In vitro growth and quantification of early (CD33⁻/CD38⁻) myeloid progenitor cells: stem cell factor requirement and effects of previous chemotherapy

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ABSTRACT

Background and Objective. All culture systems exploring the early (pre-CFU) hematopoietic compartment are generally complex, time-consuming and unsuitable for routine application. The aim of our study was to develop a stroma-free culture system to quantify early bone marrow (BM) myeloid progenitor cells.

Design and Methods. Low density, progenitor cell enriched BM cells underwent a double cytotoxic treatment with CD38 and CD33 monoclonal antibodies + rabbit complement, which depleted 99% of CFU-GM and BFU-E. Then they were cultured, both in agar and in limiting-dilution liquid culture, in the presence of 5637 cell line supernatant (containing GM-CSF, G-CSF and interleukin 1), stem cell factor (SCF) and interleukin 3 (IL3).

Results. The largest number (median 14.9 on 1 x 10⁵ cells) and size (>50,000 cells) of myelomonocytic cell clones from CD33⁻/CD38⁻ progenitors was reached after 3-4 weeks of liquid culture. SCF, but not IL3, was essential for that growth. The frequency of CD33⁻/CD38⁻ progenitors grown in liquid culture was approximately three times greater than the LTC-IC frequency in the same cell suspension. An average 93% of CD33⁻/CD38⁻ progenitors displayed HLA-DR antigens and 43% generated secondary CFU-GM. In the BM of 9/10 patients, previously exposed to chemotherapy, CD33⁻/CD38⁻ progenitor frequency was quite low (median 0.9 on 1 x 10⁶ cells), in spite of normal cellularity and morphology and sustained disease remission.

Interpretation and Conclusions. CD33⁻/CD38⁻ progenitors can be grown and quantified in a stroma-free culture system in a relatively short time. The test can reveal long-lasting, subclinical BM damage induced by chemotherapy and could also be valuable for estimating the amount of early myeloid progenitors for transplantation purposes.

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Key words: CD38 negative progenitors, complement cytotoxicity, stem cell factor, chemotherapy

The progressive development, during the past two decades, of in vitro culture techniques has allowed routine evaluation of the growth of committed hematopoietic progenitors. These cells can be quantified in bone marrow (BM) and peripheral blood for transplantation and other clinical purposes. Converse, in vitro culture and quantification of early hematopoietic progenitor/stem cells is still a difficult task. Some techniques for this purpose use pre-established BM stromal layers, without exogenous growth factors. In particular, in the past few years, a culture system has been developed that supports the growth, in microwells with irradiated BM stroma, of so called long-term culture-initiating cells (LTC-IC). The method allows quantification of these very primitive cells that share many of the features of hematopoietic stem cells. However, LTC-IC assay is a complex procedure, requiring a pre-established irradiated BM layer and a long culture time (at least five weeks), this precluding its extensive use in a clinical setting.

Stroma-free cultures require the use of combinations of growth factors and of selective procedures to suppress the overgrowth of the more abundant committed progenitors. Depletion of committed progenitors can be achieved by the use of selective culture medium, prolonged culture time, cytotoxic drugs or monoclonal antibodies against differentiation or proliferation antigens. Indeed, the earliest hematopoietic cells lack lineage-specific as well as CD38 and CD71 antigens, which are expressed by committed progenitor cells. In particular, CD38 antigen is present on almost all hematopoietic colony-forming cells. Therefore, CD38 negative progenitors represent a small fraction of very immature hematopoietic cells that lack lineage-specific antigens, and include LTC-IC. Here we describe some growth requirements of CD33⁻/CD38⁻ myeloid progenitors and describe a relatively short, stroma-free, culture method for their quantification in human adult BM. We also report the observation, using this method, of a great reduction in CD33⁻/CD38⁻ progenitor cells in the BM of patients previously treated with cytotoxic drugs, in spite of normal blood counts and BM cellularity.
Design and Methods

Cells

BM was aspirated from volunteer donors and from patients undergoing diagnostic procedures after informed consent had been obtained. Low density (<1,077 g/L) cells, obtained by Ficoll/metrizoate (Lymphoprep, Nycomed) density gradient separation, underwent a second Lymphoprep separation after phagocytosis of opsonized, heat-inactivated yeast, in order to remove mature myelo-monocytic cells. The separation procedure resulted in a 6-10 fold enrichment in myeloid and erythroid progenitors.

Complement-dependent cytotoxicity

One million low density non-phagocytosing cells (LD/phag), suspended in 0.25 mL of Iscove’s modified Dulbecco’s Medium (IMDM) with 10% fetal bovine serum (FBS), were incubated at 4°C in the presence of 10 µL of CD33 and CD38 monoclonal antibodies (MoAb). CD33 MoAb was purchased from Coulter. CD38 was initially purchased from Technogenetics, then, in further experiments, we employed the MoAb produced by the IB4 clone, kindly provided by Prof. F. Malavasi (Istituto di Genetica Medica, Università di Ancona). In some experiments, 10 µL of anti-HLA-DR MoAb (Becton-Dickinson) were also included in the cytotoxicity test. After 30’ of incubation with MoAb, 0.27 mL of rabbit complement (GIBCO), previously adsorbed on human AB group erythrocytes and diluted 1:3 with IMDM, were added (final dilution 1:6) and the incubation was continued at 37°C for 60’. Cells were then washed once and re-exposed to MoAbs and complement. Finally, cells were washed once with IMDM and resuspended in 1 mL of IMDM +10% FBS. Control cells underwent the same incubations without either antibodies or complement or both. The efficacy of cytotoxic treatment was checked by direct immunofluorescence using a phycoerythrin-labeled CD38 MoAb (Becton-Dickinson) and FACS analysis.

Colony assays

Forty microliters of control or 100 µL of MoAb-treated cells were seeded in each of two 35 mm Petri dishes containing 1 mL of IMDM +20% FBS +0.3% agar (DIFCO) +10% supernatant of 5637 cell line (as a source of GM-CSF, G-CSF and interleukin 1) ±30 ng/mL of human recombinant stem cell factor (SCF) (purchased from Amgen). In some experiments, 15 ng/mL of human recombinant interleukin-3 (IL3) (purchased from Sandoz) and 2 U/mL of human recombinant erythropoietin (Eprex, from Cilag) were also added to allow BFU-E growth. The amount of control cell suspension seeded in each dish corresponded to 4×10^6 cells, since no significant cell loss occurred during incubations or centrifugations. One hundred microliters of MoAb-treated cell suspension corresponded to 1×10^5 LD/phag cells before cytotoxicity; no correction was made for the actual number of cells which survived cytotoxicity, in order to avoid an enrichment in CD33/CD38- cells. Colonies were scored after 14 and 28 days of incubation at 37°C in 5%CO₂ atmosphere.

Liquid culture

Each of four groups of twenty-four flat bottomed microwells of a 96 microwell plate (Falcon, Becton-Dickinson) was seeded with a different amount of MoAb-treated cell suspension: 10 µL, 5 µL, 2 µL and 1 µL/microwell. These volumes corresponded, respectively, to 10,000, 5,000, 2,000 and 1,000 LD/phag cells before cytotoxicity. Each microwell was filled with 100 µL of IMDM containing 20%FBS, 10% 5637 cell line supernatant, 30 ng/mL SCF and 15 ng/mL IL3. In some experiments, either SCF or IL3 or both were omitted from the culture medium. After 7 days of culture, 50 µL of medium were removed from each well and replaced by an equal volume of fresh medium and growth factors, then, at the 14th day, 50 µL of fresh medium were added. From the 14th to the 35th day of culture, microwells were scored weekly for the presence of evident cell growth (more than 100 alive cells). The cloning efficiency, calculated on the basis of the proportion of growth-negative wells by Poisson statistics and weighted mean method, corresponded to the reciprocal of the cell concentration that determined absence of growth in 37% of wells (Figure 1). Cloning efficiency was expressed as number of clones/1×10^5 starting (pre-cytotoxicity) cells.

![Figure 1. Limiting dilution assay of CD33/CD38 progenitor cells.](image-url)
In some experiments, cells from wells likely to contain a single clone (rows with less than 30% growth-positive wells) were harvested for counting, morphologic examination and CFU-GM assay. Adherent macrophages were detached after exposure to a 5% trypsin/EDTA solution (GIBCO) for 15' at 37°C, then pooled with suspension cells from each well. Slides, obtained by spinning in a Shandon cytocentrifuge, were stained with May-Grünwald-Giemsa stain.

In order to test CFU-GM generation from CD33–/CD38– progenitors, clones were harvested after 2-3 weeks of liquid culture and each was replated in a well of a 24 well plate, containing 0.4 ml IMDM + 20% FBS + 10% 5637 cell line supernatant + 0.9% methylcellulose (Dow, 4000 cps). Secondary colonies were scored after 14 days.

**LTC-IC assay**

The same volumes of CD33–/CD38– cell suspension used for the above described limiting-dilution cultures were seeded into 96 flat bottomed microwells, each containing BM stroma, subcultured from previously established irradiated layers, and 100 µL of IMDM containing 5637 SN±IL3: some myeloid colonies (median value 11 on 1×10^5 LD/phag– cells, range 3.5-67.5) constantly appeared from cells treated with MoAbs and complement (CD33–/CD38– cells), reaching a median of 5% (range 1-19%) of control CFU-GM values. The increment in colony growth from CD33–/CD38– cells induced by SCF was highly significant (p: 0.0044) and reached a median value of 910% (Table 1); conversely SCF increased by a median of only 32% (p: 0.013) the number of colonies arising from total LD/phag– cells (control cultures) (Table 1). Colonies arising from CD33–/CD38– cells reached their maximum size after 3 weeks, surviving until the 4th week; their number was unaffected by the presence or absence of IL3 (data not shown).

**Limiting-dilution liquid cultures**

Cell growth became evident, in some microwells, after the second week of culture. The frequency of CD33–/CD38– progenitor cells giving rise to colonies in liquid culture was calculated between the 21st and 28th day of culture, when the greater proportion of growth-positive wells was reached (Figure 2). That frequency was found to range, in normal BM samples, between 8.9 and 84.3 on 1×10^5 LD/phag– cells (median value 14.9) (Table 2). In 14/15 tests the frequency of CD33–/CD38– progenitor cells appeared to be somewhat higher (median +49%) in limiting-dilution liquid culture than in agar medium colony-assay, in the presence of the same growth factor combination (compare Table 2: controls to Table 1: CD33–/CD38– progenitors in the presence of SCF). The largest clone size was usually reached after 4 weeks of culture, most clones including several thousands of cells (5,000-50,000) of the granulo-monocytic lineage. Only a few clones contained relatively small numbers of cells (100-500),

<table>
<thead>
<tr>
<th>Progenitors</th>
<th>Total CFU-GM</th>
<th>CD33 / CD38 progenitor cells in agar medium.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Colony values</td>
<td></td>
</tr>
<tr>
<td>Total CFU-GM</td>
<td>- 115 104 37-240</td>
<td></td>
</tr>
<tr>
<td>Total CFU-GM</td>
<td>+ 147 136 48-326</td>
<td>p 0.013</td>
</tr>
<tr>
<td>CD33 / CD38</td>
<td>- 3 1 0-11</td>
<td></td>
</tr>
<tr>
<td>CD33 / CD38</td>
<td>+ 22 11 3-67</td>
<td>p 0.004</td>
</tr>
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</table>

*Total CFU-GM values expressed as colonies/1×10^5 untreated (controls) LD/Phag cells; CD33 / CD38 progenitors expressed as colonies/1×10^5 LD/Phag cells exposed to CD33 and CD38 MoAb + complement, as described in the Design and Methods section.*

*

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usually macrophages. Erythroid cells were observed occasionally in some microwells during the 2nd week of culture, when EPO was present in the culture medium, but later disappeared.

Growth factor requirement
The omission of SCF from the culture medium resulted in more than one log decrease in the cloning efficiency of CD33–/CD38– cells in liquid culture, whereas only a minimal, not significant growth reduc-

tion was determined by the absence of IL3 (Figure 3). SCF alone did not induce any clone growth (data not shown). Further characterization of CD33–/CD38 myeloid progenitors was performed by limiting-dilution liquid culture in medium containing 5637 SN + SCF + IL3.

Table 2. Frequency of total CFU-GM and CD33–/CD38– progenitors among BM cells of controls and chemotherapy-treated patients.

<table>
<thead>
<tr>
<th>Case #</th>
<th>Total CFU-GM/100,000 cells</th>
<th>Case #</th>
<th>Diagnosis</th>
<th>Total CFU-GM/100,000 cells</th>
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<tr>
<td>1</td>
<td>200</td>
<td>1</td>
<td>NHL</td>
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<tr>
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<td>290</td>
<td>2</td>
<td>MM</td>
<td>99</td>
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<td>3</td>
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<td>138</td>
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<td>4</td>
<td>240</td>
<td>4</td>
<td>MM</td>
<td>105</td>
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<td>142</td>
<td>5</td>
<td>HD</td>
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<td>71</td>
<td>6</td>
<td>AML</td>
<td>95</td>
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<td>62</td>
<td>7</td>
<td>MM</td>
<td>73</td>
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<td>8</td>
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</tr>
<tr>
<td>9</td>
<td>185</td>
<td>9</td>
<td>AML</td>
<td>23</td>
</tr>
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<td>81</td>
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<td>NHL</td>
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<td>14</td>
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<tr>
<td>Median</td>
<td>160</td>
<td>Median</td>
<td>99</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*Normal donors or untreated patients without disease involving the myeloid lineage; **Low density cells depleted of phagocytic cells (LD/Phag). NHL: non-Hodgkin’s disease; MM: multiple myeloma; AML: acute myeloid leukemia; ALL: acute lymphoid leukemia.

Figure 2. Time-course appearance of clones from CD33–/CD38– progenitor cells in liquid culture. LD/Phag–BM cells underwent cytotoxic treatment with CD33 and CD38 MoAb + complement, and culture at limiting-dilution in the presence of 5637 SN, SCF and IL3. Clone numbers were scored at weekly intervals and their frequencies were calculated as described in the Design and Methods section. Curves refer to different BM samples.

Figure 3. Growth of CD33–/CD38– progenitor cells in liquid medium with different growth factor combinations. LD/Phag–BM cells underwent cytotoxic treatment with CD33 and CD38 MoAb + complement. They were then cultured at limiting-dilution in liquid medium containing 5637 SN with/without SCF and/or IL3. Clones were scored after 4 weeks of culture and their frequencies were calculated as described in the Design and Methods section. Bars represent the mean (±SD) value of three experiments with different BM samples.
Expression of HLA-DR molecules

The expression of HLA-DR molecules by CD33/CD38 progenitors was checked in four experiments by a parallel cytotoxicity test that included anti-HLA-DR together with CD33 and CD38 MoAbs. Anti-HLA-DR MoAb reduced the frequency of CD33/CD38 clonogenic cells by more than one log (average recovery 7.5±1.3%).

CFU-GM generation from CD33/CD38 progenitors

Seventy-six clones, from 3 experiments, were replated in methylcellulose medium for CFU-GM assay. Thirty-three clones (43%) showed the presence of CFU-GM, as demonstrated by the growth of variable numbers of colonies (1-98, median value: 5). Most of these colonies survived in semisolid culture until the third week; they were, however, of smaller size (50-500 cells) than primary clones. In one experiment secondary colonies were harvested, pooled and re-plated but no tertiary colonies were detected (data not shown).

LTC-IC

In three experiments a direct comparison was made between the frequency of CD33/CD38 progenitor cells growing in stroma-free liquid culture and LTC-IC. As shown in Table 3, a fair correlation was found between the two values, LTC-IC corresponding to 29±2% of CD33/CD38 progenitor cells.

CD33/CD38 progenitors in the BM of chemotherapy-treated patients

The frequency of CD33/CD38 progenitors and of total CFU-GM was evaluated in BM samples from 10 patients affected by different hematologic malignancies (Table 2). All patients had undergone different cytotoxic treatments 2-60 months before the test and were in complete remission, with normal blood counts and BM morphology. The proportion of LD/phag- cells was also similar (10-15%) in chemotherapy-treated and in control BM samples (data not shown). However, all but one patient displayed a quite low frequency of CD33/CD38 progenitor cells (median value 1 on 1×10^4 LD/phag- cells, range 0.3-71); the difference from values observed with normal BM samples was highly significant (p=0.0009). Conversely, total CFU-GM frequency was only moderately reduced in patients' (median value 99 on 4×10^4 LD/phag- cells, range 23-172) compared to control BM samples (median 181 on 4×10^4 LD/phag- cells, range 62-326) (p=0.04). The size of clones from both total CFU-GM and CD33/CD38 progenitors was not different in control and patients' cultures.

Discussion

The aim of our study was to establish a stroma-free culture system capable of quantifying, in 3-4 weeks, early myeloid progenitor cells (pre-CFU-GM) in normal BM and in chemotherapy-treated patients.

Table 3. Comparison of CD33/CD38 progenitor growth in stroma-free liquid culture and in LTC-IC assay.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Clones* in stroma-free culture</th>
<th>LTC-IC°</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13.2±2.5</td>
<td>4.6±1.2</td>
</tr>
<tr>
<td>2</td>
<td>58.9±10.7</td>
<td>17.9±2.8</td>
</tr>
<tr>
<td>3</td>
<td>49.4±8.8</td>
<td>11.4±2.1</td>
</tr>
</tbody>
</table>

CD33/CD38 cells, obtained by complement-dependent cytotoxicity from three normal BM samples, were cultured at limiting-dilution in stroma-free liquid culture, in the presence of SF-587 and IL3, and in stroma-containing microwells for LTC-IC assay. *values refer to the number of colonies on 1×10^5 pre-cytotoxic LD/Phag- cells, evaluated as described in the Design and Methods section. °values refer to the number of LTC-IC on 1×10^4 pre-cytotoxic LD/Phag- cells, evaluated as described in the Design and Methods section.

These progenitors can be identified by immunofluorescence as CD33/CD38 cells. However, only 10-25% of cells with such a phenotype are clonogenic in vitro and identifiable as LTC-IC or blast colony-forming cells. In our experience, gives more reproducible results than cytotoxic drugs such as cyclophosphamide metabolites. CD38 antigen was reported to be expressed by all lineage-committed hematopoietic progenitors, but we also added CD33 MoAb in the cytotoxicity assay to ensure the maximal killing of mature progenitor cells. Indeed, the 99% reduction of myeloid and erythroid colonies in standard culture conditions was indicative of a virtually complete depletion of CD33+ and CD38+ cells. This also avoided the need for morphologic analysis and/or recloning of all the clones grown in liquid culture in order to prove their origin from early progenitor cells, as required by some blast cell colony assays.

The optimal growth of CD33/CD38 progenitor cells was obtained in liquid culture, in the limiting-dilution assay. The long growth time and the large size of clones suggested that they originated from early progenitor cells. This was also confirmed by the presence, in 43% of clones, of CFU-GM capable of giving rise to secondary colonies, in agreement with results obtained by another group with CD34+/CD38- cells.

SCF was absolutely essential for CD33/CD38 cell growth, both in agar and in liquid cultures, whereas IL3 did not play a significant role. Therefore, a combination of growth factors, and particularly the presence of
SCF and 5637 SN (containing GM-CSF, G-CSF and IL1), were required for an optimal growth of CD33/CD38 progenitors. SCF was reported to improve CFU-GM and BFU-E growth greatly in the presence of suboptimal concentrations of CSF and EPO. However, SCF activity on CFU-GM is less evident in the presence of optimal CSF combinations, as observed in our experiments with control cells stimulated by 5637 SN (Table 1). Conversely, SCF was absolutely essential for CD33/CD38 progenitor cells, in spite of the optimal 5637 SN concentration. In a previous report, CD38-progenitor cells were efficiently stimulated to form blast colonies by a growth factor mixture that did not contain SCF but included IL6, IL3, GM-CSF, G-CSF and EPO. Thus, IL6 may substitute SCF in stimulating CD38 progenitors, since it was described to synergize with IL3 in driving quiescent early hematopoietic progenitor/stem cells into proliferative activity. More recent results have, however, reiterated the necessity of SCF, together with Flt3-ligand, IL-3, IL-6 and G-CSF for optimal CD34+/CD38 cell growth and LTC-IC expansion in vitro.23,24

More than 90% of CD33+/CD38− progenitor cells were found to express HLA-DR antigens. Moreover, no erythroid cells could be generated in long-term culture. Thus, CD33+/CD38− progenitor cells probably represent a more differentiated cell population than LTC-IC, that lack or minimally express HLA-DR antigens.23,24 Indeed, it was reported that LTC-IC represent only 10-20% of CD34+/CD38− BM cells. However, we found an almost constant ratio of LTC-IC/CD33+/CD38− progenitor cells in three of our experiments (Table 2), in spite of a wide variation in their absolute numbers. It is, therefore, possible that a minority of progenitors that formed clones in our culture system actually represented LTC-IC.

The frequency of CD33+/CD38− progenitors among LD/phag BM cells was found to be greatly reduced in 9/10 patients who had received cytotoxic chemotherapy, in spite of normal blood counts and BM cellularity. Since the proportion of LD/phag cells and total cellularity were comparable to those observed in normal BM samples, a true depletion of CD33+/CD38− progenitors occurred in the BM of these patients. The frequency of total CFU-GM too was somewhat reduced in BM of most chemotherapy-treated patients, a finding which is in agreement with results from another group.23 The differences from control values were not, however, so evident as those observed with earlier progenitors. It is possible that long-term damage to the hematopoietic system caused by cytotoxic drugs may become evident as a reduction of earliest progenitor/stem cells before a clear depletion of CFU-GM and BM cellularity occurs. Indeed, the only patient with normal values of both total CFU-GM and CD33+/CD38− progenitors had been off therapy for four years, after receiving a short-term course of chemotherapy (MACOP-B).27 This chemotherapy is unlikely to be very toxic for early hematopoietic stem cells because of the use of a single alkylating agent (cyclophosphamide) to a low cumulative dosage. However, the small number of patients and the heterogeneity of their diseases do not allow definitive conclusions to be drawn.

Nevertheless, the quantification of CD33+/CD38− progenitors could represent a useful test for ascertaining sub-clinical long-term BM toxicity of anti-neoplastic drugs. Indeed, we have recently confirmed a long-lasting depletion of both CD33+/CD38− progenitors and LTC-IC in most patients successfully autotransplanted with BM or peripheral blood progenitor cells (manuscript in preparation).

Moreover, compared to those of other methods exploring the early hematopoietic compartment, the simpler procedure and shorter incubation time of our assay make it valuable for transplantation purposes too, in estimating the amount of immature myeloid progenitors in BM harvests and leukapheresis collections. Indeed, the amount of CD33−/CD38− progenitor cells autotransplanted has recently been found to predict early myeloid regeneration more accurately28 than total CD34+ cells.29,30

Contributions and Acknowledgments

DF was the main investigator: he designed the study and wrote the paper; BO and BB performed most of the cytotoxicity and cell culture experiments; CC performed LTC-IC assays and helped in cell separation procedures; FG did immunofluorescence tests for CD38 MAb titration and cytotoxicity evaluation. The first and last authors had the main roles in performing this study, the order of the other authors was decided on the basis of the amounts of contribution they gave to the experiments.

The authors wish to thank Dr. Chiara Argentino (Divisione di Ematologia, Universita di Torino) and Dr. Gabriele Gallone (Azienda Ospedaliera “Maria Vittoria”, Torino) for their help with the statistical analysis.

Funding

This study was supported by grants from the “M inistero della Pubblica Istruzione” (M.P.I. 40%) and “Associazione Italiana per la Ricerca sul Cancro” (A.I.R.C), Italy.

Disclosures

Conflict of interest: none.
Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received November 19, 1998; accepted March 3, 1999.

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