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Original Article

Prevalence of the *BCR/ABL* fusion gene and T cell stimulation capacity of dendritic cells in chronic myelogenous leukemia

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Abstract: Dendritic cell (DC) vaccines are promising for immunotherapy, and their production using $CD34^+$ hematopoietic stem cells (HPSCs) from patients with chronic myelogenous leukemia (CML) and healthy donors is well established. However, the generation of $CD1a^+CD14^-$ DCs and their functional properties in patients with CML remain elusive. Here, we aimed to study the biology of DCs generated from $CD34^{-/low}$ HPSCs and evaluate the status of their BCR/ABL translocation, ability to stimulate T cells, and capacity of endocytosis compared to DCs derived from $CD34^+$ HPSCs from both patients with CML and healthy donors. $CD1a^+CD14^-$ DCs were generated from $CD34^{-/low}$ HPSCs and evaluated morphologically and functionally. $CD34^+$ cells are frequently selected for transplantation and the entire $CD34^{-/low}$ HPSC fraction is wasted. Here, we anticipated the $CD34^-$ HPSC subset to constitute an invaluable source for acquiring DCs for immunotherapy. $CD34^+$ and $CD34^-$ HPSCs were sorted from the bone marrow samples of CML patients and healthy donors and differentiated *ex vivo* in a similar way. DCs from $CD34^-Lin^-$ and $CD34^+Lin^-$ HPSCs expressed comparable surface markers (CD80, CD83, CD86, HLA-DR, CD40, and CD54). Functional analysis revealed that DCs acquired from both subsets retained a potent allogeneic T cell stimulatory capacity and an efficient phagocytic ability and showed a similar *BCR/ABL* translocation status. In conclusion, DCs were successfully differentiated from the $CD34^-Lin^-$ cell subset and showed potent functional capacities, indicating their potential for application in immunotherapy and basic research.

Keywords: $CD34^+$, $CD34^-Lin^-$, dendritic cells, hematopoietic stem cell, immunotherapy, chronic myelogenous leukemia

Introduction

Chronic myelogenous leukemia (CML) is a malignancy characterized by the uncontrolled clonal expansion of myeloid progenitor cells that predominantly express the BCR/ABL fusion protein [1-4]. The latter is responsible for increased tyrosine kinase activity, which leads to excessive generation of immature myeloid cells [5]. Tyrosine kinase inhibitors (TKIs), including imatinib, nilotinib, dasatinib, and bosutinib, have revolutionized the management of CML and improved the outcome of CML patients, and are considered first-line medications [3, 6-8]. However, relapse occurs in a minor percentage of patients who are mostly in the advanced stage of the disease following the cessation of TKI medication [9-12]. The clearance of residual leukemia and disease-free remission may be achieved through efficient immune responses after reducing the leukemia blasts to a lowest level using TKI treatment [13-15]. CML stem cells demonstrate natural resistance against virtually all TKIs as well as host immune cells, and it might not be easy to completely eradicate leukemia stem cells (LSCs) [1, 2, 16-19]. Additionally, innate immune cells, including natural killers (NKs), dendritic cells (DCs), and plasmocytoid DCs (pDCs), are dysfunctional in CML [14, 20]. Hematopoietic stem cell transplantation (HSCT) is often recommended for qualified patients with obstinate LSCs [21]. Targeted immunotherapy has recently been encouraged due to the graft-versus-leukemia (GVL) effect observed after allogeneic HSCT [22-24]. Additionally, pa-

tients with CML have been successfully treated with donor leukocyte infusions (DLIs) from the original bone marrow (BM) donors [24-27]. Yet, DLIs can be accompanied by graft-versus-host disease (GvHD), BM aplasia, and infections [28, 29]. In contrast, cytotoxic T lymphocytes against leukemic cells can distinguish an anti-leukemic response from GvHD [25]. Furthermore, immunotherapy with DC-based vaccines can provoke a response from effector and memory T cells against tumor-specific antigens. Recently, it has been reported that antileukemic immunity is regulated by pDCs generated from low-level *BCR/ABL1*-expressing hematopoietic stem cells (HPSCs) [30]. Clinical trials of vaccines containing *ex vivo* generated DCs pulsed with tumor antigens provide additional evidence for acquired therapeutic immunity and have suggested that their efficacy is enhanced [31-36]. However, the role of DCs generated from CML stem cells in eliciting CML-specific T cell immunity remains controversial [20, 33, 37-39]. Currently, there are no efficient vaccines for chronic diseases and cancers that elicit strong cellular immune responses; in particular, cytotoxic T cells are known to eradicate infected cells or LSCs. Therefore, to construct an efficient vaccine, a deeper understanding of the mechanisms leading to strong cellular immune responses is crucial. DCs were reported to play a major role in stimulating immune responses to foreign antigens by acting as the main host in the uptake, transport, processing, and presentation of antigens. Additionally, DCs deliver antigens from the periphery to the secondary lymphoid organs and are the only cells that are capable of inducing a primary T cell response [2, 3, 40, 41]. Intracellular proteins can be processed and presented on the cell surface by major histocompatibility complex (MHC) molecules and DCs can be differentiated from HPSCs through the hematopoiesis process. Several reports have shown that it is possible to generate, differentiate, and mature DCs from patients with CML and normal donors in culture [4, 33, 38]. DCs produced from myeloid leukemia blasts can be utilized for the production of cellular vaccines and present a cluster of endogenously expressed leukemia antigens to immune cells; however, discrepancies in DC functions and maturation have been reported. BCR/ABL-expressing DCs have either a normal maturation status or a lower expression of co-stimulatory molecules compared with normal DCs [20, 38, 42, 43]. Alternatively, others have reported that DCs derived from both the BM of normal subjects and patients with CML differentiate and mature in culture in a similar way but differ in their function, whereas DCs derived from patients with CML show decreased activity when tested with allogeneic T cells in a mixed lymphocyte reaction (MLR) [43]. Furthermore, the existence of a CD34$^-$ HPSC fraction has been well documented both in humans and murine models and are reported to possess hematopoiesis reconstitution capabilities as well as stem cell traits [44-48]. Conventionally, human DCs have been generated *in vitro* from CD34$^+$ HPSCs isolated from cord blood (CB) [49, 50], BM [51, 52], and peripheral blood (PB) [53] using a cocktail of exogenous human granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α), and interleukin 4 (IL-4). Furthermore, most clinical and experimental protocols have used undifferentiated BM or CD34$^+$ HPSCs for the differentiation of DCs. Studies that have investigated the use of CD34$^-$ HPSCs from healthy donors as a source for the generation of DCs are rare and there is no information in the literature regarding the generation of DCs from CD34$^-$ subsets derived from patients with CML [54]. Langerhans cells (CD1a$^+$CD14$^-$) are more potent in stimulating cytotoxic T cells [55], whereas CD1a$^-$CD14$^+$ myeloid dendritic cells (mDCs) only secrete IL-10 and are thought to take part in B-cell responses [56]. Occasionally, CD34$^+$ HPSCs are sorted and used for BM transplantation, while CD34$^-$ HPSCs are discarded. Consequently, we hypothesized that the CD34$^{-/low}$ HPSC subsets could be stored and used latter as a valuable source for the generation of effective vaccines for targeted immunotherapy against residual leukemia in case of patient relapse. Moreover, we intended to study the biology of DCs generated from CD34$^{-/low}$ HPSCs, evaluate their BCR/ABL translocation status, T cell stimulation capacity, endocytosis capacity, and compare them with DCs derived from CD34$^+$ HPSCs from both patients with CML and normal donors.

Materials and methods

Recruitment of BM donors and patients with CML

The BM transplant recipients and the donors were recruited and evaluated at the Oncology

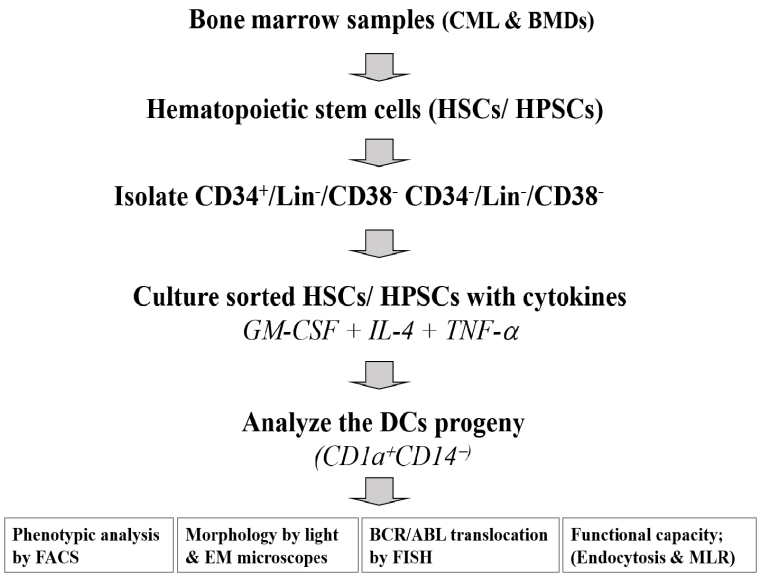

Figure 1. Schematic diagram of the experimental layout. Bone marrow (BM) samples were obtained from ten patients with CML and healthy donors. PBMCs were isolated from 5 mL BM samples using Ficoll-Paque. DCs were differentiated from the $CD34^{-}Lin^{-}$ and $CD34^{+}Lin^{-}$ HPSC subsets by culturing these with a cocktail of cytokines GM-CSF, IL-4, and TNF-α for 14 days. Their morphology and phenotypic features were verified using light and electron microscopes. Their surface phenotypes were assessed using fluorescence-activated cell sorting analysis. Mixed lymphocyte reaction (MLR) and endocytosis of polystyrene beads were performed to test their stimulatory and phagocytosis capacity, respectively.

Center, King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia. This study was approved by the Institution Research Advisory Council (RAC), Institute Ethics Committee (REC) of the King Faisal Specialist Hospital and Research Center. Written informed consent was obtained from all the patients with CML and BMDs. BM samples were obtained from the posterior iliac crest by a well-trained hematologist, as per the KFSH-RC protocol; 10-15 mL per single aspiration was drawn from each site for a maximum of four sites per donor (approximately 15 ml/KG of recipients weight). The collected BM samples were diluted in phosphate-buffered saline (PBS) containing heparin (125 U/mL of BM). For our research, we used the remaining BM samples in bags following HSCT. A schematic diagram of the study design is illustrated in **Figure 1**.

Mononuclear cells (MCs) preparation

Heparinized BM samples (5 mL) were obtained from previously untreated, Ph chromosome-positive patients with CML in the chronic phase (n = 10) and normal bone marrow donors (n=10). Mononuclear cells (MCs) were prepared from BM as described previously [43]. The BM samples obtained from patients with CML were diluted (1:4, i.e., 5 mL of BM in 20 mL of RPMI-1640 medium (Sigma-Aldrich; St. Louis, Mo, USA)) and laid over 10 mL of Ficoll-Paque PLUS (Cytiva, Marlborough, MA, USA) before centrifugation. MCs were harvested and then washed three times in RPMI-1640 medium supplemented with 5% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA). Consequently, cells were counted using a hemacytometer, and viability was calculated using the trypan blue exclusion method.

Flow cytometry analysis and isolation of $CD34^{+}$ and $CD34^{-}$ HSCs/HPSCs

Flow cytometry and lineage depletion were performed as described previously with some modifications [36-39]. Briefly, 1×10^{8} MCs were enriched for Lin^{-} cells using immunomagnetic separation (STEMCELL Technologies, Vancouver, Canada). A cocktail of antibodies against glycophorin A, CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b, and CD41 was used to label lineage-positive cells. Then a second step of sorting by FACS was conducted to confirm the purity of HPSCs. CD34 phycoerythrin (PE)-Cy5 (clone 581, class III) was added to the ready available Lin antibody, which were labeled with fluorescein-6-isothiocyanate (FITC)-conjugated antibodies against Lin antigens (CD2, CD3, CD4, CD7, CD8, CD10, CD11b, CD14, CD19, CD20, CD56, CD235a; BD Biosciences, San Jose, CA, USA), and CD38 APC (BD Biosciences) or isotopic control antibodies. Cells were sorted using BD FACSAriaTM Cell Sorter, BD Biosciences. At least 10,000 events were gated for analysis with cell purity reaching > 97%. Primary human HPSCs expressing the $CD34^{+}$ or $CD34^{-}Lin^{-}$ markers were recovered.

DCs generation and differentiation from unfractionated, CD34+/Lin-, and CD34-/Lin- HPSCs

DCs were generated from three different cellular sources: unfractionated, $CD34^+/Lin^-$, and $CD34^-/Lin^-$ HPSCs. DC generation from unfractionated HPSCs was conducted as previously described with some modifications [43]. In brief, 1×10^7 MCs were extracted, and the remaining cells were reserved for further fractionation. T cells negatively selected from PBMCs were washed twice in washing media (RPMI 1640 with 2% fetal calf serum). Then, sorted HPSCs (1×10^6) were cultured for 14 days at 37°C in a 5% CO_2 incubator. RPMI-1640 medium supplemented with 20% fetal calf serum (Gibco, Grand Island, NY, USA), L-glutamine (2 mmol/L) at a concentration of 2 mmol/L, and 1% penicillin-streptomycin were added. The following cytokines were added to the culture: 200 ng/mL GM-CSF, 10 ng/mL TNF-α, and 200 ng/mL IL-4. All cytokines were purchased from Sigma-Aldrich. The DCs were maintained at 37°C in an incubator with humidified air supplemented with 5% CO_2. Depending on the number of cultured DCs, one-third of the media volume was refreshed every 3-4 days. Differentiated DCs were harvested and counted after 12-14 days. A series of different assays were performed thereafter.

Phenotypic analysis of DCs using flow cytometry

The DCs collected after the magnetic separation were immune-labeled. Flow cytometry was conducted as previously described [57]. A panel of FITC- or PE-conjugated antibodies directed against CD3, CD4, CD8, HLA-DR, CD1a, CD54, CD11a, CD14, CD33, CD80, CD86, CD83, CD40, CD16, and CD19 was used. The analysis of the stained DCs was performed using a BD LSR Flow Cytometer (BD Biosciences) and the data obtained were analyzed using BD FACSDiva Software (BD Biosciences).

Cytochemical and immune fluorescence staining

DC morphology was revealed and validated using light and electron microscopes. DCs were harvested onto glass slides after 14 days of culture using Cytospin for cytochemical and immune fluorescence staining. The dried slides were stained with myeloperoxidase and non-specific esterase and counterstained with Wright-Giemsa, as described before [43].

Electron microscopy

The DC cultures generated from the $CD34^+$ and $CD34^-$ HPSCs were harvested and fixed for 1 h in 2% paraformaldehyde at 4°C and inserted in acryl resin (LR White; Agar Scientific, Essex, UK) and then sectioned. Sections were cut using an ultramicrotome equipped with a diamond knife; the sections were collected in a water bath as they fall from the ultramicrotome. The sections were mounted onto nickel grids and stained using anti-fluorescein mouse monoclonal antibody (Roche, Basel, Switzerland), diluted to 1:100, followed by incubation with diluted goat anti-mouse Ig-coated colloidal gold particles (Ylem, Milan, Italy). Then observed using Transmission Electron Microscope (TEM) (Joel 1230).

MLR

Heparinized blood was collected from MHC-mismatched healthy volunteers. PBMCs were harvested via centrifugation on Ficoll-Paque gradients, T cells were negatively selected and then used as targets. An MLR assay was performed as described previously [43]. A total of 5×10^4 sorted T lymphocytes were incubated with DCs. $CD1a^+$ DCs irradiated with 3,000 cGY were added at different concentrations (500; 1,000; 2,500; 5,000) to each well (Nunclon Sphera, Thermo Fisher Scientific, Waltham, MA, USA). The controls used matched the number of T lymphocytes without the addition of DCs. After 5 days, 5-bromo-2-deoxyuridine (BrdU) (Oncogene Research Products, San Diego, CA, USA) ELIZA assay was performed. A proliferation assay was applied to measure the stimulation capacity of DCs in allogeneic MLR, and the results were analyzed and compared between normal BM donors and patients with CML.

Fluorescence in situ hybridization (FISH)

Part of the DCs harvested after 14 days of culture were assigned for FISH analysis. In brief, cells were washed twice in PBS and then stained with CD1a-FITC/CD14 PE-conjugated antibodies. Finally, the DCs with the $CD1a^+$ $CD14^-$ phenotype were sorted and prepared for FISH analysis using a standard cytogenetic method, as described before [43].

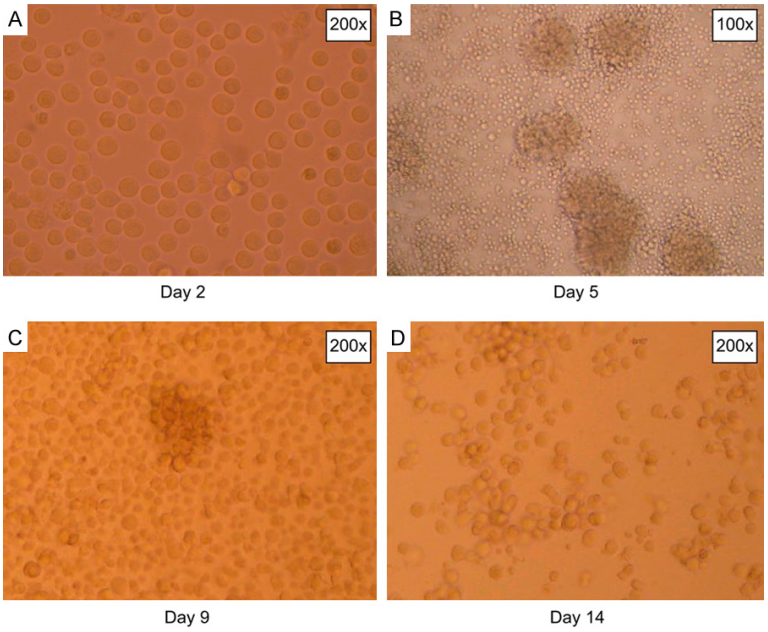

Figure 2. The morphology of the DCs generated from CD34-Lin- HPSCs from a patient with CML during 14 days of culture using a light inverted microscope (A-D). (B) Small groups of DCs were spotted after 5 days of culture. (C) The DC cluster increased in size and released some individual floating cells after 9 days of culture. (D) Large mature DCs were observed after 14 days.

Phagocytosis

The phagocytic capacity of DCs from different HPSC sources (undifferentiated, CD34+, and CD34-) was assessed via endocytosis of fluorescent polystyrene beads (2 μm, 50 × 10^6 beads/mL). Beads were incubated with the DCs at different maturation statuses for 4 h and then washed twice to remove the excessive released particles and reduce the extra-cellular fluorescence signal. The internalization of the fluorescence beads was visualized using a FLUOstar OPTIMA (BMG Labtech, Saitama, Japan) with excitation and emission wavelengths of 480 and 520 nm, respectively, or analyzed using the FACS Caliber System (BD Biosciences). The internalization of particles was confirmed using fluorescence microscopy. The nuclei and cytoplasm were stained with DAPI and hematoxylin, respectively. The control DCs were pretreated with a 2.5% sodium azide solution.

Statistical analysis

For continuous data, satisfaction of normality assumption was first examined. If the normality assumption was satisfied, continuous data were summarized using means with standard deviations and compared among different groups using Student's *t*-test or analysis of variance for two or more than two groups, respectively. However, if the normality assumption was violated, data were summarized using medians with ranges and compared using Mann-Whitney rank-sum and Kruskal-Wallis tests for two groups or more than two groups respectively. Categorical variables were summarized using frequencies with percentages and compared using Chi-squared test. *P*-value < 0.05 was considered significant. Statistical analyses were conducted using SigmaStat and SigmaPlot (Systat Software, San Jose, CA, USA).

Theory

DC-based vaccines are powerful tools in the field of personalized therapy and can be highly effective in initiating effective T cell immune responses against tumor-specific antigens, especially when generated from leukemia blasts. They are superior to others as they facilitate effective HLA-restricted reactions and can migrate to tumor sites. CD1a+CD14- DCs generated in this study using CD34- HPSCs from healthy BMDs or patients with CML can be useful for patients with refractory leukemia and for basic research.

Results

DCs cultured from BM

In this study, we generated and evaluated phenotypical and functional roles of the subset of DCs with the CD1a+CD14- phenotype (**Figure 1**). In addition to the unfractionated HPSCs, DCs were also generated and differentiated from CD34+Lin- and CD34-Lin- HPSC subpopulations. The three different populations of sorted and unsorted HPSCs were cultured in the presence of a cytokine cocktail (GM-CSF, TNF-α, and IL-4) and monitored daily for 14 days. Small clusters of typical DCs with cytoplasmic extensions were visible after 3-4 days of culture (**Figure 2A**) and were augmented in number and size a few days later (**Figure 2B**).

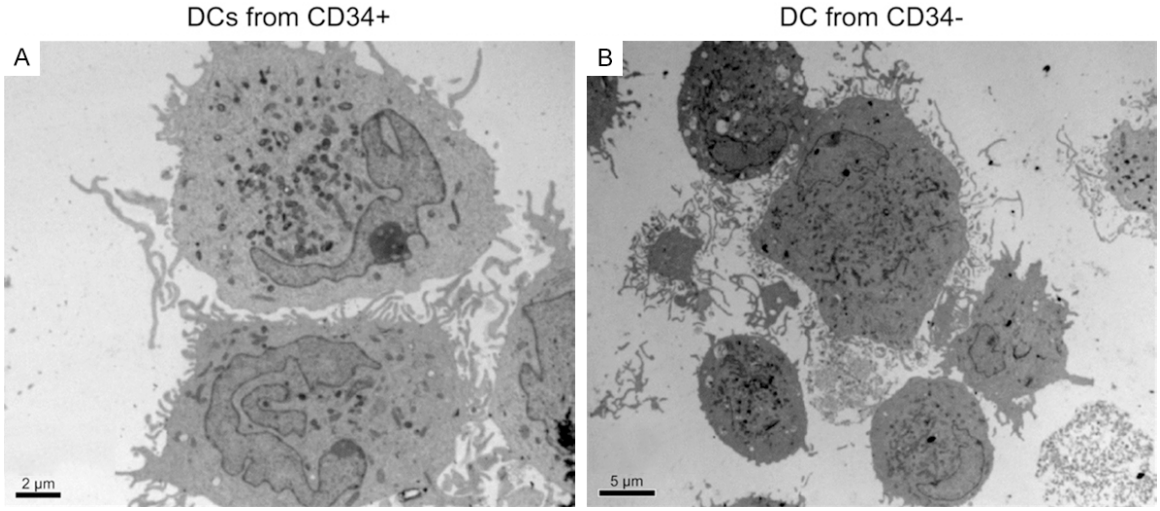

Figure 3. Ultra-detailed morphological features of the DCs derived from (A) $CD34^+Lin^-$ and (B) $CD34^-Lin^-$ HPSCs from a patient with CML were documented and confirmed via TEM.

Discrete aggregates with typical DC appearance could be visualized after 9 to 12 days of culture and floating individual cells could be observed in the media (**Figure 2C** and **2D**). Most cells were found to be negative to weakly positive for myeloperoxidase (MPO) and non-specific esterase (NSE), whereas macrophages and granulocytes were observed to be strongly positive for MPO and NSE (data not shown). We could generate and differentiate DCs of the $CD1a^+CD14^-$ phenotype from the $CD34^-Lin^-$ HPSC subsets obtained from BMDs at a purity of 1.0% to 49.0% (average, 18.3%), from $CD34^+Lin^-$ HPSCs at a purity of 2.0% to 46.0% (average, 21.9%), from the $CD34^-Lin^-$ HPSCs collected from patients with CML at a purity of 1.0% to 31.6% (average, 19.9%), and from the $CD34^+Lin^-$ HPSCs subset at a purity of 1.0% to 52.9% (average, 13.3%). Conversely, undifferentiated HPSCs yielded a purity of 0.9% to 62.5% (average, 21.9%) and 5.0% to 29.0% (average, 17.2%) in patients with CML. We started the culture with 6×10^6 HPSCs obtained from the patients with CML and with 6×10^6 HPSCs obtained from normal BMDs. Interestingly, the overall number of HPSCs declined to more than 50% after 14 days of culture compared with the original number of cells. The average number of recovered cells of the $CD34^+$ and $CD34^-$ phenotype from normal donors was 3×10^4 - 43×10^4 (median: 6×10^4) and 21×10^4 - 164×10^4 (median: 97×10^4) and those from patients with CML was 1×10^5 - 2.4×10^5 (median: 1.6×10^5) and 6.6×10^5 - 124×10^5 (median: 33.25×10^5) respectively.

Morphology analysis of DCs using light and electron microscopes

The differentiation and morphology of the DC cultures were tracked for 14 days. Both light and TEM were used to visualize and verify the $CD1a^+/CD14^-$ phenotype, as shown in **Figures 2** and **3**. After 12 to 14 days, floating and semi-adherent cells generated from the $CD34^+Lin^-$ and $CD34^-Lin^-$ HPSC subsets obtained from patients with CML and BMDs presented typical DC morphology with kidney-shaped nuclei and the presence of dendrites (**Figure 3A** and **3B**). No differences were detected between the ultra-morphology of the DCs generated from the $CD34^+Lin^-$ HPSC subset and that of DCs from the $CD34^-Lin^-$ HPSC fraction as per electron microscopy observation. Cells displayed cytoplasmic projections in which labeled dense granules could also be observed (**Figure 3A** and **3B**).

Characterization of DCs generated from $CD34^+Lin^-$ and $CD34^-Lin^-$ HPSCs from patients with CML

Different regions containing small and large cells were observed in the scatter gram and comprised cells expressing CD1a or CD14 or both antigens at different proportions. Consequently, to assess the proportion of the $CD1a^+/CD14^-$ subpopulation using flow cytometry, various regions were gated on the scatter gram. A subpopulation of DCs with the $CD1a^+/CD14^-$ phenotype was identified to be $CD45^+$ and negative for lineage (Lin^-) and for the

Table 1. The frequencies of the DC phenotypes (mean ± SD) generated from $CD34^+/Lin^-$, $CD34^-/Lin^-$, and $CD2^-$ HPSCs sorted from the BM samples of patients with CML (n = 6)

DC Surface Phenotypes	$CD34^+Lin^-$ % (n = 6)	$CD34^-Lin^-$ % (n = 6)	$CD2^-$ % (n = 6)
CD1a	20.8 ± 21.8*	30.8 ± 23.5	21.9 ± 21.1
CD80	11.5 ± 14.7	13.5 ± 16.6	13.3 ± 16.2
CD86	3.9 ± 3.1	5.5 ± 4.9	15.6 ± 25.4
CD83	10.5 ± 11.2	9.8 ± 12.7	2.2 ± 2.2
HLA-DR	11.7 ± 7.9	15.1 ± 8.3	24.6 ± 27.1
CD40	7.3 ± 9.2	8.0 ± 11.4	39.0 ± 19.5
CD54	27.9 ± 28.9	41.4 ± 27.7	47.1 ± 32.8

*(mean ± SD).

Table 2. The frequencies of the DC phenotypes (mean ± SD) generated from $CD34^+/Lin^-$, $CD34^-/Lin^-$, and $CD2^-$ HPSCs sorted from the BM samples of BMDs (n=9)

DC Surface Phenotypes	$CD34^+Lin^-$ % (n=9)	$CD34^-Lin^-$ % (n = 9)	$CD2^-$ % (n = 9)
CD1a	22.0 ± 11.0	21.0 ± 18.0	17.0 ± 8.0
CD80	16.0 ± 10.0	12.0 ± 11.0	11.0 ± 8.0
CD86	10.0 ± 8.0	10.0 ± 11.0	9.0 ± 7.0
CD83	5.0 ± 3.0	7.0 ± 9.0	5.0 ± 4.0
HLA-DR	32.0 ± 24.0	18.0 ±14.0	29.0 ± 26.0
CD40	25.0 ± 22.0	29.0 ± 33.0	24.0 ± 29.0
CD54	34.0 ± 22.0	36.0 ± 27.0	54.0 ± 29.0

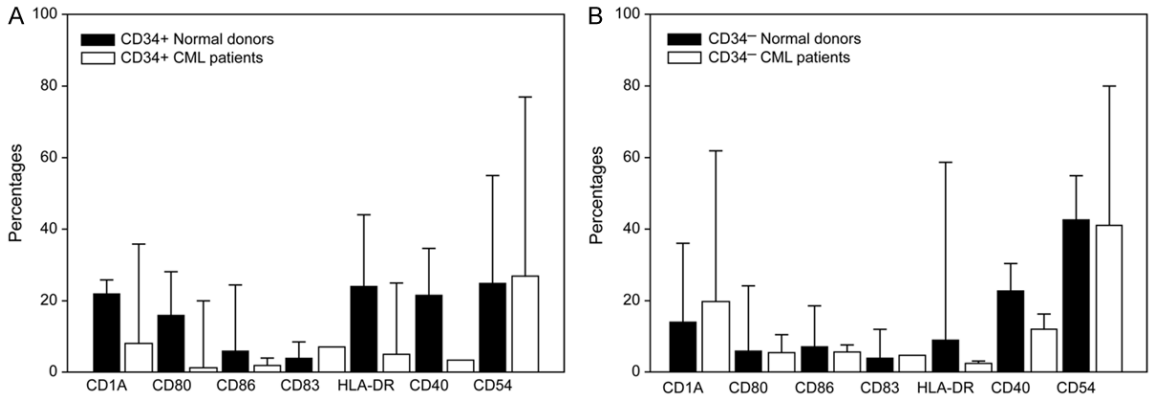

Figure 4. Comparison of the surface phenotypes of the DCs derived from (A) $CD34^+$ and (B) $CD34^-$ HPSCs from healthy BMDs (black bars) and from patients with CML (white bars), respectively.

markers that are used to detect granulocytes (CD15), B cells (CD19), T cells (CD2, CD3, and CD8), and NK (CD16). DCs expressing $CD1a^+CD14^-$ generated from $CD34^-Lin^-$ HPSCs were compared to their counterparts generated from $CD34^+Lin^-$ HPSCs from patients with CML (**Table 1**). The proportion of $CD1a^+$ DCs generated from the $CD34^-$ subsets was higher than that generated from $CD34^+$ subsets of BM obtained from patients with CML (**Table 1**). Additionally, DCs generated from $CD34^-Lin^-$ HPSCs expressed higher DC phenotypic markers and co-stimulatory molecules compared to their counterparts generated from $CD34^+Lin^-$ HPSCs (**Table 1**). However, statistical analysis did not show any significant differences in the mean expression of the DC surface phenotypes produced from the three different subpopulations of HPSCs (unfractionated, $CD34^+Lin^-$, and $CD34^-Lin^-$) (**Table 1**).

Characterization of DCs generated from $CD34^+Lin^-$ and $CD34^-Lin^-$ HPSCs from normal donors

In line with our findings described above, DCs generated from the $CD34^-$ lineage that were obtained from normal BMDs were found to dis-

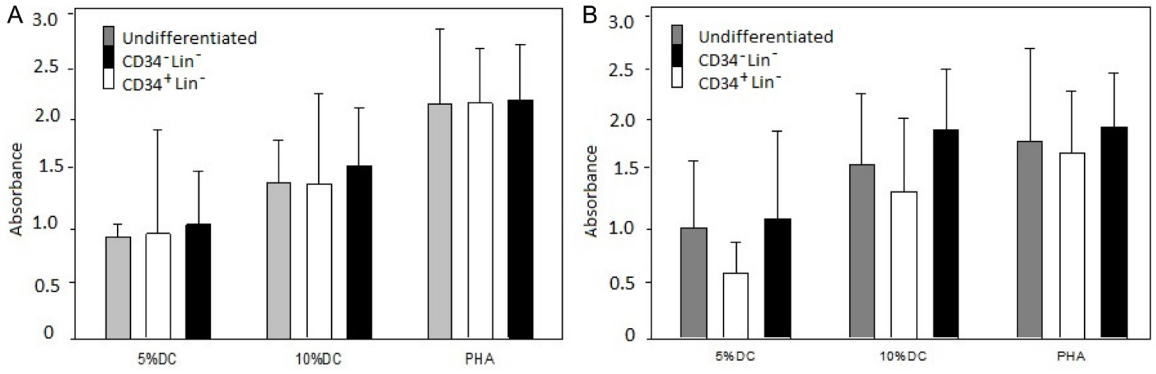

Figure 5. A. The stimulatory capacity of the DCs generated from the undifferentiated, CD34-, and CD34+ HPSC subsets obtained from BMDs, measured using MLR. B. Stimulatory capacity of the DCs generated from the three different lineages incubated at different ratios with T cells obtained from the CML patients, as illustrated by an increase in OD.

play comparable CD1a, CD80, CD86, and CD40 expression levels compared to those displayed by the DCs generated from the CD34+ lineage (**Table 2**). Conversely, DCs generated from the CD34+ HPSCs derived from BMDs displayed higher expression level of HLA-DR compared to those from CD34- HPSCs (**Table 2**).

Comparison of DCs generated from HPSCs of patients with CML and BMDs

Most DCs expressed considerable levels of MHC molecules (**Tables 1** and **2**). When the DCs generated from CD34+ from the patients with CML and healthy BMDs were compared, we observed that the expression of the DC phenotypic markers and costimulatory molecules (HLA-DR, CD40, CD83, CD86, and CD54) was lower in patients with CML compared to that in normal donors (**Figure 4A**). Conversely, higher expression levels of CD1a and CD80 were found in patients with CML than those in normal donors (**Figure 4B**). Consistent with the picture shown in **Figure 4A**, we also observed that DCs from CD34- from CML patients, tended to display lower HLA-DR, CD40, CD83, CD86 and CD54 expression levels compared to those from BMDs but statistically not significant.

DC immunostimulatory function analysis using MLR

To evaluate the DC stimulatory function, DCs differentiated from the three different HPSC subpopulations (undifferentiated, CD34-Lin-, and CD34+Lin-) collected from patients with CML and healthy BMDs were used. Equal numbers of CD1a+CD14- DCs were tested for their allogeneic T cell proliferation responses and these responses were compared. In general, different ratios of DCs derived from BMDs were observed to evoke equivalent T cell proliferative responses as measured by the BrdU assay (**Figure 5A**). Further, we found that the CD1a+ DCs generated from the CD34- HPSCs of normal donors displayed significantly higher proliferation than their counterparts derived from patients with CML. Nonetheless, comparison of proliferative responses of the CD1a+ DCs derived from different sources showed that those derived from the CD34- HPSC subset from patients with CML induced significantly higher proliferative responses than those generated from the CD34+ or undifferentiated lineages, especially when lower DCs ratios were compared (**Figure 5B**).

FISH analysis

To quantify the frequency of DCs with and without the *BCR/ABL* translocation, FISH analysis was performed using the sorted CD1a+/CD14- DCs differentiated from the CD34- and CD34+ HPSC lineages derived from six newly diagnosed patients with CML. A heterogeneous mixture of *BCR/ABL* mutated and normal DCs was uncovered from the DCs generated from different patients with CML (**Figure 6A**). We observed that in the first patient, most DCs derived from CD34- HPSCs were negative for *BCR/ABL* translocation, which was not the case for those derived from CD34+ HPSCs. However, the second and third patients showed comparable percentages of DCs derived from both CD34- HPSCs and CD34+ HPSCs that carried the Philadelphia chromosome (Ph). Further-

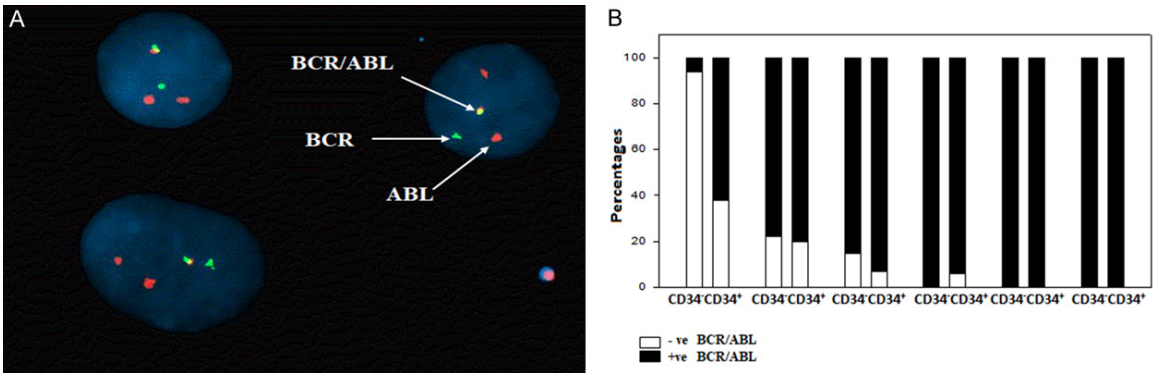

Figure 6. A. Spread of the *BCR/ABL* signals using a FISH probe and CD1a+/CD14- DCs generated from CD34-Lin- HPSCs. The arrows display the presence of Ph (*BCR/ABL*). The *BCR* is indicated by the green fluorescence, the red fluorescence shows *ABL*, and the yellow fluorescence indicates fusion of the *BCR/ABL* signal. B. A graph summarizing the levels of *BCR/ABL*-positive (black) and *BCR/ABL*-negative DCs (white) differentiated from the two CD34- and CD34+ HPSC subsets.

more, in the last two patients, all DCs had the *BCR/ABL* translocation. And all DCs differentiated from the CD34-Lin- HPSCs had the *BCR/ABL* translocation in the last three patients, whereas only 6% and 62% of the DCs from CD34-Lin- HPSCs and CD34+Lin- HPSCs, respectively, had the *BCR/ABL* translocation in the first patient (**Figure 6B**).

Phagocytosis of DCs

The phagocytic capacity of the DCs differentiated from the different HPSC sources (undifferentiated, CD34+, and CD34-) was assessed using fluorescently labeled polystyrene beads (2 μm, 50 × 10^6 beads/mL). Beads were incubated with the DCs generated from different HPSC sources at different differentiation levels for 4 h, and then washed to remove unbound particles. Then, the internalization of the beads was examined using a fluorescent microscope to assess the phagocytic capacity of the differentiated DCs (**Figure 7**). Immature DCs showed the highest levels of phagocytosis compared to mature ones (**Figure 7A** and **7B**).

Discussion

We aimed to explore the possibility of generating DCs from CD34-Lin- HPSCs sorted from BM samples from patients with CML and from BMDs for targeted immunotherapy and to further understand their functional abilities. DCs were successfully differentiated *ex vivo* from the CD34-Lin- HPSC subsets harvested from patients with CML and healthy BMDs by the addition of GM-CSF, IL-4, and TNF-α to the conditioned media. Comprehensive phenotypic and functional analyses of the CD1a+CD14- DCs were performed since those markers have been reported to be potent stimulators of naïve cytotoxic T cells [4, 5, 55]. Conversely, CD1a-CD14+ mDCs only secrete IL-10 and are thought to take part in B-cell responses [56]. Furthermore, we intended to evaluate the best source of HPSCs for DC generation for immunotherapy applications. The phenotypic and functional features of the DCs derived from CD34-Lin- HPSCs were compared to those derived from CD34+Lin- and unfractionated HPSCs. The CD1a+CD14- DCs derived from CD34-Lin- HPSCs were observed to be morphologically, phenotypically, and functionally competent and comparable to their counterparts differentiated from CD34+Lin- or undifferentiated HPSCs. They constitutively expressed MHC class I and II molecules, co-stimulatory molecules CD40, CD80, CD86, and leukocyte adhesion molecules ICAM-1, LFA-1, and LFA-3 on their surface membrane. Morphological investigations, conducted using an electron microscope, revealed typical dendrites and Birbeck granules.

In contrast, the introduction of TKI treatment during the last two decades has prolonged the overall life expectancy of patients with CML, and half of them can sustain remission for a long time after halting treatment. Moreover, some patients with CML at the advanced stage of the disease often suffer from residual leukemia after terminating the TKI treatment [13-15, 20, 58]. Consequently, HSCT is recommended as the best remedy for patients with refractory leukemia, but its usage is limited to young eli-

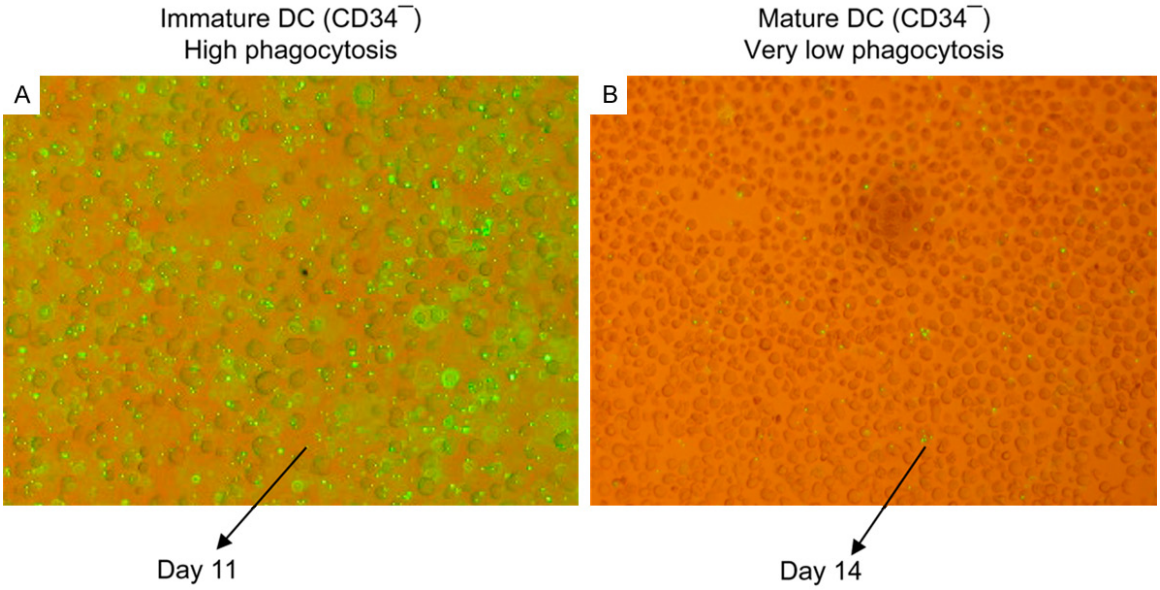

Figure 7. Efficient phagocytosis of fluorescent polystyrene beads by *ex vivo*-generated live DCs. (A) Immature and (200X) (B) mature DCs (200X) differentiated from the CD34- HPSC subset from a patient with CML.

gible patients and is often associated with GvHD and/or relapse. The addition of DLI to HSCT is a successful modality for leukemic patients, including those with CML and relapse, but it is also frequently associated with GvHD [24, 25]. Previous management may result in the clearance of the residual LSCs, avoiding a relapse [24, 59]. Furthermore, the increasing resistance to treatment with TKIs, incidence of residual leukemia, efficacy of anti-CML immune responses, and presence of the leukemia-specific *BCR/ABL* have encouraged the exploration of effective vaccine management of patients with CML for two decades [2, 60]. DCs are antigen presenters that regulate the immune response which can induce primary T cell responses [2, 3, 61-63]. Immunotherapy is an ideal approach but is limited by the need for repetitive vaccination with large numbers of DCs and by the incompatibility of human leukocyte antigen (HLA) in allogeneic HSCT. The latter might induce an exaggerated immune reaction and lead to the manifestation of GvHD after treatment. Herein, the problem of HLA incompatibility was overcomed by generating DCs from the same patient with CML or his donor and by using the CD34- fraction after the selection of CD34+ HPSCs for primary HSCT. Additionally, myeloid- and leukemia-associated antigens were reported to be expressed by myeloid blasts [64]. Several studies have reported that DCs can be differentiated and matured either from PB, CB from BMDs, and patients with CML. Herein, we confirmed the possibility of the generation of DCs from CD34+ and whole unfractionated HPSCs, in addition to their generation from the CD34- lineage, which has rarely been described in the literature [54]. Park et al. had successfully generated DCs from CB from healthy donors but DC differentiation from CD34- subsets of patients with CML has not yet been reported [54]. The DCs generated from myeloid leukemia blasts have been used for the production of cellular vaccines as they were found to present a mass of endogenously expressed leukemia antigens to T and other immune cells [43, 65, 66]. Thus, DCs with the *BCR/ABL* translocation differentiated from patients with CML are of major interest both clinically and scientifically since they represent ideal cell-based vaccines. The DCs generated from patients with CML in this study were also found to harbor the *BCR/ABL* translocation at various ratios, which implies that they could specifically stimulate specific T cells for the eradication of LSCs, as previously reported [43, 65, 66]. The efficacy of vaccination is directly linked to the nature and value of the immune responses induced by a particular vaccine. It has previously been reported that 73-100% of the DCs derived from monocytes are positive for the Ph translocation [33, 67, 68]. However,

the data comparing the ability of DCs from BMDs and patients with CML to stimulate T cells remain controversial [20, 33, 37-39]. DCs derived from both patients with CML and healthy donors are comparable in morphology, surface cell phenotypes, and function [69]. Alternatively, others have reported a variation in the function of the DCs generated and matured in culture under the same conditions between those derived from the BM of healthy subjects and patients with CML, while DCs generated from patients with CML showed a decreased activity during MLR [43]. The addition of IFN-α to DC cultures significantly upregulated the expression of MHC molecules (class I and II) and stimulatory molecules (B7.1 and B7.2) in both donor types [43].

Here, the stimulatory capacity of the DCs derived from the $CD34^+$ lineage from normal BMDs was higher than those derived from patients with CML and this is in agreement with a previous report [43]. However, the biology of the DCs generated from $CD34^-Lin^-$ HPSCs had not been investigated before in patients with CML and normal donors. Herein, we have established the generation of DCs from $CD34^-Lin^-$ HPSCs and compared them with those generated from $CD34^+Lin^-$ HPSCs using a cocktail of different cytokines. In addition, the presence of *BCR/ABL* translocation was comparable in most of the patients. However, in a few patients, the frequency of the Ph chromosome differed in the two populations of HPSCs, which required further analysis in patients with CML at different stages of the disease. Thus, variability in the incidence of the *BCR/ABL* fusion gene in DCs derived from a newly diagnosed patient with CML to another was observed using FISH analysis. This observation has been reported before and could be explained by the notion that some DCs might have evolved from a CML mutant clone, whereas others most likely developed from normal clones. The frequency of *BCR/ABL* translocation varies considerably between DCs differentiated from $CD34^+Lin^-$ and $CD34^-Lin^-$ HPSCs from patients with CML [43]. This disparity in the results could be due to the differences among patients, including the clinical stage of the disease, the diverse sources of DCs, different protocols used, the duration of culture, and the sorting methods used. The stimulatory capacity of the DCs produced from patients with CML tended to be higher than that of those derived from normal HPSCs but the difference was not statistically significant. Meanwhile, the DCs generated from $CD34^-Lin^-$ HPSCs possessed lower stimulatory capacity than those generated from $CD34^+Lin^-$ HPSCs when tested via allogeneic MLR.

Herein, we demonstrate the value of $CD34^-Lin^-$ HPSCs for the generation of competent DCs for clinical applications and basic research. Different protocols should be attempted to differentiate the different subtypes of DCs and evaluate their efficiencies. Competent DCs with typical features were successfully produced from $CD34^-Lin^-$ HPSCs from BM from both patients with CML and BMDs. Phenotypic analysis showed that the expression of typical DC surface molecules was comparable in DCs generated from both HPSC subsets. Functional studies revealed a potent T cell stimulatory capacity and efficient phagocytosis of fluorescent poly beads by DCs from the $CD34^-/Lin^-$ BM cells. A limitation of this study is that DCs were only evaluated *ex vivo* and no *in vivo* analysis was performed. Moreover, DCs should be expanded *ex vivo* on large scale for clinical trials. We attempted to expand HPSCs before differentiation, but more studies are needed as this manipulation might affect the biology of the extracted DCs.

In conclusion, we demonstrate that the $CD34^-Lin^-$ HPSCs are an indispensable multipotent source that must be explored for the generation of DCs for effective targeted immunotherapies and basic research to explore the biology of hematopoiesis. Additionally, the DCs generated in this study could be used in phase one clinical trials. Thus, further evaluation of $CD34^-Lin^-$ HPSCs for the production of DC vaccines for patients with CML is required to ensure the complete clearance of LSCs after the cessation of TKI therapy. DCs differentiated *ex vivo* are a good approach in personalized medicine and constitute a valuable vaccine for patients with CML with relapse to induce efficient immune responses for complete LSC clearance.

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Disclosure of conflict of interest

None.

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