Obtaining of CD34+ cells from healthy blood donors: development of a rapid and efficient procedure using leukoreduction filters

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BACKGROUND: Human CD34+ cells are mandatory to study many aspects of human hematopoiesis. Their low frequency in blood or marrow and ethical reasons limit their obtainment in large quantities. Leukoreduction filters (LRFs) are discarded after preparation of red blood cells. The CD34+ cell concentration in healthy donor blood is low (1×10^3-4×10^3/mL), but their number trapped in one LRF after filtration of 400 to 450 mL of blood is high (0.4×10^6-1.6×10^6).

STUDY DESIGN AND METHODS: To develop a procedure allowing obtainment of purified CD34+ cells from LRFs with a good yield, white blood cell (WBC) recoveries after a 500-mL continuous or after sequential elution (50- or 20-mL fractions) were compared. Different WBC and mononuclear cell (MNC) centrifugation methods were tested to minimize their PLT contamination before the CD34+ cell immunomagnetic selection. Cell functionality was finally analyzed under various culture conditions.

RESULTS: The 20-mL back-flushing of LRFs allowed the most efficient WBC recovery. The next steps ($110 \times g$ centrifugation, MNC separation on Ficoll, and washes) resulted in a cell suspension in which the lymphocyte recovery was approximately 76 ± 10% and the PLT contamination below 1.6%. After immunomagnetic selection, 4×10^5 to 6×10^5 cells containing approximately 85% of functional CD34+ cells were obtained.

CONCLUSION: This procedure allows the easy, rapid (<5 hr), and efficient preparation of large quantities of CD34+ cells having functional activities similar to those of CD34+ cells from other sources. Therefore, easily available and virally safe, LRFs represent an important and regular WBC source to work with human CD34+ cells, but also with other WBC types.

Despite the very low percentage of CD34+ cells in the steady-state blood of healthy donors—approximately 0.15% of mononuclear cells (MNCs)[1]—adult blood is an attractive alternative to umbilical cord blood and marrow for research and, in the future, for regenerative medicine.[2] Indeed, a blood gift bag (400 to 450 mL with 1.6×10^9 to 4×10^9 white blood cells [WBCs]), represents a potential source of large numbers of CD34+ cells (0.4×10^6 to 1.6×10^6/400 mL of blood), since leukoreduction filters (LRFs) are discarded after the preparation of therapeutic red blood cells (RBCs) devoid of WBCs and platelets (PLTs). CD34+ cell isolation from LRFs has already been an objective for us and others.[2,3] Differences in deformability,[4] electronic charges,[5] and surface antigens[6,7] between blood cell types explain that LRFs trap approximately 99% of PLT and WBCs within the filter matrix fibers.[8,9] Since LRFs are easily available, rather safe for common blood infectious agents, and contain the number of CD34+ cells found in 400 to 450 mL of steady-state adult blood, they represent an interesting source for purifying CD34+ cells as well as other WBCs types. Procedures currently available for WBC elution require back-flushing of LRFs with large volumes of buffer. Therefore, they are difficult, tedious, and time-consuming to be realized on a regular basis. This study was devoted to

ABBREVIATIONS: FRP = fraction rich in platelets; GpA = glycophorin A; HuS = human serum; LRF(s) = leukoreduction filter(s).

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develop an easy and rapid technical procedure allowing the most efficient WBC elution from LRFs, the largest PLT reduction, and the best CD34+ cells selection (84 ± 4.1%) with favorable yields (>90%).

MATERIALS AND METHODS

Elution of WBCs from LRFs and preparation of PLT-reduced MNCs (Fig. 1)

LRFs (Composelect WB, Fresenius, Paris, France), still connected to the empty primary blood donor bag, were obtained from the Regional Blood Transfusion Institute (Etablissement Français du Sang Aquitaine-Limousin, Bordeaux, France) and used within 22 hours after blood collection. After its removal from the primary bag, the LRF was fixed on a stand and back-flushed with elution buffer using either a 500-mL plastic bag (hung at 1 m above the filter) or a 50-mL sterile syringe connected to the filter exit (Fig. 1, Step 1). Elution buffers tested were composed of phosphate-buffered saline (PBS; pH 7.2) supplemented or not by ACD-A (10% vol/vol) and heat-inactivated human serum (HuS; 5%, vol/vol). After comparing the effects of elution with various buffers at 4 and 20°C on WBC recovery, PLT reduction, and lymphocyte yield, all further experiments were performed at 20°C in PBS supplemented with ACD-A and HuS. Our previously described 500-mL continuous elution method[2] was compared with sequential back-flushing with 50 or 20 mL of elution buffer using a 50-mL syringe. RBC, PLT, and WBC counts and differentials in the collected fractions were performed with an automatic multivariable hematology analyzer (CELL-DYN 3500, Abbott Laboratories, Inc., Abbott Park, IL). Since eluted fractions contained very high numbers of PLTs, we compared the efficiency of three low-speed centrifugation procedures (260, 190, and $110 \times g$; 20 min; 20°C), in terms of PLT reduction and WBC recovery. After elimination of the upper fraction rich in PLTs (FRP), RBC pellets and WBC buffy coat were then resuspended in elution buffer (qs 30 mL), layered onto Ficoll (d = 1.077, lymphocytes separation medium, Laboratories EUROBIO, Courtaboeuf, France) and centrifuged ($400 \times g$; 30 min; 20°C). After elimination of the FRP, the MNC layer was harvested and washed twice in elution buffer (qs 50 mL) by centrifugation ($260 \times g$; 15 min; 20°C). The pellet was then resuspended in 10 mL of selection buffer (PBS [pH 7.2], 2 mmol/L ethylenediaminetetraacetate [EDTA], 5 g/L HSA), washed once, and resuspended in 2 mL of selection buffer for the immunomagnetic selection of CD34+ cells.

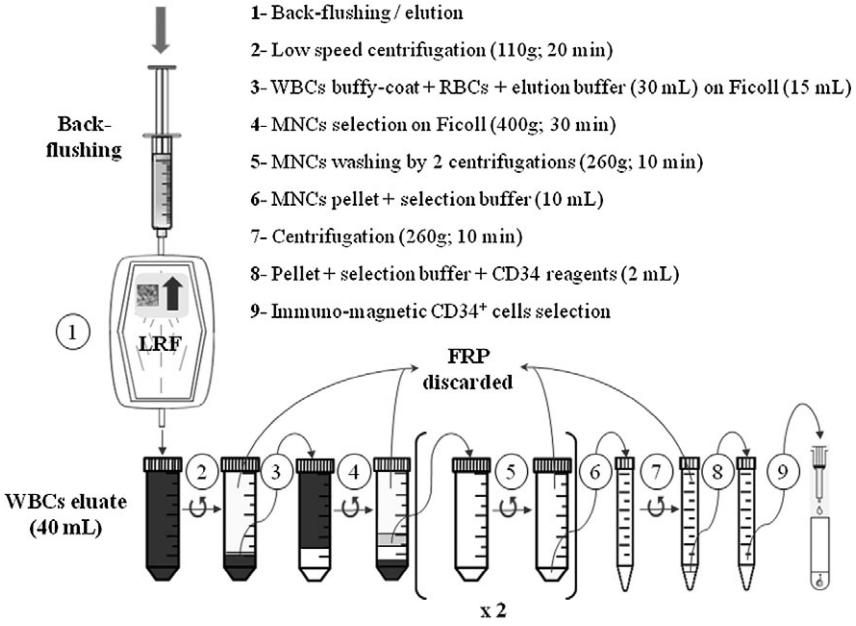

Fig. 1. From LRFs to immunomagnetic columns: a schema of CD34+ cell selection. LRFs obtained from discarded blood collecting devices were used within 22 hours after blood collection. For WBC elution, LRFs were back-flushed with two injections of 20 mL of elution buffer, using a 50-mL sterile syringe (Step 1). The black arrow on LRF indicates the donor blood filtration way. Steps 2 to 9 allowed PLT reduction and MNC enrichment. Finally, CD34+ cells were selected by a double immunomagnetic procedure according to the manufacturer's protocol (Miltenyi Biotec). All steps were performed at 20°C.

CD34+ cell selection

CD34+ cells were isolated with a two-step "indirect" immunomagnetic procedure (Indirect kit CD34 microbeads kit, Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's protocol: the first step used the "midi" column and the second used the "minicolumn" (Vario Macs device, Miltenyi Biotec). Finally, CD34+ cells were counted using Trypan blue dye staining for the identification of dead cells.

Flow cytometry analysis of CD34+ cells at seeding and after liquid cultures

The percentage of CD34+ cells along the purification procedure was measured with a flow cytometer (FACSCanto, Becton Dickinson, San Jose, CA) and analyzed through the accompanying software (FACSDiva, Becton Dickinson). Cells were first washed (PBS, Ca^{2+}/Mg^{2+} free, EDTA [10 mmol/L], fetal calf serum [FCS; 5%, vol/vol], and azide [0.05%, wt/vol]) and then labeled for 30 minutes with anti-CD34–phycoerythrin

(PE; BD Biosciences, Meylan, France) or with the corresponding isotype control (mouse γ1-PE, BD Biosciences). The same protocol was used to assess the commitment of purified CD34+ cells after 7 and 11 days of culture in Iscove's modified Dulbecco's medium (IMDM + GlutaMAX-1, Gibco, Invitrogen Corp., Cergy Pontoise, France) supplemented with FCS (10%, vol/vol), interleukin (IL)-3, IL-6, and stem cell factor (SCF), respectively at 20, 20, and 100 ng/mL, and erythropoietin (EPO) at 3 U/mL (all from PeproTech, Neuilly-Sur-Seine, France). The CD33, CD61, CD65, and glycophorin A (GpA) antigens were analyzed using fluorescent antibodies (all from BD Biosciences). The cell cycle status of freshly isolated and cultured CD34+ cells was analyzed using Topro-3 iodide (Topro) and anti-Ki-67. Topro discriminates cells with 2n (G_0 and G_1 phases), 2n to 4n (S phase), and 4n (G_2 and M phases) DNA content. The nuclear antigen Ki-67 is expressed by cells in active phases of the cell cycle (G_1 to M phases) and absent in G_0 quiescent cells. Cells were washed, fixed, and permeabilized for 30 minutes with formaldehyde (0.4%, vol/vol), saponin (0.02%, vol/vol), and Hepes (pH 8; 10 mmol/L), H_2O. Cells were washed twice and incubated with an anti-Ki-67–PE antibody (B56 clone, BD Biosciences) or with the corresponding isotypic control (clone MOPC-21, BD Biosciences) for 30 minutes. After being washed, cells were labeled with Topro and analyzed by flow cytometry.

Colony-forming ability of CD34+ cells

Colony-forming unit assays were performed with CD34+ cells freshly purified from LRFs and after 3 and 6 days of liquid culture of cells seeded (5×10^4 per mL) in IMDM plus FCS (10%) and IL-3 (20 ng/mL). For this test, 5 µL of cell suspension was added to 250 µL of Stemα.1D (Stem Alpha, Saint-Clément-les-Places, France), a semisolid methylcellulose medium (containing FCS, human transferrin, IL-3, IL-6, IL-11, SCF, EPO, granulocyte-macrophage–colony-stimulating factor, and granulocyte–colony-stimulating factor) and seeded in 24-wells plates (NUNC, Roskilde, Denmark). After 14 days of culture (37°C, 20% O_2, 5% CO_2), the colonies (>50 cells) were counted under an inverted microscope.

Statistical analysis

Mean values ± SD of the mean values were calculated from data of independent experiments. Differences between experiments were assessed using analysis of variance and considered as significant when the p value was below 0.05.

RESULTS

LRF back-flushing modalities influence the WBC elution

Preliminary tests (data not shown) showed that WBC elution from LRFs were similar at 4 and 20°C and that addition of ACD-A and HuS to PBS improved cell survival and reduced cell aggregate formation during the CD34+ cell selection procedure. All the experiments were then performed at 20°C with PBS supplemented with ACD-A and HuS.

We compared the WBC recovery reached with the 500-mL continuous elution procedure and with two sequential procedures using 10 or 5 independent injections of 50 or 20 mL, respectively. Figure 2 shows that significantly ($p < 0.01$) more WBCs were eluted in Fraction 1 of the 50- and 20-mL sequential procedures than in the total 500 mL of the continuous back-flushing. The WBC counts in the 10 or the 5 cumulated fractions from the sequential injections were respectively 2 to 2.2 times higher than in the 500-mL continuous one (Fig. 2, inset). Interestingly, the number of WBCs in Fractions 1 + 2 of the 20-mL sequential back-flushing represented more than 85% of the total number of WBCs in its whole five fractions. This number was equal to the one in Fractions 1 + 2 of the 50-mL sequential elution and higher than in the 500-mL continuous back-flushing.

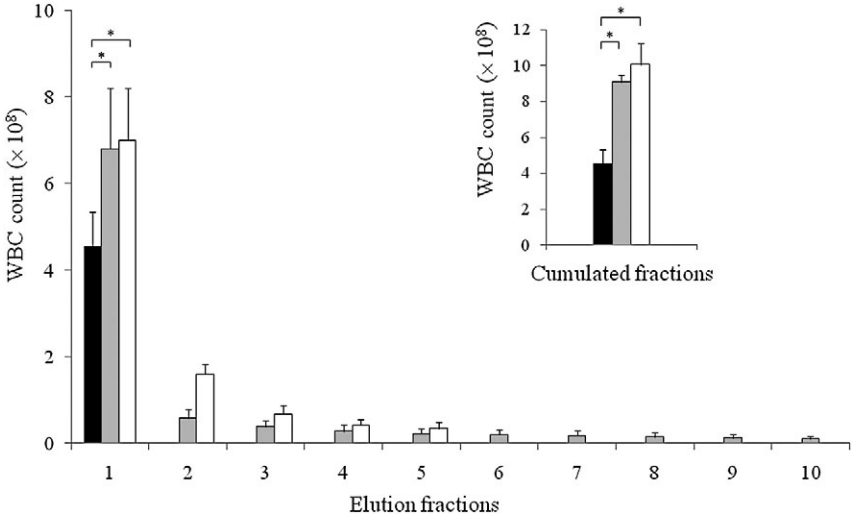

Fig. 2. LRF back-flushing modalities influence the WBC elution (n = 12). The WBC number recovered from a 500-mL continuous (■), a 50-mL sequential (10 fractions, ▒), or a 20-mL sequential elution (five fractions, □), are shown. Inset compares the number of WBCs obtained in 500 mL by continuous elution (■), in 500 mL by the 10 × 50-mL sequential elution (▒), and in 100 mL by the 5 × 20-mL fractioned elution (□). *p < 0.01.

TABLE 1. Blood cell recoveries after three different elution procedures*

Elution procedure	PLTs ($\times 10^{10}$†)	RBCs ($\times 10^{11}$†)	PMNs (%/$\times 10^{8}$†)	Lymphocytes (%‡/$\times 10^{8}$†)	CD34+ cells (%‡/$\times 10^{6}$†)
500 mL (continuous)	4.2 ± 0.7	0.7 ± 0.3	37.1/1.7 ± 0.4	55.1/2.5 ± 0.3	ND
100 mL§ (50-mL sequential)	3.5 ± 0.3	1.15 ± 0.2	28.9/2.1 ± 0.6	59.5/4.4 ± 1	0.07/0.58 ± 0.17
40 mL§ (20-mL sequential)	3.1 ± 0.9	0.7 ± 0.4	29.1/2.5 ± 0.7	64.3/5.5 ± 0.7	0.06/0.69 ± 0.11

* Results are the mean of 12 experiments ± SD.
† Absolute number of cells in each WBC subpopulation.
‡ Percentage of each WBC subpopulation among total WBCs.
§ Volume of Fractions 1 + 2.
ND = not done.

TABLE 2. PLT reduction, lymphocyte recovery, and CD34+ cell enrichment: effects of low-speed centrifugation, Ficoll, washing steps, and immunomagnetic columns*

	Low-speed centrifugation ($\times g$)				CD34 selection†	
Cell type	260	190	110	MNC Ficoll layer†	Before	After
PLTs‡ (%)	47.9 ± 0.6	36.3 ± 6.7	22.5 ± 3.5	18 ± 2.8	1.6 ± 0.9	0
WBCs‡ (%)	93.1 ± 1.4	94.5 ± 4.6	95.8 ± 4.2	66 ± 8	63 ± 7.8	0.06 ± 0.02
Lymphocytes‡ (%)	92.9 ± 2	95 ± 3.8	96 ± 3	77.9 ± 8	75.8 ± 10	0.05 ± 0.00
CD34+ cells§ (%)	ND	ND	0.06 ± 0.028	0.083 ± 0.036	0.083 ± 0.036	84 ± 4.1
CD34+ cells‖ ($\times 10^{6}$)	ND	ND	0.665 ± 0.23	0.6 ± 0.2	0.58 ± 0.17	0.41 ± 0.05

* Results were obtained after a 2 × 20-mL sequential elution and are the mean of 16 experiments ± SD.
† All these values were obtained with WBCs suspensions issued from the 110 × g centrifugation procedure.
‡ Ratio (%) of the number of cells recovered at each step to the number of cells back-flushed from LRFs.
§ Ratio (%) of the CD34+ cells number to the total number of cells at each step.
‖ Total number of CD34+ cells ($\times 10^{6}$) among WBCs at each step.
ND = not done.

The comparison of the numbers of RBCs, PLTs, polymorphonuclear leukocytes (PMNs), and lymphocytes obtained with the three elution procedures (Table 1) show that the number of lymphocytes was the highest and the number of PLTs the lowest in Fractions 1 + 2 of the 20-mL sequential procedure, an interesting result since CD34+ cells are inside the lymphocyte population. The sequential elution by two successive 20-mL fractions was then used for all the following experiments.

PLT reduction

The propensity of PLTs to form aggregates and clumps in vitro with WBCs and/or RBCs often reduces the yield of CD34+ cells during their selection, by blocking the immunomagnetic column matrix. To maximally reduce the PLT contamination of WBCs before the CD34+ cells selection, we compared three low-speed centrifugations (260, 190, and 110 × g; 20°C; 20 min). Table 2 shows that PLT reduction was 2 and 1.6 times more efficient after centrifugation at 110 × g than at 260 and 190 × g, respectively, whereas the WBC and lymphocyte recoveries remained unchanged and higher than 96%. Further reductions of the centrifugation speed and/or time led to a significant loss of WBCs. The next steps of MNCs selection by Ficoll and subsequent washes allowed the elimination of approximately 98% of RBCs, 98.5% of PLTs, and of only 24% of the lymphocytes eluted from LRFs. Importantly, with this procedure, the cell suspension loaded on the immunomagnetic column did not contain aggregates or clumps.

CD34+ cell recovery and purity rate

The 20-mL back-flushing of LRFs was also the right technical choice for the CD34+ cell recovery since their percentage and total number were similar in Fractions 1 + 2 of the 50- and 20-mL sequential elution procedures (Table 1, Column 6).

The yields of CD34+ cells (related to their initial number in LRF-eluted WBCs) were also analyzed after MNC isolation on Ficoll, after their resuspension in selection buffer and after their immunomagnetic selection (Table 2, Columns 4 to 6). As expected, CD34+ cells and lymphocyte loss were similar and low during the low-speed centrifugation, Ficoll, and washing steps of the procedure. The CD34+ cell yield after the selection step was lower and variable from one LRF to another, ranging from 54% to 72% with a mean purity of 84 ± 4.1%. Most importantly, the total number of CD34+ cells recovered after the complete selection procedure ranged from 0.36×10^{6} to 0.46×10^{6}. The PLT contamination of the cell suspension was quite null and PLT satellitism on CD34+ cells, measured by CD34/CD41 double labeling of purified cells, was always below 0.7 ± 0.5% of the total number of CD34+ cells (data not shown).

TABLE 3. Functional capacities of CD34+ cells purified from LRFs*

Day	Growth rate†	Mortality rate‡ (%)	Colony-forming ability§ (%)
0			11 ± 2.5
3	2 ± 0.1	9.5 ± 1.2	11.6 ± 4.3
6	6.5 ± 0.2	14 ± 1.7	4.1 ± 0.5

* Growth and mortality rates were obtained from liquid cultures seeded at 1×10^5/mL. Colony-forming ability was obtained from methylcellulose semisolid cultures, seeded by 250 eluted CD34+ cells or by 5 µL of liquid cultures. Results are the mean of seven experiments ± SD.
† Growth rate: number of living cells after culture/number of seeded cells.
‡ Mortality rate: dead cells number/total cells number (dead + alive) at each time point.
§ Colony-forming ability: colonies number/seeded cells number.

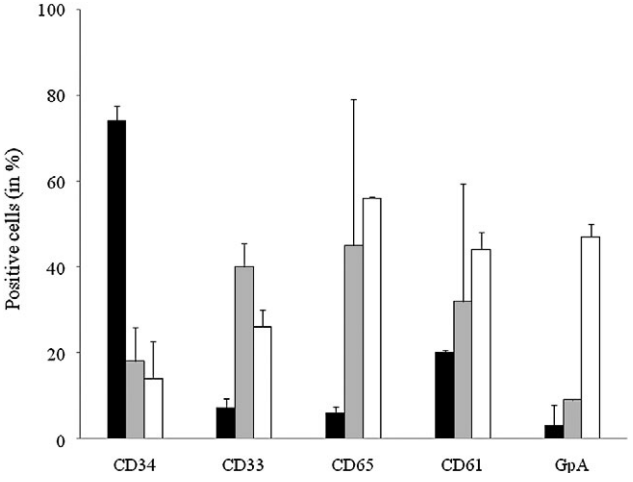

Fig. 3. Myeloid differentiation of LRFs CD34+ cells in culture (n = 3). Analysis of CD34 (stem/progenitor cells antigen), CD33 (early myeloid differentiation antigen), CD65 (monocytic antigen), CD61 (PLT/megakaryocytes antigen), and GpA (late erythroid antigen) was performed on freshly isolated CD34+ cells (■) and after 7 (▒) and 11 days (□) of liquid culture in IMDM supplemented by FCS, IL-3, IL-6, SCF, and EPO.

Functional analysis of LRF CD34+ cells: growth rate, colony formation, cell cycling, and differentiation

The growth and mortality rates of CD34+ cells after 3 and 6 days of liquid culture with IL-3 (Table 3, Columns 2 and 3) as well as the frequency of progenitors (Column 4) were very similar to those previously found for steady-state blood CD34+ cells selected with other methods.[1-3] Their cell cycling was also similar to the one of CD34+ cells from other sources.[10] Indeed, before liquid culture, 45 ± 4.5% of the CD34+ cells were in G_0 phase, 55 ± 4.5% in G_1, and none in S, G_2, or M phases (n = 6). In contrast, after 3 days of culture in IMDM supplemented by FCS (10%) and IL-3 (20 ng/mL), cells entered in the active cell cycle phases, as evidenced by 1) the decrease of the proportion of cells in the G_0 phase to 33 ± 2.3% and to 42 ± 3.2% for the G_1 phase and 2) the increase of the proportion of cells in S and G_2/M phases, respectively, to 13 ± 2.1 and 12 ± 1.4% (data not shown).

When cultured for 7 to 11 days in IMDM plus IL-3, IL-6, SCF, and EPO, the CD34+ cells progressively differentiated along the granulomonocytic and erythroid pathways (Fig. 3). In particular, 47.5 ± 2.9% of cells expressed the GpA after 11 days of culture, against 3 ± 4.8% initially and 9 ± 0.2% after 7 days. Altogether, these results demonstrate that CD34+ cells isolated from LRFs of adult blood donors present functional properties very similar to those of CD34+ cells obtained from other sources.[1-3,10]

DISCUSSION

LRFs are a convenient and abundant source of CD34+ cells since they contain a high number of WBCs and are discarded after the preparation of RBCs. We and others already used LRFs to purify CD34+ cells for research purposes.[2,3] However, WBC recovery required back-flushing of LRFs with a large volume of buffer and resulted in a heavy PLT contamination, making this technique tedious and time-consuming for research laboratories lacking materials for processing large volumes of blood-derived products. The method described here is easy and neither more expensive nor longer (<5 hr from LRF elution to purified CD34+ cell recovery) than other procedures commonly used for the isolation of CD34+ cells. Our first objective was reached with the 20-mL sequential elution procedure, which reduced by 12.5 times the volume necessary to extract an equal or higher number of WBCs and CD34+ cells. This low elution volume allows the concomitant use of several LRFs and the pooling of WBCs to purify large numbers of CD34+ cells required for biochemical analyses. Our second objective was to dramatically reduce the PLT contamination, which is a frequent source of aggregates and clumps with RBCs and WBCs leading to a lower quality and yield of the CD34+ cell selection and to uncontrolled disturbances of the hematopoietic progenitors growth. A $110 \times g$ low-speed centrifugation, followed by four other centrifugation steps along the CD34+ cell isolation, led to the total elimination of PLTs as evidenced by the very low percentage of CD34+/CD41+ cells present after the immunomagnetic selection step. CD34+ cells obtained with this convenient, rapid, and efficient procedure have satisfying functional properties as evidenced by their cell cycle entry, ex vivo expansion, colony-forming ability, and lineage differentiation.

In conclusion, the procedure described here to purify CD34+ cells from LRFs is an interesting alternative to other methods and sources. Indeed LRFs are an easily available and safe source of cells whose fibers trap the

WBCs contained in 400 to 450 mL of blood from one healthy donor. Thus, one LRF allows the isolation of 0.36×10^6 to 0.46×10^6 CD34+ cells. Since 6 to 10 filters can be processed together, a high number of CD34+ cells (2.2 to 4.8×10^6) can be purified in the mean time, as required for some biochemical analyses. Mixing of CD34+ cells from several donors may be even interesting to moderate individual differences. Finally, the initial steps of this procedure could also be used to isolate other subpopulations of WBCs present in the eluted fractions.[11,12]

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest relevant to the manuscript submitted to **TRANSFUSION**.

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