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## **Proliferation and differentiation potential of CD133+ and CD34+ populations from the bone marrow and mobilized peripheral blood**

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► **To cite this version:**

Irena Koutna, Martina Peterkova, Pavel Simara, Stanislav Stejskal, Lenka Tesarova, et al.. Proliferation and differentiation potential of CD133+ and CD34+ populations from the bone marrow and mobilized peripheral blood. *Annals of Hematology*, 2010, 90 (2), pp.127-137. 10.1007/s00277-010-1058-2 . hal-00573459

**HAL Id: hal-00573459**

**<https://hal.science/hal-00573459>**

Submitted on 4 Mar 2011

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1 Proliferation and differentiation potential of CD133+ and CD34+ populations from the bone  
2 marrow and mobilized peripheral blood.  
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## Abstract

*Objective:* CD34 is the most frequently used marker for the selection of cells for bone marrow (BM) transplantation. The use of CD133 as an alternative marker is an open research topic. The goal of this study was to evaluate the proliferation and differentiation potential for hematopoiesis (short and long term) of CD133+ and CD34+ populations from bone marrow and mobilized peripheral blood.

*Patients and Methods:* Eight cell populations were compared: CD34+ and CD133+ cells from both the BM (CML Ph-, CML Ph+ and healthy volunteers) and mobilized peripheral blood cells. Multicolor flow cytometry and cultivation experiments were used to measure expression and differentiation of the individual populations.

*Results:* It was observed that the CD133+ BM population showed higher cell expansion. Another finding is that during a six-day cultivation with CFSE, more cells remained in division D0 (non-dividing cells). There was a higher percentage of CD38- cells observed on the CD133+ BM population. It was also observed that the studied populations contained very similar but not the same pools of progenitors: erythroid, lymphoid, and myeloid. This was confirmed by CFU-GM and CFU-E experiments.

The VEGFR antigen was used to monitor subpopulations of endothelial sinusoidal progenitors. The CD133+ BM population contained significantly more VEGFR+ cells.

*Conclusion:* Our findings suggest that the CD133+ population from the BM shows better proliferation activity and a higher distribution of primitive progenitors than any other studied population.

*Keywords:* CD34, CD133, HSCs transplantation, VEGFR

## Introduction

1  
2 In recent years, the CD34+ antigen has been the most frequently used marker for the  
3  
4 identification of hematopoietic stem cells (HSCs). The main source of those cells for bone  
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6 marrow (BM) transplantations is peripheral blood stem cells (PBSCs) obtained using so-  
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8 called mobilization of hematopoietic stem cells from healthy donors (in cases of allogeneic  
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10 transplantation) or patients in remission (in cases of autologous transplantation). BM  
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12 transplantation is becoming an increasingly less significant method [1]. CD34+ cells represent  
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14 a heterogeneous population consisting mainly of lineage-committed progenitors and only a  
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16 small hematopoietic cell population [2]. The percentage of cells capable of rapid  
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18 reconstitution of sinusoidal endothelial cells (which is mandatory for the regeneration of so-  
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20 called vascular niches) is thus low [3].  
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26 Recently, the importance of hematopoietic stem cell populations defined by other molecules  
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28 (e.g., adhesive or chemokine receptors) has increased. Such populations can play an important  
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30 role during stem cell homing to the BM and during permanent hematopoiesis reconstitution.  
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33 One previously identified but frequently discussed hematopoietic stem cell marker is the  
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35 molecule CD133 [4]. CD133 is reported to be a structural homolog of murine promin-1. It is a  
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37 marker for stem cells with pluripotent plasticity, and CD133+ cells have been shown to be  
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39 capable of induced differentiation to endothelial cells, neural cells, hepatocytes, myocytes,  
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41 and osteoblasts [5].  
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46 Providing patients with a pool of cells that is capable of invoking both short-term and long-  
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48 term hematopoiesis reconstitution is the most important phase of both autologous and  
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50 allogeneic BM transplantation.  
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53 This study is focused on comparing eight populations of hematopoietic progenitors obtained  
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55 using magnetic separation: CD133+ and CD34+ cells from both BM (CML Ph+, CML Ph-  
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57 and healthy volunteers) and PBSCs. Flow cytometry was used for that purpose.  
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1 The first part of the study examined the proliferation potential of all studied populations. Cell  
2 expansion was monitored together with the differentiation potential of individual populations.  
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4 The main reasons for performing these experiments were 1) to provide an overview of the  
5 populations' ability to grow, expand, and proliferate and 2) to determine the share of non-  
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7 dividing -quiescent cells in these populations. Quiescent stem cells are defined as stem cells  
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9 not currently undergoing repeated cell cycles, but that might be stimulated in the future. The  
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11 presence of such a cell type is very important for long-term hematopoiesis reconstitution. The  
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13 distribution of the CD38- population was measured during the isolation of individual  
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15 populations as well as during short-term cultivation [6].  
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21 Another topic studied in this article is the distribution of both markers (CD133 and CD34) in  
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23 individual populations. We sought to determine the degree of overlap between these  
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25 populations. Recent studies [7] aimed at regeneration of sinusoidal BM cells have shown that  
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27 endothelial progenitor cells (EPCs) are very important for graft engraftment. The  
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29 determination of the EPC perceptual distribution was thus also included in this study. This  
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31 determination was achieved by monitoring the distribution of cells expressing a VEGFR2  
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33 marker. The expression of this particular marker in the hematopoietic progenitor population  
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35 defines the subpopulation of EPCs [8, 9].  
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41 In order to study the percentage of progenitor cells, the following lineage commitment  
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43 markers were analyzed: CD71 for the erythroid lineage, CD33 for the myeloid lineage, and  
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45 CD10 for the lymphoid lineage. The presence of those progenitors in grafts is very important  
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47 from the perspective of short-term hematopoiesis reconstitution. The ability of individual  
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49 populations to reconstitute hematopoiesis was also proven using CFU-GM and CFU-E  
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51 experiments.  
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## Materials and Methods

### *Patients and samples*

BM samples were taken from 12 patients diagnosed with chronic myeloid leukemia (CML) and 7 healthy volunteers. The CML samples included seven patients with de novo CML and five patients in complete molecular remission (i.e., with no leukemia cells detected by PCR and FISH). Molecular emission was evaluated according to the recommendations of European Leukemia Net. Regular evaluation of BM-treated CML patients at exact intervals was used to monitor the treatment success rate. Cytogenetic monitoring was performed at months 3, 6, 12 and 18. Molecular monitoring was performed every 3 months. Patients were analyzed on month 3 (hematologic response [HR]), month 6 (HR or cytogenetic response [CgR]), month 12 (CgR [Philadelphia chromosome-positive (Ph+) < 35%]) and month 18 (complete CgR, major molecular response [MMoIR]) [10].

CML de novo diagnosed patients played a very important role in the study: large amounts of both CD34+ and CD133+ cells were obtained from their BM samples (see results). Thus, most of the experiments mentioned in the results section were done simultaneously using one sample and even multiple repetitions.

Twelve samples of mobilized PBSCs were taken from volunteers. All samples were taken after obtaining written, informed consent. All samples were processed immediately after collection.

### *Isolation of CD34+ and CD133+ cells*

Mononuclear cells (MNCs) were isolated from the BM and PBSCs using Histopaque-1077 density gradient centrifugation (Sigma-Aldrich, St. Louis, USA). After washing three times

1 with phosphate-buffered saline (PBS), the MNCs were incubated with an erythrocyte lysis  
2 buffer (ammonium chloride lysis buffer) to remove residual erythrocytes.  
3

4 MNCs from the BM and PBSCs were divided into two equal parts: the first one was separated  
5 into CD34+ cells, and the second one was separated into CD133+ cells. For CD34+ and  
6 CD133+ selection, the MNCs were subjected to immunomagnetic separation using magnetic-  
7 activated cell sorting (MACS) with a CD34 or CD133 MicroBead Kit (Miltenyi Biotec,  
8 Bergisch Gladbach, Germany). Positive selection of CD34 and CD133 was performed  
9 according to the instructions of the manufacturer (Miltenyi Biotec, Bergisch Gladbach,  
10 Germany). The cells were labeled with anti-CD34 or anti-CD133 antibodies, directly coupled  
11 to magnetic microbeads, and incubated for 30 min at 4°C. The cells were washed with PBS  
12 and then counted in a Bürker counting chamber using a Countess Automated Cell Counter  
13 (Invitrogen, Carlsbad, USA).  
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28 We obtained eight different cell populations: three of BM CD34+ (source: CML Ph-, CML  
29 Ph+ and healthy volunteers) three of BM CD133+ (source: CML Ph-, CML Ph+ and healthy  
30 volunteers), one of PBSC CD34+ and one of PBSC CD133+ cells. These cell populations  
31 were compared with each other.  
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#### 41 *Lymphocyte depletion*

42 The mononuclear portion of leukocytes of the BM of healthy donors or naive de novo CML  
43 patients consists of immature myeloid cells, lymphocytes and monocytes in various  
44 percentages [11]. To enrich the primitive hematopoietic cells in the mononuclear cell fraction  
45 for FACS analysis, we depleted the lymphocytes by magnetic labeling. CD2 Microbeads  
46 (MACS<sup>®</sup> Cell Miltenyi Biotec, Bergisch Gladbach, Germany) were used to deplete the T cells  
47 and NK cells. CD19 Microbeads (MACS<sup>®</sup> Cell Miltenyi Biotec, Bergisch Gladbach,  
48 Germany) to deplete the B cells.  
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5 *Cell viability*  
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7 Cell viability was evaluated through the trypan blue exclusion test using the Countess  
8 Automated Cell Counter. A single sample measurement using this counter provided the  
9 following data: live and dead cell concentration/ml, total cell concentration/ml, viability,  
10 mean diameter, and cell images.  
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16 For identification of apoptotic and necrotic cells, we also used an annexin V-FITC Kit  
17 (Miltenyi Biotec, Bergisch Gladbach, Germany) and propidium iodide (PI) (Sigma-Aldrich,  
18 St. Louis, USA) according to the manufacturer's directions. The viability of CD34+ and  
19 CD133+ cells just after isolation was  $99 \pm 0.5\%$ .  
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29 *CFSE staining*  
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31 CD34+ and CD133+ cells were stained with 1  $\mu$ M 5(6)-carboxyfluorescein diacetate N-  
32 succinimidyl ester (CFSE; Sigma-Aldrich, St. Louis, MO, USA) as described previously [12].  
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34 These cells were cultured for six days in StemSpan Serum-Free Expansion Medium (SFEM;  
35 StemCell Technologies, Köln, Germany) with a cytokine mixture. After three and six days,  
36 the cells were harvested, washed once in PBS, and stained with anti-CD34-PE or anti-CD133-  
37 PE antibodies for flow cytometric analysis. We also cultured the cells in the presence of 100  
38 ng/ml demecolcine (Sigma-Aldrich, St. Louis, MO, USA), which arrests the cell cycle. The  
39 cultures containing demecolcine were used to establish a non-dividing cell population (CFSE  
40 maximum) during the six-day interval.  
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56 *Multicolor flow cytometric analysis*  
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1 The percentage of human CD133, CD34, CD38, VEGF R2, CD10, and CD33 cells was  
2 determined using antibodies labeled with fluorescein isothiocyanate (FITC), phycoerythrin  
3 (PE), or allophycocyanin (APC). The anti-CD133 antibody conjugated to PE (clone:  
4 AC133/2) and the anti-CD38 antibody conjugated to APC were obtained from Miltenyi  
5 Biotec (Bergisch Gladbach, Germany). The anti-CD34 antibody conjugated to PE or FITC  
6 was from Becton-Dickinson (Heidelberg, Germany). The anti-human VEGFR R2/KDR  
7 conjugated to FITC was from RD Systems (Minneapolis, MN, USA). The anti-CD10  
8 antibody conjugated to PE and the anti-CD33 antibody conjugated to FITC were obtained  
9 from Immunotech (Prague, Czech Republic). The immunofluorescent staining was performed  
10 according to the manufacturer's instructions. The cells were incubated with appropriate  
11 antibodies for 30 min at 4°C in the dark. Subsequently, the cells were washed once with PBS.  
12 Flow cytometric analysis was performed on the BD FACS Canto II flow cytometer (Becton-  
13 Dickinson, Heidelberg, Germany) using BD FACSDiva software (Becton-Dickinson,  
14 Heidelberg, Germany). A minimum of 15,000 events were analyzed in each sample. In the  
15 tables, the medians and range were used to describe data collected in flow cytometry. For  
16 flow cytometric analysis, the cells were taken from cell culture on days 0, 3, and 6.

#### 41 *In vitro cultivation*

42 Liquid culture: CD34+ or CD133+ cells were maintained in SFEM supplemented with the  
43 StemSpan CC100 cytokine mixture. The concentration of cytokines in the CC100 cocktail  
44 used for cultivating enriched HSCs was 100 ng/mL for SCF and FLT-3 ligand (Flt-3-L), and  
45 20 ng/mL for IL-3 and IL-6.

46 (StemCell Technologies, Köln, Germany) at 37°C in 5% CO<sub>2</sub> and 100% humidity. The cells  
47 were cultivated for 6 days in 4-well multidishes from Nunclon (Thermo Fisher Scientific,  
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Langensfeld, Germany), with  $2.0 \times 10^4 - 10^5$  cells/ml added to each well. The medium was changed after three days.

Semi-solid culture: CD133 and CD34 cells were resuspended in SFEM at  $5 \times 10^3$  cells/ml and mixed with 4 volumes of methylcellulose-based semi-solid culture medium (Human MethoCult; StemCell Technologies, Köln, Germany) containing stem cell growth factor (SCGF; 20 ng/ml) and granulocyte colony-stimulating factor (G-CSF; 6000 U/ml) for generating CFU-GM and SCGF (20 ng/ml) and erythropoietin (EPO; 1.5 U/ml Sigma-Aldrich, St. Louis, USA) for generating CFU-E. Aliquots of the mixtures ( $10^3$  cells/1 ml) were plated in dishes and incubated for 15 days at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C.

### *Statistical analysis*

Data are presented as medians. To determine statistical significance, the nonparametric Mann-Whitney U test was applied. The criterion for statistical significance was defined as  $p < 0.05$ . The software available at <http://faculty.vassar.edu/lowry/utest.html> was used for the computation of statistical values.

## Results

### *In vitro expansion*

Cell growth, absolute cell counts in the culture and population viability was monitored in parallel. At the beginning of cultivation, a given amount of cells was added to the culture. Cell growth measurements for individual populations are shown in Table 1. The median values for each group were based on 7 CML Ph+, 5 CML Ph- and 7 healthy volunteer samples for the BM and 12 samples for PBSC groups. There were no significant differences in proliferation

1 activity among all of the studied sources (CML Ph+, CML Ph- and healthy volunteers) of the  
2 CD133 cells. The same was observed for the CD34 cells.  
3

4 Although the concentrations of cells (cell/ml) added to the cultures differed at the beginning  
5 of each experiment, this difference did not have a statistically significant effect on the  
6 proliferation and viability of the resulting culture. A significant increase in proliferation  
7 activity was observed in cultures of CD133+ cells from the BM ( $p=0.006$ ). The increase was  
8 more than two times higher (as seen in Table 1) than that observed in all CD34+ cultures.  
9 Interestingly, the differences among populations of the same cell type were insignificant.  
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#### 24 *CFSE experiments*

25 Using the CFSE method, we were able to monitor changes in the number of divisions in  
26 individual cultures. As clearly shown in Table 2 and Figure 1, it was easy to determine the  
27 number of cell divisions and the number of cells remaining in the non-dividing state (i.e.,  
28 division 0).  
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36 From the perspective of cell counting, it was obvious that there was a high percentage of  
37 CD133+ cells from the BM that were not dividing, even after 6 days of culture. Despite this  
38 fact, those cells highly expressed the CD133 marker. Such cells were also found in the  
39 CD34+ BM and the CD34+ PBSC populations but at much lower concentrations (as seen in  
40 Table 2). It can thus be assumed that the distribution of non-dividing cells (which are assumed  
41 to be primitive progenitors) in the CD133+ BM population is significantly higher than in  
42 other populations.  
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53 To confirm this assumption, we performed an independent experiment to monitor the  
54 CD133+/38- and CD34+/38- populations in parallel. Particular divisions were monitored on  
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1 culture day 5. We found 70% of cells were viable in the CD133+ BM population and 68%  
2 were viable in the CD34+ BM population.  
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4 The most of cells in the D0 phase were CD133+/38- cells, while the populations of CD133+  
5 and CD38- were minor. In subsequent generations, there were less CD133+ cells. The  
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7 distributions of the individual cell types in the D0-D3 divisions on culture day 5 are clearly  
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9 visible in Figure 2.  
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### 14 *CD38+ antigen determination*

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16 The CD38- population was monitored in order to determine the distribution of primitive HSCs  
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18 in individual populations. Numbers of the CD133+/38- and CD34+/38- cells from the BM and  
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20 PBSCs were monitored using flow cytometry on days 0, 3 and 6 during cultivation. The  
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22 results are shown in Table 3. The differences between the BM populations are also  
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24 documented in Figure 3. During CD38+ antigen determination in both types of CML samples  
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26 (Ph+ and Ph-), it was observed that the CD133+ population generally contained more CD38-  
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28 cells than the CD34+ population. The measurements on samples from healthy volunteers  
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30 confirmed the same trend: the CD133+ population contained 9.2% CD38- cells and the  
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32 CD34+ population contained only 3.5% on day 0.  
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### 43 *Mutual distribution of individual populations*

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45 The amounts of cells isolated from 5 ml of BM or PBSCs were quantified. The numbers of  
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47 CD133+ cells isolated from the BM of de novo diagnosed CML (CML Ph+) patients were  
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49 268,000-873,000 (median 634,000), and the numbers of CD34+ cells isolated from the BM of  
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51 de novo diagnosed CML patients were 658,000-1,600,000 (median 1,197,000).  
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2 The cell counts for the patients in molecular remission (CML Ph-) were lower: the numbers  
3 of CD133+ cells from the BM were 43,000-112,000 (median 76,000) and the numbers of  
4 CD34+ cells were 105,000-256,000 (median 183,000).  
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7 The cell counts for the healthy volunteers were also different: the numbers of CD133+ cells  
8 from the BM were 180,000-320,000 (median 221,000) and the numbers of CD34+ cells were  
9 225,000-550,000 (median 413,000).  
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13 The numbers of CD133+ cells isolated from PBSCs were 78,000-288,000 (median 108,000)  
14 and the numbers of CD34+ cells isolated from PBSCs were 122,000-608,000 (median  
15 495,000).  
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20 For both BM and PBSCs, the cell yields during CD133+ antigen isolation were about 50% of  
21 that during CD34+ antigen isolation.  
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25 To validate the presence of two times more CD34+ cells in both bone marrow and PBSC, we  
26 performed FACS analysis directly on the harvested samples. Because the distributions of both  
27 CD133+ and CD34+ cells are between 0.3% and 1.2%, the significance of the FACS analysis  
28 would not be sufficient. Therefore, we decided to deplete the lymphocyte fraction in order to  
29 obtain an enriched population. The measurements of the CD133+ and CD34+ distributions  
30 were performed on BM samples of one de novo CML (one sample), one CML in molecular  
31 remission, and one healthy donor and two samples of PBSC. The distributions in the enriched  
32 population were  $25.3\% \pm 4.8\%$  for CD34+ and  $13.1\% \pm 3.4\%$  for CD133+.  
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45 By comparing the expression of the CD133 and CD34 antigens using multicolor flow  
46 cytometry, we observed that CD133+ cells were clearly not in the subgroup of CD34+ cells  
47 [13]. For example, the BM sub-population sorted from healthy volunteers using the CD133  
48 antigen contained only 72% of CD34 cells. Table 4 shows the changes in the individual  
49 markers (CD133 and CD34) immediately after isolation and during cultivation.  
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1 From the discussion above, it is obvious that BM cells and PBSCs differ significantly. The  
2 CD133+ and CD34+ PBSC populations demonstrate a relatively large overlap; however, for  
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4 the BM, the CD133+/34- subpopulation represents more than 30% of the entire CD133+  
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6 population (Table 4).  
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### 10 11 *Analysis of the distribution of lineage-committed progenitors*

12 Flow cytometry was used to study the distributions of the following antigens: CD71 for  
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14 erythroid progenitors, CD10 for lymphocytic progenitors and CD33 for myeloid progenitors.  
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16 For the CD71 antigen, only day 0 was included in the study because EPO was not included in  
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18 the cultivation medium; therefore, differentiation into the erythroid line was not supported.  
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20 The average median of these populations was  $5.4\% \pm 1.3\%$  (data not shown). The distribution  
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22 was thus sufficient in all populations to permit the successful reconstitution of erythropoiesis  
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24 in the presence of the relevant cytokines. Significant differences among the populations were  
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26 not observed during analysis of the myeloid and lymphoid line markers. The p-values for  
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28 comparisons between the individual populations were between 0.625 and 0.961. During  
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30 cultivation, similar increases were observed across all populations (Figure 4).  
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39 The CFU-GM and CFU-E formations were used to compare the colony-forming capacities of  
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41 CD133 and CD34 cells derived from the BM of the same healthy volunteer. In five separate  
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43 experiments, cells were cultured for 15 days in methylcellulose-based semi-solid medium.  
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45 CD133 and CD34 cells displayed non-significant differences in CFU-GM formation (median  
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47 number of colonies 43, cf. 39).  
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51 At the beginning of the experiment, all BM populations showed equal distributions of the  
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53 CD71 marker. However, they showed different numbers of colonies during CFU-E  
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55 experiments. Even though the differences in the number of colonies generated from CD133  
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1 and CD34 cells were not significant (at  $p < 0.05$ ), larger numbers of these colonies were  
2 found in CD133 cells (median number of colonies was 36, cf. 25)  
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### 6 *Analysis of VEGF distribution*

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9 As stated above, the presence of sinusoidal progenitor cells seems to be one of the most  
10 important factors for successful transplantation. The VEGFR antigen was used to monitor this  
11 subpopulation. As shown in Table 5 and Figure 5, the amount of cells bearing this antigen  
12 was significantly ( $p=0.0093$ ) higher in the CD133+ BM populations compared to the CD34+  
13 BM population. Furthermore, these results indicate that the concentration of these cells is not  
14 high enough to negatively affect the reconstitution of hematopoiesis.  
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### 26 Discussion

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28 Currently, the transplantation of hematopoietic stem cells is one of the most extensively used  
29 treatments for multiple types of leukemia.  
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33 The transplantation of hematopoietic stem cells depends on four main factors: homing, which  
34 is influenced by the presence of adhesive molecules in the graft; graft engraftment, which is  
35 an immunological process; reconstitution of the blood cell population; and reconstitution of  
36 the stem cell population, which is related to the presence of stem cells in the graft.  
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38 Unfortunately, although these four factors have been extensively studied, the probability of  
39 non-relapse mortality is still far from zero. This problem provides the motivation for further  
40 investigation of the suitability of the most frequently used source of hematopoietic stem cells,  
41 CD34+ cells from PBSCs. Many studies that aimed to define the total amount of CD34+ cells  
42 [14, 15] can be found in the literature.  
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55 This study focused on the CD133 and CD34 populations from BM and PBSCs. These  
56 populations were compared in terms of their mutual distributions. An attempt was made to  
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1 evaluate the distributions of cells for the fast and permanent reconstitution of hematopoiesis  
2 and the presence of cells capable of reconstituting sinusoidal endothelial cells.  
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4 The first aim of this article was to determine the amount of cells that could be obtained from a  
5 given volume of BM or PBSCs and the expansion potential of the individual populations.  
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7 Because the sample used for isolation was split into two identical portions before MACS  
8 separation, it was possible to determine the population distributions in the samples. As  
9 described in the Results section, the cell yields for the CD133+ antigen were always about  
10 50% of those for the CD34+ antigen in both the BM and PBSCs. Thus, this population is  
11 minor compared with the CD34+ population. A comparison of the relationships between these  
12 two populations resulted in the hypothesis that there is a functional hierarchy between CD133  
13 and CD34 [16]. This hypothesis was also based on the expression profiles for the two  
14 populations [17]. A comparison of the expansion potentials was also very interesting.  
15 CD133+ cells had a more than two-fold higher expansion potential than the CD34+ cells,  
16 even for a short-term 6-day cultivation. Furthermore, it was clear that the CD133+ population  
17 also contained a high percentage of CD34- cells that are capable of expressing CD34+ during  
18 cultivation. Therefore, those cells can be regarded as a more primitive cell type.  
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21 Some authors have suggested that the CD34- cell subpopulation is fully capable of  
22 reconstituting hematopoiesis [18, 19]. It is widely accepted that the more primitive population  
23 of HSCs does not express the CD38 antigen [20]. This antigen is expressed even on lineage-  
24 committed progenitors and especially on immune system cells, in which it is responsible for  
25 cell-to-cell interactions [21]. In this study, we demonstrated that the CD133 population  
26 (especially from the BM) contained a significantly higher amount of CD133+/CD38- cells  
27 compared with the CD34+ population. In the CD38- population, quiescent stem cells should  
28 also be present. Over long periods of time, those cells remain in the BM in a quiescent, non-  
29 dividing state and are localized in the stem cell niches. They are also responsible for  
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1 hematopoietic homeostasis and the creation of a stem cell pool hierarchy by asymmetric self-  
2 renewal, which produces lineage-restricted, short-term repopulating cells and long-lasting  
3 repopulating cells (LTRCs). Recent studies have also shown that these cells have very  
4 important functions in repopulation dynamics, turnover, hierarchical structure, and the cell  
5 cycle status of human HSCs in the recipient BM microenvironment [22]. Using the CFSE  
6 experiments, we demonstrated that a high percentage of cells in the CD133+ BM population  
7 neither divided nor were differentiated, even under the pressure of growth factors. They  
8 remained fully viable and retained CD133+ antigen expression. The presence of these cells is  
9 very important from the perspective of permanent hematopoiesis reconstitution after  
10 transplantation.  
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24 An equally important prerequisite for successful transplantation is the presence of lineage-  
25 committed progenitors in the graft. This study demonstrated that lymphoid, erythroid, and  
26 myeloid progenitors were similarly present in all populations. Subsequent CFU-GM  
27 experiments also did not show any significant differences in the numbers of colonies  
28 generated. However, the CFU-E experiments showed differences: the CD133+ population  
29 was able to generate a slightly larger number of colonies. Finally, the content of those cells  
30 was sufficient for high quality, short-term hematopoiesis reconstitution.  
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41 CD133 is a marker of pluripotent plasticity. CD133-positive cells are capable of  
42 differentiating into various cell and tissue types, including a wide variety of hematopoietic  
43 and non-hematopoietic types [16, 23, 24]. This characteristic could indicate that this cell pool  
44 is not appropriate for transplantation. However, this population also contains endothelial  
45 progenitors that play an important role during successful transplantation [7]. High dose  
46 chemotherapy to a large extent destroys the architecture of the BM. When transplanted cells  
47 do not find relevant niches in the BM or cells capable of reconstituting the BM architecture  
48 are not present in the graft, the graft itself is not able to engraft [25]. The CD133+ population  
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1 contains a higher percentage of EPCs than the CD34+ population. EPCs support the creation  
2 of sinusoidal endothelial cells. Endothelial cells are a mandatory component of the BM niche  
3 and play an important role in neoangiogenesis [9]. During the evaluation of the VEGFR  
4 antigen (EPC marker) on BM cells, significant differences were not observed among the  
5 CD34 sub-populations (CML Ph+/Ph-, healthy volunteers). Significant differences were also  
6 not observed among CD133 sub-populations (CML Ph+/Ph-, healthy volunteers) [26].  
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10 The number of EPCs was measured in the CD34+ and CD133+ populations, and a significant  
11 difference was observed. The high pluripotent plasticity of CD133+ VEGFR+ and CD34+  
12 VEGFR+ cells was demonstrated using cultivation experiments. These cells did not  
13 proliferate into endothelial cells in the presence of relevant growth factors. During cultivation,  
14 the VEGFR antigen disappeared. Because an increase in apoptotic cell death corresponding to  
15 the percentage of EPCs was not observed during culture (97% viability), we suggest that the  
16 cultivation environment induced proliferation into the hematopoietic pathway.  
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31 Many recent studies have reported that the CD133+ cell population is either a subpopulation  
32 of CD34 cells [27-30] or an independent population that is fully capable of supporting or  
33 reconstituting hematopoiesis for both patients and SCID mice [31].  
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39 There are articles in which CD133 was reported to be a marker for a new selection strategy  
40 for the purification of autologous grafts and transplantation [32]. In an article by Isidori [33],  
41 the capacity of positively selected autologous CD133+ hematopoietic stem cells (HSCs) to  
42 reconstitute lymphomyelopoiesis in chronic lymphocytic leukemia is described. The authors  
43 state that the reinfusion of highly purified CD133+ HSCs facilitated a rapid and sustained  
44 recovery of hematopoiesis after myeloablative treatment in resistant/relapsed CLL patients.  
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53 The aim of the study by Hess et al. [34] was to identify an alternative HSC population to the  
54 standard CD34+ marker and show that cell selection based on ALDH activity and CD133  
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1 expression provides a novel method for purification of HSCs with long-term repopulating  
2 capacity  
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4 Even when samples from patients with CML (Ph+, Ph-) were compared with samples from  
5 healthy volunteers, significant differences were observed in both cellular proliferation and the  
6 expression of relevant CD antigens in the individual populations.  
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11 In the PBSC populations, significant differences between CD133 and CD34 were not  
12 observed. The statistical significance (p) values of the monitored parameters were near 0.06.  
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16 In contrast, the differences for the BM were significant at  $p=0.015$ .  
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18 This work demonstrates that the CD133+ cell population of BM shows many differences  
19 compared with the other populations included in the study. During the experiments described  
20 above, the CD133+ BM population had better cultivation and expansion potentials than the  
21 CD34+ population.  
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28 The population also contained a higher percentage of non-dividing CD38- cells. Those cells  
29 can be assumed to be more primitive component of the bone marrow, which may be important  
30 for long term hematopoiesis reconstitution. During analysis of the CFU-GM and CFU-E  
31 experiments, we did not observe significant differences between the CD133+ and CD34+  
32 cells; therefore, from the perspective of short-term hematopoiesis, both populations are  
33 comparable.  
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44 Furthermore our study confirmed that the CD133+ BM population contains a significantly  
45 higher percentage of the so called endothelial progenitors in the CD133 population. Those  
46 cells play a crucial role in the reconstitution of the BM architecture and thus in engraftment.  
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51 In summary, the CD133+ selected population has many advantages over other studied  
52 populations.  
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There are many studies available in which the CD133+ enriched population was used for transplantation. For example, a report by Lang et al. showed the transplantation of a combination of CD133+ and CD34+ selected progenitor cells from alternative donors [28]. The patients showed rapid platelet recovery, whereas T cell regeneration was variable. The implication of these results is that CD133+ selection can be feasibly used for transplantation from alternative donors, and further trials should be conducted with total CD133+ separated grafts.

The results presented in this work confirm that the CD133+ population is suitable for transplantation. However, an important limiting factor is also presented. The content of the CD133+ cells in bone marrow is significantly lower than that of CD34+ cells. The logical solution therefore is the ex-vivo expansion, which should enable the application of the mentioned method for transplantation.

#### Acknowledgments

This work was generously supported by the Ministry of Education of the Czech Republic grant MSM-0021622430, NPVII (2B06052). We would like to thank the Dept. of Internal Medicine – Hematooncology, Hospital Brno, Jihlavská 20, 625 00 Brno, Czech Republic, especially Zdenek Koristek M.D. and Zdenek Racil M.D. for the collection of blood and bone marrow samples.

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Figure legends:

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5 Figure 1. The flow cytometric analysis of experiments with CFSE. A) CD34+ cells from BM  
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7 of healthy volunteers after three-day cultivation with CFSE. B) CD133+ cells from BM of  
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9 healthy volunteers after three-day cultivation with CFSE C) CD34+ cells from BM of healthy  
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11 volunteers after six-day cultivation with CFSE. D) CD133+ cells from BM of healthy  
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13 volunteers after six-day cultivation with CFSE. The demecolcine control is highlighted only  
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15 in subfigure A for better orientation in figures. CD133+ and CD34+ cells were stained with a  
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17 PE-conjugated antibody, and CFSE intensities were measured by the FITC channel as  
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19 described in the materials and methods section.  
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24 Figure 2. The distribution of analysed populations in individual divisions after five days of  
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26 cultivation.  
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29 Figure 3. The flow cytometric analysis of CD133+/CD38- and CD34+/CD38- populations  
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31 from bone marrow obtained from healthy volunteers. CD133+ and CD34+ cells were stained  
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33 with PE-conjugated antibody and CD38+ with APC-conjugated antibody as described in the  
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35 materials and methods section.  
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41 Figure 4. The visualization of CD33+ and CD10+ cells in the analyzed populations on day 0  
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43 and after 3 and 6 days of cultivation. The numbers for BM populations from healthy  
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45 volunteers are represented by their median value.  
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51 Figure 5. The graphic visualization of VEGFR+ cells from healthy volunteers' BM in all  
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53 analyzed populations on day 0 and after 3 and 6 days of cultivation.  
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Tables

Table 1. The amount of input cells in the culture and fold increase of cell amount after three and six days of cultivation. The measured population viability in Day 0 was 99% in all cases.

Type of isolation		Day 0 Cell count median (cell/ml)	Day 3 Fold increase (median) Average population viability (%)	Day 6 Fold increase (median) Average population viability (%)	
BM	CD133+	CML Ph <sup>+</sup>	65,000 (44,000-85,000)	4.5 (2.5-6.0) 94%	24 (18.0 -32.0) 91%
		CML Ph <sup>-</sup>	29,000 (10,000*-32,000)	4.0 (1.5-5.0) 94%	19 (12.0 -29.0) 91%
		healthy volunteers	60,000 (40,000-80,000)	4.5 (2.5-5.0) 94%	21 (16.0 -30.0) 91%
	CD34+	CML Ph <sup>+</sup>	97,000 (80,000-110,000)	4.8 (3.0-4.3) 96%	11 ( 5.0- 16.0) 88%
		CML Ph <sup>-</sup>	37,000 (30,000-60,000)	3.2 (1.2-4.0) 96%	9 ( 5.0- 14.0) 88%
		healthy volunteers	90,000 (80,000-100,000)	4.8 (3.0-4.3) 96%	10 ( 7.0- 15.0) 88%
PBSC	CD133+ PBSC	32,000 (10,000*-94,000)	3.9 (2.2-4.8) 92%	13( 8.0-19.0) 90%	
	CD34+ PBSC	41,000 (30,000-85,000)	3.8 (1.9-4.7) 93%	11 (9.0-14.0) 90%	

\*The amount of cells bellow 30,000 was not included in the calculation of fold increase.

Table 2. The percentage of cells (median) in individual divisions after a six-day cultivation  
\*healthy volunteers - H.V.

Type of isolation			Percentage of cells in individual divisions				
			D0	D1	D2	D3	D4
BM	CD133+	CML Ph <sup>+</sup>	12.6 (8.9-14.2)	16.1 (12.9-18.3)	21.3 (17.9-23.2)	27.7 (22.8-31.1)	15.4 (8.9-16.2)
		CML Ph <sup>-</sup>	10.4 (9.2-13.0)	18.4 (8.9-22.2)	24.2 (18.2-26.6)	23.6 (16.8-26.2)	14.3 (10.3-17.2)
		*H.V.	12.6 (8.3-15.6)	15.8 (11.7-19.6)	22.8 (14.9-29.3)	26.9 (18.9-30.0)	15.8 (9.6-17.2)
	CD34+	CML Ph <sup>+</sup>	5.2 (4.4-7.6)	21.3 (18.1 -39.5)	32.7 (29.4-34.9)	25.1 (19.2-28.4)	11.5 (8.6-15.8)
		CML Ph <sup>-</sup>	4.9 (2.7-7.3)	22.1 (17.2 -37.5)	29.3 (27.2-41.6)	26.2 (14.9-28.7)	8.8 (5.3-10.8)
		*H.V.	7.1 (6.5-10.1)	16.2 (12.3 -20.1)	27.4 (24.1-31.2)	26.4 (15.4-30.9)	11.3 (5.6- 12.3)
PBSC	CD133+	8.1 (6.1-11.3)	19.3 (14.3 -29.4)	34.6 (27.4-41.3)	24.8 (16.2-29.4)	8.6 (4.2-9.7)	
	CD34+	4.3 (2.5-5.8)	30.2 (13.7 -36.2)	33.8 (27.2-40.6)	20.5 (15.4-26.7)	9.4 (6.8 -11.4)	

Table 3. The percentage of CD38 in analyzed populations on day 0 and after 3 and 6 days of cultivation.

\* "H.V." stands for healthy volunteers

Content of CD 38 <sup>-</sup> in individual populations	BM (median %)						PBSC (median %)	
	CD133+/CD38 <sup>-</sup>			CD34+/CD38 <sup>-</sup>			CD133+/CD38 <sup>-</sup>	CD34+/CD38 <sup>-</sup>
	CML Ph <sup>+</sup>	CML Ph <sup>-</sup>	H.V.*	CML Ph <sup>+</sup>	CML Ph <sup>-</sup>	H.V.*		
Day 0	8.4	6.6	9.2	3.1	2.9	3.5	5.4	1.2
3	5.1	3.8	4.2	2.1	1.5	2.5	1.6	1.2
6	2.6	2.3	2.4	1.6	1.4	1.2	0.7	0.6

Table 4. The percentage of mutual distribution of CD133+ and CD34+ antigens of analyzed populations on day 0 and after 3 and 6 days of cultivation. For example cells isolated using CD133 antigen from BM of CML Ph+ patients contained only 52% of CD34+ cells in day 0.

\* "H.V." stands for healthy volunteers

CD34+ antigen	BM						PBSC	
	CD133+ isolation (median)			CD34+ isolation (median)			CD133+ isolation (median)	CD34+ isolation (median)
	CML Ph <sup>+</sup>	CML Ph <sup>-</sup>	H.V.*	CML Ph <sup>+</sup>	CML Ph <sup>-</sup>	H.V.*		
Day 0	52%	64%	72%	98%	95%	98%	74%	97%
3	56%	59%	61%	74%	69%	59%	62%	68%
6	51%	54%	47%	42%	52%	45%	56%	42%
CD133+ Antigen								
Day 0	94%	97%	96%	45%	51%	53%	97%	62%
3	65%	62%	67%	28%	29%	34%	67%	46%
6	42%	48%	29%	19%	19%	18%	39%	29%

Table 5. The percentage of VEGFR-positive cells in all analyzed populations on day 0 and after 3 and 6 days of cultivation.

\* "H.V." stands for healthy volunteers

VEGF+ antigen	BM (median %)						PBSC (median %)	
	CD133+			CD34+			CD133+	CD34+
	CML Ph <sup>+</sup>	CML Ph <sup>-</sup>	H.V.*	CML Ph <sup>+</sup>	CML Ph <sup>-</sup>	H.V.*		
Day 0	17.1	13.8	12.1	6.6	5.2	7.4	11.8	10.6
3	2.4	2.1	1.9	1.3	1.6	2.3	1.2	1.5
6	0.5	0.7	0.3	0.8	0.9	0.7	0.5	0.5

Figure 1  
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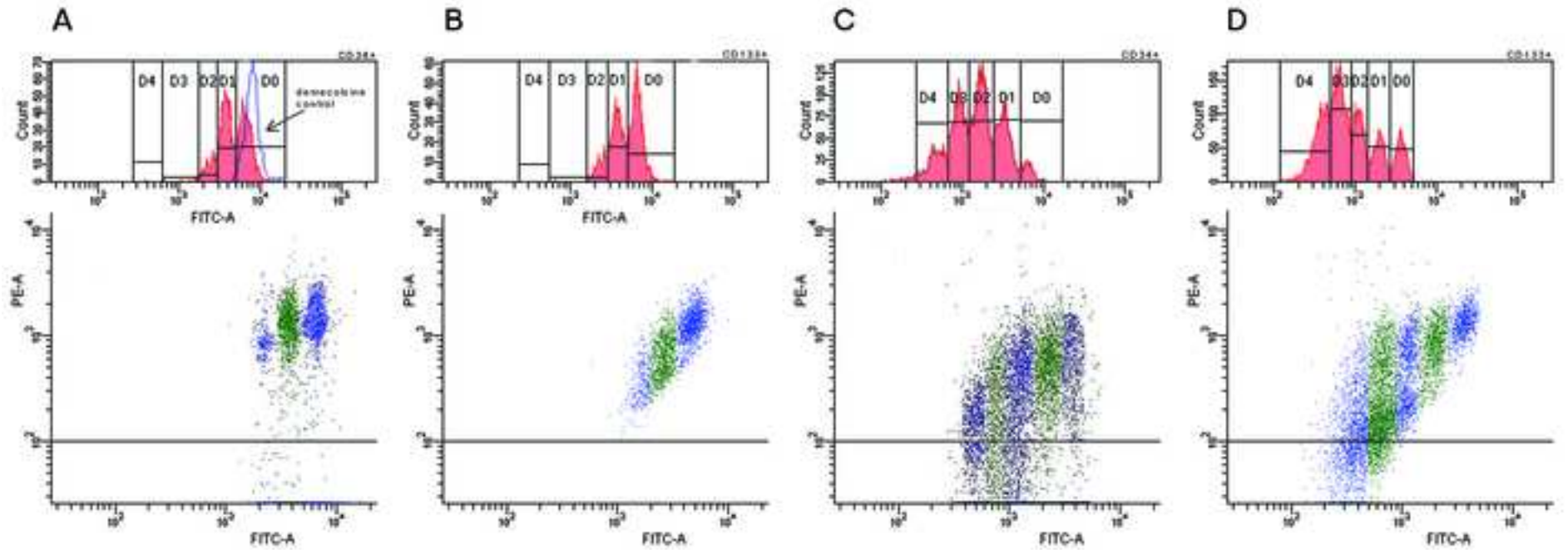




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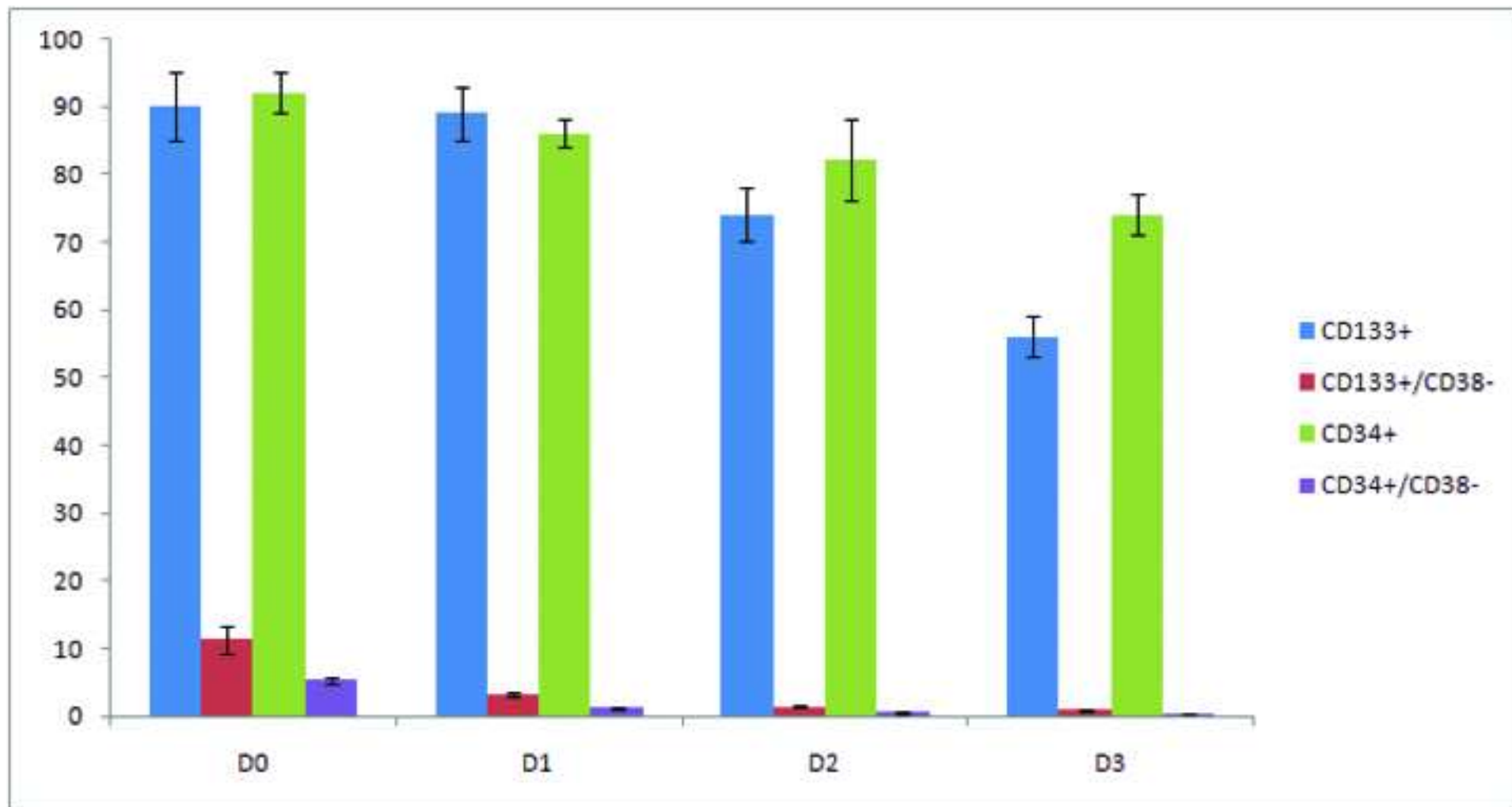
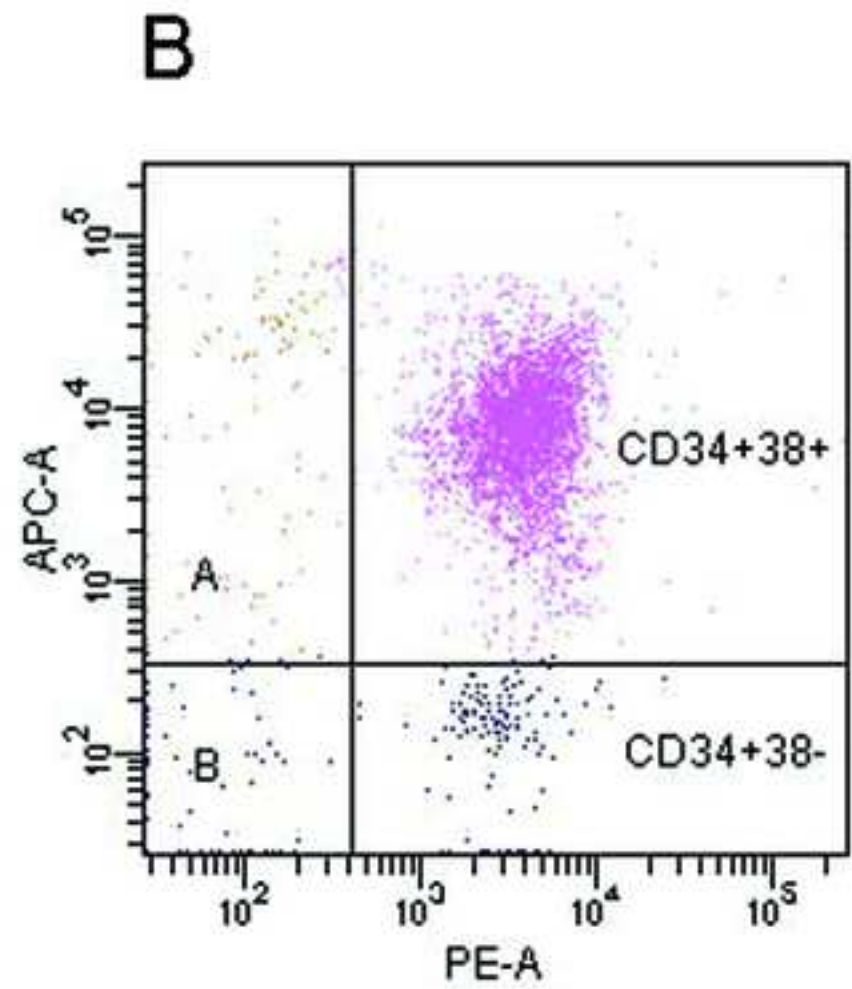
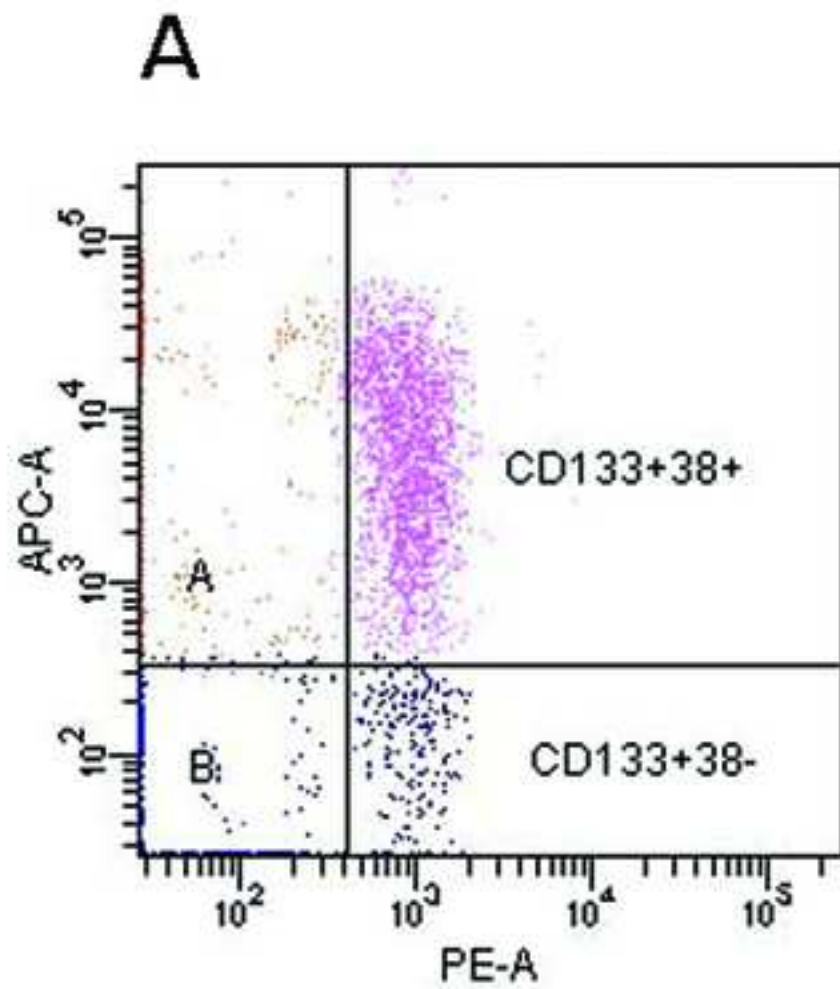


Figure 3  
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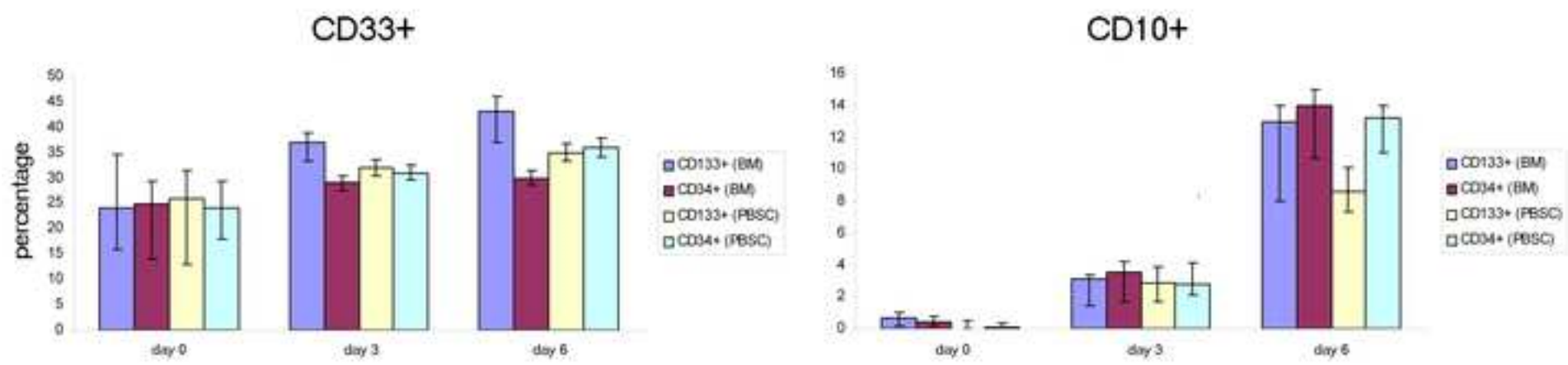


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