Whole-blood leukoreduction filters are a source for cryopreserved cells for phenotypic and functional investigations on peripheral blood lymphocytes

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BACKGROUND: Leukoreduction of blood is now widely performed by blood banks, and the possibility of recovering 10^8 to 10^9 white blood cells (WBCs) from leukoreduction filters, which are usually discarded, represents a promising source for normal human cells. Previous studies with these filters to prepare WBCs have performed their experimentation with fresh cells only. Whether these filter-derived cells could also be used to prepare frozen cell banks to facilitate work organization and open new avenues for their utilization as references in physiological studies and clinical investigations was investigated.

STUDY DESIGN AND METHODS: Blood samples or whole-blood leukoreduction filters were obtained, after informed consent, from volunteers or blood donors, respectively. The proportions of CD3+, CD14+, CD16+, CD19+, and CD45+ cells within peripheral blood mononuclear cells (PBMNCs) were determined by flow cytometry from all samples. B cells were isolated and their functional responses were evaluated in vitro.

RESULTS: The yield of PBMNCs recovered from whole-blood leukoreduction filters was lower (50%) than the one with fresh blood samples but still provided 2×10^8 to 4×10^8 PBMNCs per unit. After one cycle of freezing-thawing, the proportions of B- and T-cell populations were similar to normal blood values. Purified B cells issued from whole-blood leukoreduction filters displayed normal phenotypes and functions.

CONCLUSION: Leukoreduction filters represent a valuable source of PBMNCs. These cells could be easily recovered to prepare frozen cell banks useful in basic phenotypic and functional analyses involving the main subsets of B cells and the global T-cell population.

Since June 1999, Héma-Québec, as many other blood suppliers in Europe and the United States, has prepared leukoreduced blood products by filtration. Filters, usually discarded, turned out to be an interesting source of white blood cells (WBCs), as reported elsewhere.[1-6] WBCs recovered from leukoreduction filters were reported to be useful in the preparation of large quantities of DNA[2] from peripheral blood mononuclear cells (PBMNCs) or purified human B cells.[3] Recently, two studies also showed that physiologically functional monocytes[2] and endothelial progenitors cells[4] representing, respectively, approximately 10 and less than 1 percent of total PBMNC populations, can be successfully isolated from these leukoreduction filters.

Human peripheral blood cells are widely used for physiological and clinical investigations and can be utilized as reference material for clinical diagnosis. In our laboratory, we are using an in vitro system involving CD40-CD154 interaction to study human B cells. In vivo the CD154 molecules, which are expressed on activated T cells[7] interact with CD40 expressed on B cells. This in vitro model reproduces T-dependent activation of B cells[8,9] allowing their proliferation and differentiation into antibody-secreting cells.[10,11] Usually, the study of

ABBREVIATIONS: 7-AAD = 7-aminoactinomycin D; FBS = fetal bovine serum; IMDM = Iscove's modified Dulbecco's medium; PBS-glc = phosphate-buffered saline with 10 mmol per L glucose.

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human B cell physiology requires large volumes of blood samples to enable the isolation of 10^7 B cells, which represent only 5 to 15 percent of total PBMNC.

The logistics for the collection of large volumes of blood samples from volunteers are complex. Indeed, accessibility to blood samples relies on recruitment, selection, and availability of volunteers, all supervised by a coordinator nurse, involving supplemental cost. In addition, blood samples must be tested for blood-borne pathogens, which could interfere with cell experimentation and analysis. The preparation of WBCs from whole-blood leukoreduction filters could help to solve these problems given that informed consent and transmissible diseases testing are done on each donation and can be associated to each sample.

Cryopreservation of PBMNCs is already in use in clinical investigations;[12] thus, the use of leukoreduction filters to prepare cryopreserved samples of PBMNCs could be an asset for human investigations combining safety and convenience.

Previous studies with leukoreduction filters as a source of human WBCs[1-4] were based on the immediate utilization of freshly prepared cells. We thus investigated whether whole-blood leukoreduction filters could also be used to prepare frozen cell banks as a source for viable and physiologically active cells. In this study, we have compared populations in PBMNC samples isolated from whole-blood leukoreduction filters and blood samples. We also compared B-cell populations isolated from fresh or frozen PBMNCs obtained either from blood or from whole-blood leukoreduction filters. Finally, B-cell proliferation and secretion of immunoglobulins were analyzed for all these samples after an in vitro stimulation by CD154 in the presence of cytokines.[9,10]

MATERIALS AND METHODS

Blood samples and whole-blood collection

Blood samples were collected in heparinized tubes (Vacutainer, BD Labware, Franklin Lakes, NJ) or blood collection bag containing additive solution-3–citrate phosphate double dextrose (Leukotrap blood collection system, Pall Medical, Covina, CA). Whole-blood units were collected from donors with Leukotrap blood collection system, kept refrigerated, and leukoreduced by filtration at 1 to 6°C. The inline whole-blood leukoreduction filters (WBF2, Pall Medical) of the collection system were recovered within 2 to 8 hours after blood collection. All blood samples or WBF2 filters were obtained after informed consent from volunteers or blood donors, respectively.

Preparation of PBMNC banks

After collection in tubes or bags as described above, blood samples were diluted (1:1) with phosphate-buffered saline (PBS; 10 mmol/L potassium/sodium phosphate buffer with 136 mmol/L NaCl, pH 7.4, Dulbecco's PBS [Invitrogen, Grand Island, NY]) supplemented with 10 mmol per L glucose (PBS-glc) before preparation of PBMNCs. The cells were extracted from WBF2 filters by back-flushing with a 60-mL syringe filled with PBS-glc containing 5 mmol per L ethylenediaminetetraacetate (EDTA) as reported elsewhere.[3] PBMNCs were isolated over Ficoll-Paque following the manufacturer's instructions (Amersham/Pharmacia Biotech, Baie D'Urfé, Canada). PBMNCs were washed in PBS-glc and diluted at 5×10^7 cells per mL in cooled freezing medium containing 50 percent Iscove's modified Dulbecco's medium (IMDM), 40 percent fetal bovine serum (FBS), and 10 percent dimethy sulfoxide. Cryovials containing the PBMNC suspension were transferred into Nalgene cryofreezing containers (Nalgene Nunc, International, Rochester, NY) at –80°C for 18 to 24 hours and then transferred to a liquid nitrogen freezer. Before use, frozen PBMNCs were partially thawed in a 37°C water bath and rapidly diluted with more than 10 vol of warmed PBS-glc containing 50 percent FBS. PBMNCs were washed once with PBS-glc before their utilization.

Physiological stimulation of B cells

B cells were purified from PBMNCs by negative selection with a CD19 cocktail according to the manufacturer's instructions (StemSep, Stem Cell Technologies, Vancouver, British Columbia, Canada). Purified B cells (4×10^5 cells/mL) were seeded in plates (Primaria, BD Labware) coated with 0.75×10^5 cells per cm^2 gamma-irradiated (75 Gy/7500 rad) L4.5 cells, which are expressing CD154.[9] Human B cells were cultured in IMDM supplemented with 10 percent heat-inactivated ultralow immunoglobulin (IgG) FBS, 10 µg per mL insulin, 5.5 µg per mL transferrin, 6.7 ng per mL sodium selenite, antibiotics (all from Invitrogen) and 100 U per mL interleukin (IL)-4 or 50 µ per mL IL-10 (R&D Systems, Minneapolis, MN). Cultures were fed by replacing half of the culture medium every 2 to 3 days, whereas irradiated L4.5 cells were renewed every 4 to 5 days. Cell counts and viability were evaluated in triplicate by trypan blue dye exclusion.

Flow cytometry analysis

Peridinin chlorophyll protein–cyanin 5.5-conjugated anti-CD19; allophycocyanin-conjugated anti-CD14, anti-CD19, or anti-IgM; phycoerythrin (PE)-conjugated anti-CD45, anti-CD27, or anti-IgD; fluorescein isothiocyanate (FITC)-conjugated anti-CD3, anti-CD16, anti-CD27, or anti-IgG; Alexa 488–conjugated anti-CD16; and Alexa 488-allophycocyanin-, PE-, and FITC-conjugated isotype controls were used in quadruple- or triple-staining procedures. All antibodies were IgG_1 mouse monoclonal antibodies obtained from BD Biosciences (Mountain

View, CA). All staining was done with 1 μg of each antibody for 10^5 cells at 4°C. Cells were fixed with 2 percent paraformaldehyde. In all analyses, more than 95 percent of the cells were double-negative when using isotype-matched control antibodies. Regions containing dead cells were delineated with 7-aminoactinomycin D (7-AAD) staining, following the manufacturer's instructions (BD Biosciences). Acquisition of data was performed by gating more than 80,000 cells for PBMNCs or more than 5000 for purified B cells, with a flow cytometer and its software (FACSCalibur and CellQuest Pro, BD Biosciences). Data were subsequently analyzed with a computer program (FCS Express II, De Novo Software, Thornhill, Ontario, Canada).

Determination of IgG and IgM secretion rates

Cells were harvested at the end of the culture period, washed with PBS-glc, and seeded at 1×10^6 to 2×10^6 cells per mL in IMDM alone. Supernatants were collected after 18 to 20 hours and IgG and IgM concentrations were determined by enzyme-like immunosorbent assay (ELISA).[10]

RESULTS

Whole-blood leukoreduction filters: a source for normal human lymphocytes

The total number of PBMNCs isolated from blood samples collected in heparinized tubes, whole-blood bag, and back-flushed from WBF2 filters was evaluated before and after freezing (Table 1). For comparison, data were converted at the level of 1 unit of blood. The yield of PBMNCs recovered from WBF2 filters was low (<50%) when compared to blood samples obtained in tubes or bags. No difference was observed whether the PBMNCs were back-flushed from WBF2 filters rapidly (<2 hr) or after 4 to 8 hours after blood donation (<8 hr). Viability and recovery of PBMNCs as well as the proportion of B cells, however, were similar for all samples after thawing. In addition, the proportion of B cells, 5 to 20 percent, within all PBMNC samples was in accordance to normal blood values.[13] Despite the low yield, up to 2×10^8 to 4×10^8 PBMNCs were recovered from each WFBF2 filter. Additionally, the recovery and viability of PBMNCs from WBF2 filters were similar to blood samples following one freezing-thawing cycle.

PBMNCs from WBF2 filters show normal proportion of lymphocyte populations

To further characterize the PBMNC populations back flushed from WBF2 filters, we have analyzed by flow cytometry the cells recovered from 11 filters (Table 1; <8 hr) after one freezing-thawing cycle. For comparison, we have also analyzed PBMNCs freshly recovered from three WBF2 filters and three blood samples (tubes) and frozen PBMNCs recovered from three blood samples (tubes). Expression of CD3, CD14, CD19, CD27, CD45, IgG, and IgD was monitored (Fig. 1). The proportion of CD3+, CD14+, or CD19+ cells within the CD45+ populations as well as the proportion of CD27+, CD27–, IgG+, and IgD+ cells within the CD19+ populations were determined. The proportions of CD3+CD45+ (range, 57 ± 3-76 ± 7%) and CD19+CD45+ (range, 7 ± 1-8 ± 5%) cells in thawed and fresh PBMNC samples prepared from WBF2 filters, and blood samples were comparable and in accordance to blood normal content.[13,14] Unexpectedly, the CD14+CD45+ population was almost undetectable within the thawed (<2 ± 1%) or fresh (<1 ± 1%) PBMNC populations recovered from filters. In comparison, fresh and thawed PBMNCs prepared from blood samples showed 2 ± 1 and 9 ± 3 percent CD14+CD45+cells, respectively. Finally, the proportions of naïve CD19+CD27– (range, 64 ± 10-70 ± 11%), memory

TABLE 1. Preparation of PBMNC cell banks from WBF2 filters and blood samples: yield, viability, and recovery after one freezing-thawing cycle

	Blood samples*		Before thawing	After thawing		
Blood sample type	Processing time (hr)	Number	Number of PBMNCs/unit ($\times 10^8$ cells)	Recovery (%)	Viability (%)	CD19+ cells (%)
Fresh†						
Heparinized tubes	<2	19	840 ± 311	NA	NA	5 6 ± 2 9†
Frozen						
Heparinized tubes	<2	46	904 ± 406	88 ± 42	95 ± 4	8 4 ± 5.2
Whole-blood bag unit	<2	10	886 ± 309	84 ± 27	68 ± 21	7.5 ± 1.6
Filter—WBF2	<2	14	204 ± 74‡	87 ± 27	94 ± 6	8.3 ± 4.3
Filter—WBF2	<8	14	284 ± 117‡	80 ± 30	88 ± 9	7.1 ± 5 0

* Blood samples collected in heparinized tubes, whole-blood bag units, and WBF2 filters were used to prepare PBMNCs. Cell count and viability were determined by trypan blue exclusion. Before freezing, viability was greater than 99 percent for all samples. The proportion of B cells (CD19+ cells) was determined by flow cytometry.

† Proportion of CD19+ cells was determined in fresh samples.

‡ Difference between WBF2 filter and any blood samples (fresh or frozen, tube or bag) was significant ($p < 0.005$) with the t test.

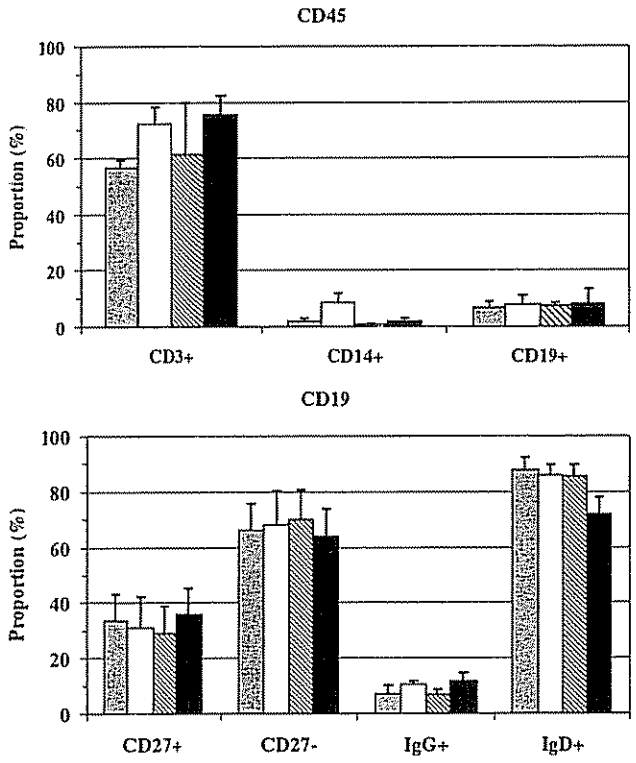

Fig. 1. PBMNCs recovered from WBF2 filters show normal proportion of B and T cells. PBMNCs prepared from 11 filters (WBF2/frozen, ■) and three blood samples (blood/frozen, □) were analyzed, after one freezing-thawing cycle, for the proportions of CD3+, CD14+, and CD19+ cells within the CD45+ cell populations (top) and CD27+, CD27–, IgG+, and IgD+ cells within the CD19+ cell populations (bottom). PBMNCs freshly prepared from three filters (WBF2/fresh, ▨) and three blood samples (blood/fresh, ▤) were analyzed for the same markers. For each PBMNC sample, 80,000 to 200,000 events were gated according to size and granularity, and the purity of CD45+ cells within PBMNCs was greater than 95 percent in all samples. Filters were processed between 4 and 8 hours after collection.

CD19+CD27+ (range, 29 ± 10-36 ± 10%), CD19+IgG+ (range, 7 ± 2-12 ± 3%), and CD19+IgD+ (range, 72 ± 6-88 ± 4%) B-cell subpopulations were comparable in fresh and thawed samples prepared from WBF2 filters and blood samples and their proportions were in accordance with blood normal content.[15,16]

The profiles of CD3+, CD14+, CD16+, and CD19+ within CD45+ cells was also determined with 7-AAD staining to gate only viable cells. Further analyses were done in three thawed and fresh PBMNC samples prepared from WBF2 filters and blood samples collected in tubes (Fig. 2). According to 7-AAD staining, the gate used to analyze the PBMNCs (Gate R1) contained at least 96 to 99 percent of viable cells, and all these cells expressed CD45. The proportion and expression level of CD3+, CD16+, and CD19+ cells matched blood normal values[13] in all samples. As observed above, the CD14+CD45+cell population was almost undetectable in thawed and fresh samples prepared from WBF2 filters whereas this population was observed in fresh and thawed PBMNCs prepared from blood samples. Additionally, the proportion of CD16+CD45+, representing mainly natural killer cells, were in a range of 7 to 14 percent (data not shown) in accordance with reference range expected for blood.[13]

B-cell populations from WBF2 filters or blood samples correspond to blood normal values

B cells were isolated from thawed PBMNCs prepared from blood samples or WBF2 filters with a negative selection system. The purity and phenotype of these B cells obtained from both sources were compared in three independent experiments with flow cytometry analysis. In all samples, high purity of CD19+CD45+ B cells (>95%) was reached and normal phenotypes and proportions of naïve (CD27–), memory (CD27+), and IgG+ cells (Fig. 3) were observed. Intensity of CD27 and IgG expression was also similar in both samples. Finally, PBMNCs prepared from WBF2 filters were sufficient to isolate up to $12 \times 10^6 \pm 4 \times 10^6$ B cells per unit (data not shown).

B cells from both sources similarly proliferate and differentiate to secrete immunoglobulin

For these experiments, B cells were isolated from frozen and fresh PBMNCs prepared from blood samples or from frozen PBMNCs prepared from WBF2 filters. The capacity of B cells to respond to in vitro CD40 stimulation by proliferation was evaluated with CD154+ cells and IL-4 or IL-10 as indicated (Fig. 4). All three B-cell samples were able to proliferate at a similar rate in the presence of IL-4 or IL-10. No differences were observed between frozen and fresh samples or between WBF2 filters and blood samples. In addition, the evaluation of IgG and IgM secretion rates after CD40 stimulation indicate that the same level of differentiation was reached in the three B-cell samples after stimulation in the presence of IL-10 (Fig. 5). These results (Figs. 4 and 5), representative of more than three independent experiments, indicate that the purified B cells issued from PBMNCs prepared from filters were functionally similar to B cells isolated from fresh or frozen PBMNCs prepared from blood samples.

DISCUSSION

Overall our results indicate that whole-blood leukoreduction filters are suitable for the preparation of frozen PBMNC banks. Even though the recovery yield was lower for leukoreduction filters than blood samples, the fact that these filters, usually discarded, can represent a source of 10^8 PBMNCs to prepare frozen cell banks prevailed over

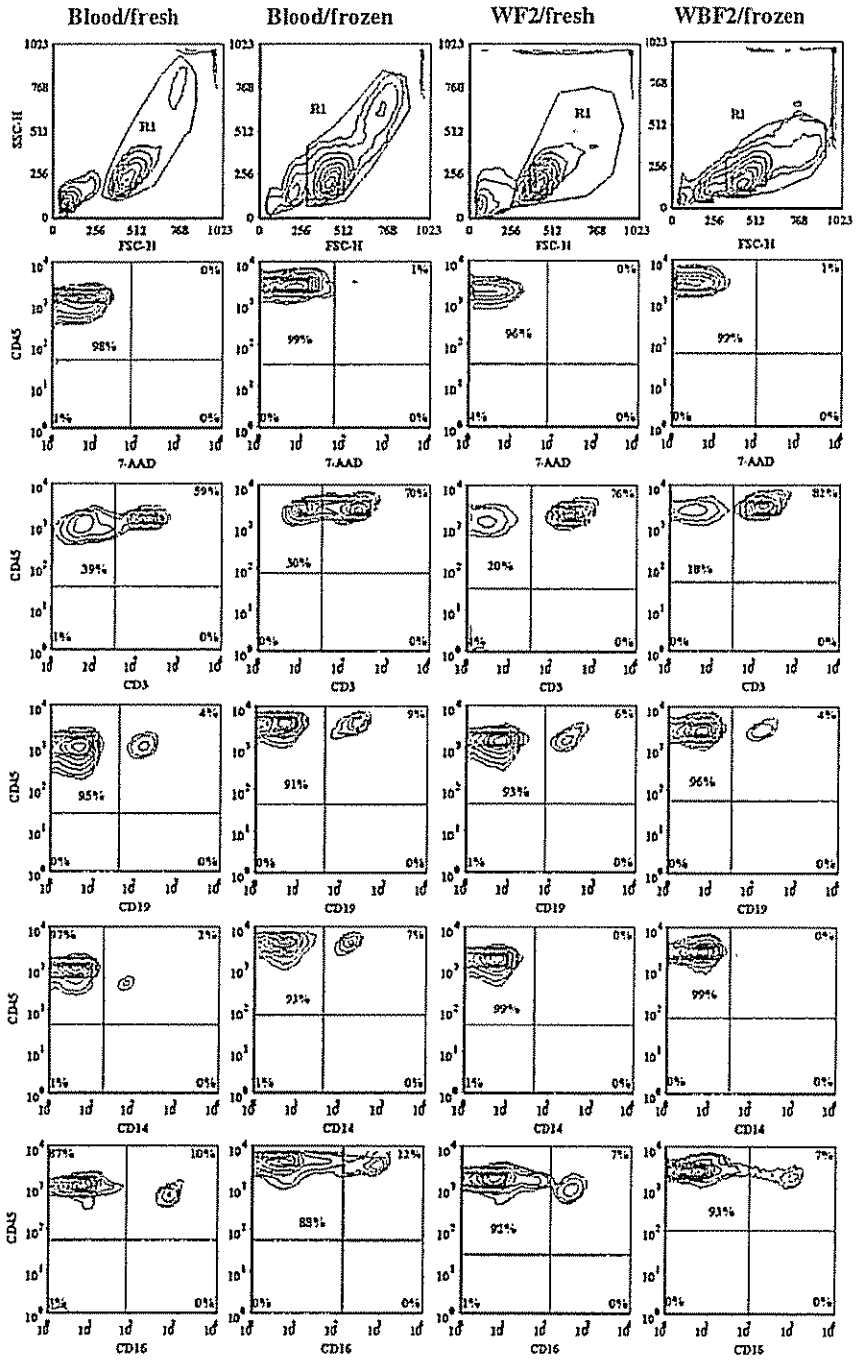

Fig. 2. CD45+ cells prepared from WBF2 filters correspond to normal phenotypes. PBMNCs were analyzed for their proportion of cells expressing CD3, CD14, CD16, and CD19 corresponding, respectively, to T cells, monocytes, natural killer, and B cells. 7-AAD was used to assess the viability of the thawed PBMNCs and to gate only the viable cells for further analysis. The profiles, presented in contour plots, showed the populations observed after analysis performed on 100,000 to 150,000 events in Gate R1 as indicated. These results are representative of analyses performed on three independent samples for each source of fresh or thawed PBMNCs prepared from blood samples (blood/fresh and blood/frozen) and WBF2 filters (WBF2/fresh and WBF2/frozen). WBF2 filters were processed between 4 and 8 hours after collection.

this limitation. In addition, after thawing, these samples provide viable cells, which were physiologically active, according to the B-cell populations.

The yield of PBMNCs, excluding granulocyte contaminations, recovered after back-flushing of leukoreduction filters was reported to be 50×10^6 to 200×10^6 PBMNCs per RCM1 unit.[3] A higher yield, $664 \times 10^6 \pm 223 \times 10^6$ PBMNCs was obtained from WBF2 leukoreduction filters but with a special collection unit designed by the manufacturer.[6] Therefore, low recovery yield when compared to blood samples was not unexpected. Additionally, it could be related to the usually high efficiency of whole-blood leukoreduction filters to bind and retain WBCs.[17,18]

Cryopreservation of PBMNCs does not influence the major CD4 and CD8 T-cell populations in human immunodeficiency virus (HIV)-positive and HIV-negative samples, but it was reported to induce changes in the expression of CD62L and CCR5 on the surface of T cells.[12] In this study, the effect of the freezing-thawing process was not investigated for T-cell subsets but for B-cell subsets. Nevertheless, our results show that the proportion of B and T cells were, as expected, approximately 10 and 75 percent of PBMNCs after one freezing-thawing cycle, respectively. In addition, the B-cell subsets and B-cell functions were not influenced by one freezing-thawing cycle or by the source of PBMNCs. Normal amounts ($12 \times 10^6 \pm 4 \times 10^6$ cells) of B cells per WBF2 filter were isolated from these PBMNCs. Targeting more specifically B-cell populations for their use in physiological experiments, we reported here that the main subsets of B cells according to CD27, IgG, and IgD expression were similar to blood normal values.[15,16]

We observed low recovery of CD14+cells from WBF2 filters compared to blood samples as observed previously on fresh PBMNC samples.[4] Conversely, WBF2 filters were used with success to isolate CD14+ cells from PBMNCs, for the generation of human dendritic cells.[2] Indeed, this study was performed with PBS containing dextran, saccha-

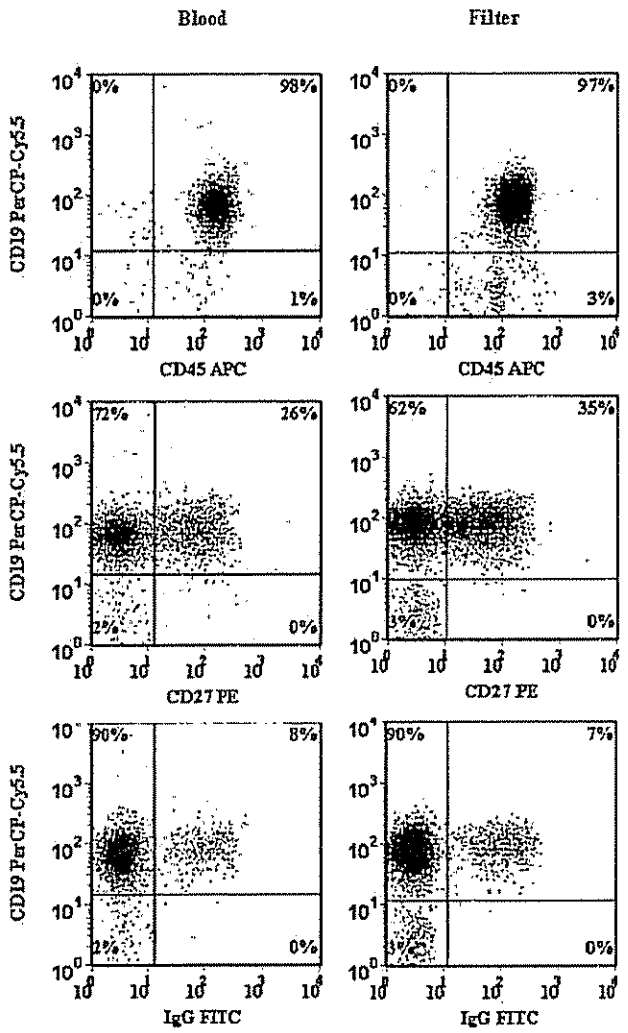

Fig. 3. B-cell populations prepared from blood samples or WBF2 filters showed normal patterns. Purified B cells were prepared from PBMNCs with StemSep from both sources and analyzed by flow cytometry for CD19, CD27, CD45, and IgG expression in quadruple staining. These results are representative of three independent experiments. PerCP-Cy5.5 = peridinin chlorophyll protein–cyanin 5.5; APC = allophycocyanin.

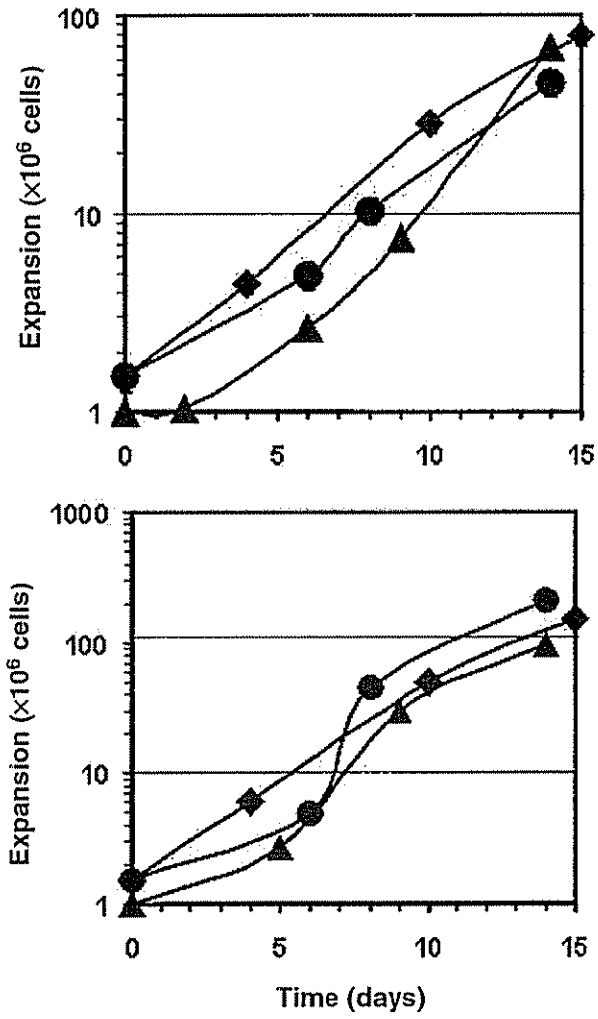

Fig. 4. CD40 stimulation of B cells is similar in all samples. B cells purified from fresh (▲) or frozen PBMNCs (●, ◆), which were prepared from blood (▲, ●) or WBF2 filters (◆), were stimulated with gamma-irradiated CD154+ L4.5 cells in the presence of 100 U per mL IL-4 (top) or 50 U per mL IL-10 (bottom) for 14 to 15 days as indicated. Cell count was determined at indicated days and used to evaluate total expansion according to expansion rate and seeding cell density. Viability was greater than 95 percent for all samples during the culture period. These results are representative of at least three independent experiments for each condition. Error bars can be smaller than symbols.

rose, and human serum albumin to back-flush PBMNCs from WBF2 filters. Therefore, the important loss of CD14+CD45+ cells in our assays with the same WBF2 filters could be attributed to the use of PBS-glc containing EDTA. These observations indicated that the solution used to back-flush the cells can influence the status of the monocytes trapped on the filters. As reported for a human monocytic cell line,[19-21] variation in environment per se could have enhanced CD14+ cell adherence[17] and activation and have rendered the monocytes more fragile during the preparation.

Blood samples are used to establish diagnosis in various diseases associated to peripheral blood cells. Many immune deficiencies and disorders are characterized by important alterations of blood cell phenotypes in comparison to healthy controls. One method to investigate such disorders is multiparameter flow cytometry analysis, which is part of the French-American-British classification for acute myeloid leukemia.[22,23] Flow cytometry is also important for the diagnosis of lymphomas[24,25] and to evaluate disequilibrium or displacement in B-lymphocyte populations.[26-29] Frozen PBMNC banks, as normal refer-

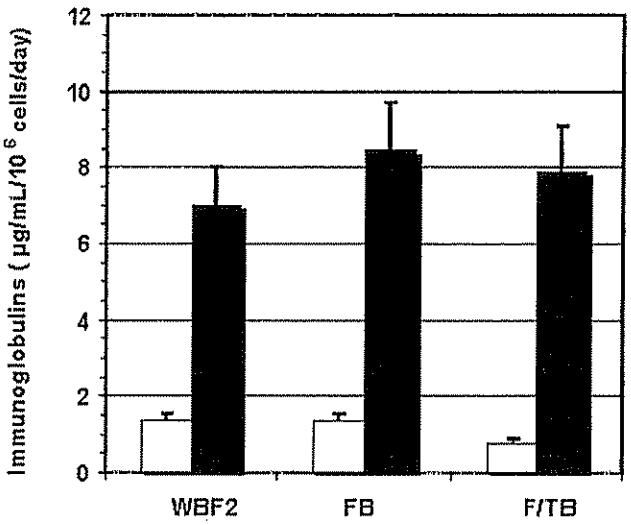

Fig. 5. CD40-stimulated B cells issued from WBF2 filters or blood samples secrete IgG (□) and IgM (■) at similar rate. As above, B cells were isolated from fresh (FB) or frozen PBMNCs (F/TB), which were prepared from blood samples, or frozen PBMNCs prepared from filters (WBF2). B cells stimulated as described in the legend to Fig. 4 in the presence of IL-10 for 14 to 15 days were harvested, washed, and seeded at 1×10^6 to 2×10^6 cells per mL in IMDM alone. Supernatants were harvested after 18 to 20 hours of incubation at 37°C. IgG and IgM secretion were determined by ELISA.

ence, could thus be an asset for establishment of diagnosis in these diseases.

In conclusion, these results suggest that frozen PBMNCs prepared from WBF2 filters can be used in physiological studies or as internal controls for immunophenotyping when global T- and B-cell populations or B-cell subpopulations are under investigations. More specifically, B cells isolated from such samples are as physiologically active as freshly purified B cells.

Few studies aim at the characterization of cells recovered from leukoreduction filters[1-6] and only three reports investigated the physiological status of targeted populations among freshly isolated MNCs.[2,4,5] Until now, no information was available on the physiological status of B cells recovered from leukoreduction filters as well as on the physiological status of frozen cells prepared from these filters. Here, we report for the first time that frozen cell banks prepared from leukoreduction filters provided viable cells with active physiological status with B cells as indicator. Finally, given that whole-blood leukoreduction filters are discarded after filtration, their use to sample human peripheral lymphocytes provides an extra value to blood donation.

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