced drug sensitivity could proceed from a physiological mechanism involved in hematopoietic stem cell pro-
tution from toxic injuries. Regarding the mechanism of the DNR resistance of the CD34+/CD38– subpopulation, we detected a lower apoptosis induction in this subset as compared with the CD34+/CD38+ counterpart, in addition to a lower Fas expression and decreased Fas-L induction. It is tempting to partially link DNR resistance to decreased function of the Fas/Fas-L system because its involvement in anthracycin-induced apoptosis in leukemia cells has been suggested (25). Nonetheless, this latter point is controversial (37) because other authors consider that drug-induced apoptosis is associated with enhanced Fas-L expression but does not rely on the Fas/Fas-L system (27, 28, 38).

We detected Fas-L expression in most leukemias we tested. This is, to our knowledge, the first description of Fas-L expression in AML primary samples because previous data in leukemic cells were obtained either in myeloid cell lines (39, 40) or in lymphoid malignancies (reviewed in Ref. 41). The significance of Fas-L expression by leukemic cells is of interest; it could contribute to Fas-mediated destruction of antitumor-reactive T lymphocytes in solid malignancies (42–44) and myeloma (45). Because lower Fas-L expression is observed in the CD34+/CD38– subpopulation, this mechanism is unlikely to participate in early leukemic progenitor immune escape. In addition to lower Fas-L expression, the CD34+/CD38+ subpopulation had also decreased Fas expression, as described previously (29). This may have several consequences. Reduced expression of both Fas/Fas-L may protect AML blasts from “paracrine suicide.” Then, reduced Fas expression may also impair Fas-mediated apoptosis induction in AML cells by activated T lymphocytes that express Fas-L (26). The clinical significance of Fas expression in leukemia is controversial (37). Some authors failed to find an absolute correlation between Fas expression and Fas-mediated apoptosis in AML (29), suggesting either a dysregulation of the apoptotic pathway as in some lymphoid malignancies (46, 47) or a cell cycle-dependent refractoriness (26). In our CD34+/CD38– and CD34+/CD38+ subpopulation comparative analysis, the differential Fas expression has functional significance, as demonstrated by higher Fas-mediated apoptosis in the CD34+/CD38+ subset. Nonetheless, other mechanisms of apoptosis than Fas triggering have been described, such as tumor necrosis factor-α (48), IL-1β (49), nitric oxide (50), reactive oxygen intermediates (51), or granyme B and perforin (52). All these mechanisms could participate in leukemic cell eradication and will require further investigation.

The CD38– leukemic precursors demonstrated a significantly lower stimulatory activity in MLR (MHC-matched or MHC-unmatched) than the CD38+ counterpart, both regarding T-lymphocyte proliferation and cytokine secretion (IL-2 or IFN-γ). The differential immune recognition was not directly mediated via CD38 interactions, because the acquisition of CD38 antigen also correlated with higher expression of molecules involved in leukemia immune recognition, such as MHC-II, LFA-3, B7-1, and in particular B7-2 (53), the defective expression of which impairs alloantigen presenting functions of normal human hematopoietic cells (54–56). In addition to being a defective target for immune response, the immature CD34+/CD38– leukemia cells could help mature blasts to evade the immune system via an indirect mechanism, i.e., T-cell tolerance for leukemic cells (57). This hypothesis is supported by decreased B7-2 expression in CD34+/CD38– cells, because this molecule is critical in T-lymphocyte signaling and may determine whether antigen stimulation results in immunity or tolerance (58). Nonetheless, tolerance induction is improbable because recent data suggest that even low-level B7 molecule expression may prevent it (59). A comparable differential alloimmune recognition was observed in nonleukemic CD34+ progenitors between CD38+ and CD38– cells, leading to the description of a novel hematopoietic cell subpopulation with decreased alloimmune reactivity, in addition to the CD18− and B7-2− subsets (54, 55). This suggests that the decreased alloimmune stimulation potential of leukemia CD38– precursors is a physiological mechanism developed to protect normal hematopoietic stem cells against inappropriate immune reactions. This immune privilege could favor the engraftment of allogeneic stem cell or bone marrow transplants by limiting the efficiency of the “host versus graft” reaction against allogeneic hematopoietic precursors.

This reduced response of T cells against the most immature leukemia progenitors raises the question of the means to improve their immunogenicity. Recently, stimulation by CD40L of tumor cells was demonstrated to be an efficient model to improve the immunogenicity of lymphoid malignancies (60) or myeloid leukemia (53) and the transformation of the latter into mature DC (14–16), which are prototypic antigen-presenting cells. Many data indicate that the efficient development of antileukemic T-cell response depends on the presence of DCs generated from leukemia (14–16). We failed to generate DCs from CD34+/CD38– leukemic progenitors, in contrast with the CD34+/CD38+ counterpart. This phenomenon could be differentiation dependent and related to lower B7-2 expression in the leukemic CD34+/CD38+, because normal CD34+/B7-2+ hematopoietic progenitors are able to differentiate into DCs (54, 55) but not their B7-2– counterpart. In line with this hypothesis of a differentiation-dependent blockade of DC transformation, we obtained DCs from CD34+/CD38+ normal hematopoietic progenitors but not from their
CD34+/CD38− counterpart. Because the DC transformation of early progenitors may require early-acting cytokines (although we obtained this differentiation from the CD34+/CD38− subpopulation), we tested other cytokines, such as tumor necrosis factor-α and stem cell factor, which failed to induce DC differentiation of CD34+/CD38− leukemic progenitors (data not shown). The possibility to transform immature leukemic progenitor cells into DCs is a challenge, the importance of which is underlined by the fact that leukemic very immature progenitors retain identical susceptibility to LAK and CTL cytotoxicity. This suggests that the initial steps of leukemia immature progenitor recognition could constitute one of the most potent limitations to the development of an efficient antileukemic immune response.

Our observations shed light on the persistence of leukemia stem cells, despite the rapid and efficient treatment-induced clearance of most circulating and bone marrow blasts. Our data could help to refine some leukemia prognosis factors (CD34 expression/drug resistance) by focusing on the CD34+/CD38− leukemic population. Very recently, a new class of human hematopoietic cells with SCID repopulating activity has been identified, which is restricted to a Lin−CD34+/CD38− population without detectable surface markers (61). Whether these cells participate in leukemogenesis is unknown. The chemosensitivity, immune recognition, and the potential for DC transformation of these CD34−SCID-repopulating cells are important questions for human leukemia physiopathology, treatment, and stem cell transplantation.

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CHEMOSENSITIVITY AND IMMUNOGENICITY OF LEUKEMIA PRECURSORS


Human Acute Myeloid Leukemia CD34+/CD38− Progenitor Cells Have Decreased Sensitivity to Chemotherapy and Fas-induced Apoptosis, Reduced Immunogenicity, and Impaired Dendritic Cell Transformation Capacities
