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Research paper

Filter Buffy Coats (FBC): A source of peripheral blood leukocytes recovered from leukocyte depletion filters

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Abstract

In compliance with federal regulations, blood banks routinely use leukocyte depletion filters to eliminate contaminating leukocytes from blood products such as red blood cell and platelet concentrates. We developed and optimized conditions to elute leukocytes adsorbed to these filters; resulting in leukocyte suspensions which we termed Filter Buffy Coats (FBCs). These Filter Buffy Coats can replace standard buffy coats for various research applications.

After optimizing both the filter elution medium as well as elution protocols, we compared commonly used leukocyte depletion filters from four different manufacturers. Relative fractions as well as total recoveries of leukocyte subsets, such as lymphocytes, monocytes and granulocytes, found in Filter Buffy Coats were identified and compared among the filters as well as to standard buffy coats and whole blood. Flow cytometric analysis of Filter Buffy Coats confirmed the presence of T- and B-lymphocytes, NK cells and monocytes. Furthermore, a significant quantity of $CD34^+$ hematopoietic stem or progenitor cells (HSC/HPC) was detected in Filter Buffy Coats prepared from different filters, thus making FBCs a valuable source for research on HSC/HPC. Colony assays revealed that most of these $CD34^+$ cells are functional. Using immunomagnetic cell sorting (MACS), we isolated a variety of leukocyte populations from FBC mononuclear cells (Filter-PBMCs) including T lymphocytes ($CD4^+$, $CD8^+$, $CD3^+$), B lymphocytes ($CD19^+$), NK cells ($CD56^+$), HSC/HPC ($CD34^+$, $CD133^+$) or dendritic cells (BDCA-4^+). Functional properties of Filter-PBMCs, as well as of some of these isolated leukocyte populations, were confirmed using standard assays

In summary, Filter Buffy Coats are a valuable and convenient source of different peripheral leukocyte populations and can replace standard buffy coat preparations for research applications.

Keywords Buffy coat; Leukocyte depletion filter; Filter Buffy Coat; PBMC; $CD34^+$ cells

1. Introduction

Allogeneic leukocytes present in blood cell products such as red blood cell and platelet concentrates are known to cause a variety of adverse transfusion reactions like HLA-induced incompatibilities or viral infections. Furthermore, leukocytes contained in blood

Abbreviations: FBC, Filter Buffy Coat; MNCs, mononuclear cells; HSC/HPC, hematopoietic stem cells/hematopoietic progenitor cells; PDCs, plasmacytoid dendritic cells

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products can release compounds (such as enzymes or cytokines) upon disintegration, which negatively affect the quality and shelf life of the blood products (Goldman and Delage, 1995; Dzik, 1996; British Committee for Standards in Haematology, 1998; Seghatchian, 2003). Thus, leukocytes should be removed from blood products prior to use and in Germany leukocyte depletion by in-line filtration has been implemented as a regulatory requirement by the national authorities. Subsequently, a variety of different leukocyte depletion filters have been developed and are now routinely used at blood banks for leukocyte removal. These filters are capable of reducing the number of leukocytes in blood products by a factor of up to 10,000. These circumstances led to a shortage of standard buffy coats derived from whole blood after component fractionation by centrifugation, which has been widely used in the past as a leukocyte source in scientific and technical development.

We developed a method to elute leukocytes aseptically from leukocyte depletion filters, thus creating a convenient source of leukocytes (termed Filter Buffy Coat, or FBC) for research purposes. In this study, we show that viable peripheral blood leukocytes can be retrieved from a variety of leukocyte depletion filters currently implemented in blood banking. Filters back-flushed with physiological buffer solutions generated Filter Buffy Coats containing viable peripheral blood mononuclear cells in sufficient amounts for scientific purposes, with cellular compositions similar to standard buffy coats. Furthermore, a European patent application has been submitted.[1] By using immunomagnetic cell sorting (MACS), we isolated a variety of leukocyte populations from Filter Buffy Coat mononuclear cells (Filter-PBMCs), including T- and B-lymphocytes, hematopoietic progenitor cells, monocytes, dendritic cells and NK cells. Further characterization of Filter-PBMCs as well as of MACS isolated cell populations revealed that these cells are functional.

2. Materials and methods

2.1. Leukocyte depletion filters

The following four different leukocyte depletion filters were used to prepare Filter Buffy Coats, to analyze leukocyte recovery and to characterize cellular fractions: Compoflex T3908 (Fresenius Hemocare, Friedberg, Germany), Leukoflex LST-1 (MacoPharma, Tourcoing, France), Leukotrap WBF-3 (Pall Medical, Ascoli, Italy), and Optipure RZ 2000 (Baxter, Unterschleißheim, Germany). These filters are used in routine blood banking and are designed either as hard case (WBF-3, RZ 2000, T3908) or soft case (LST-1) filters. Each filter contains about 30 to 40 different filter layers consisting of unwoven PVC, PP or PET fibers (Bruil et al., 1995). After initial experiments with all four filters, optimal back-flushing conditions were developed with T3908 and LST-1 filters. For all of the following experiments, leukocyte depletion filters were used after leukodepleting 500 ml of whole blood from normal healthy donors.

2.2. Filter elution medium

Chemicals (research purity grade) were obtained from Merck (Darmstadt, Germany).

PBS buffer (pH 7.2–7.4) was prepared according to standard procedures (Mishell and Shiigi, 1980). Alternatively, Dulbecco's PBS (D-PBS) without $MgCl_2$ and $CaCl_2$ (Invitrogen, UK) was used for back-flushing of filters. To obtain the filter elution medium, 5 mM Na_2–EDTA and 2.5% [w/v] sucrose was added to the PBS. Sterile filtration of the filter elution medium was performed prior to use. The pH of the filter elution medium was determined by glass electrode measurement according to the European Pharmacopeia.

2.3. Filter back-flushing

Filters were flushed at room temperature with leukocyte elution medium by attaching a sterile 100 ml syringe to the tubing of the blood bag system using a luer lock adapter. The flow direction of the leukocyte elution medium during filter back-flushing was opposite to the primary blood flow during the leukocyte depletion step. Filter back-flushing was conducted in steps of 50 ml each up to a total volume of 200 ml elution medium between 2 and 8 h after the leukocyte depletion step of whole blood. Applying high pressure and thus increasing the speed of back-flushing was avoided since this may lead to cell loss, cell disruption and, in the case of soft case filters, even to filter leakage. It took approximately 1 to 3 min to flush hard case filters (T3908, WBF-3, RZ 2000), although partially clogged filter units may take longer. Soft case filters, e.g. LST-1, can inflate during back-flushing and thus lead to reduced cell recoveries and increased flushing times. Therefore, soft case filters were trapped in an

[1] European patent application No. 03012551.2: Methods of Preparing Peripheral Stem Cells from Leukocyte Reduction Filters and the uses thereof. Blutspendedienst des Bayerischen Roten Kreuzes.

external tight fitting hard case to permit the same flushing performance as with hard case units. To enhance leukocyte yields, residual buffer in the filter was pressed out by flushing the system with air at the end of the elution procedure. All these steps were performed aseptically in a "closed" blood bag system and the eluted cells were collected in the primary whole blood collection bag, which can be used as sterile transport container. Before performing cell counts and further experiments, the blood bag containing leukocytes was gently mixed about 20–30 min using an overhead or similar mixer.

2.4. Leukocyte counts in Filter Buffy Coats

Leukocyte counts were performed by light scattering using the automatic multiparameter hematology analyzers CELL-DYN 3200 or 3500 (Abbott Lab. Inc., Illinois, USA) to assess successful cell flushing. All reagents for the hematology analyzers (e.g. cell lysing reagents, sheath reagent) were purchased from Abbott. Absolute cell counts of total white blood cells (wbc), lymphocytes, monocytes, neutrophilic, eosinophilic and basophilic granulocytes from Filter Buffy Coats were determined simultaneously by the hematology analyzers on the basis of light scattering characteristics. Using these data, yields and purities of the leukocytes were calculated.

2.5. Flow cytometric characterization of FBCs

Cell fractions of interest from the recovered FBC leukocytes were characterized by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany) and a variety of specific cell markers. To detect lymphocyte subsets, the following surface markers were used to assign cells to the different cell lineages: $CD3^+/CD45^+$ (total T lymphocytes), $CD3^+/CD4^+/CD45^+$ (T helper lymphocytes), $CD3^+/CD8^+/CD45^+$ (cytotoxic T lymphocytes), $CD19^+/CD45^+$ (B lymphocytes), $CD16^+/CD56^+/CD45^+$ (NK cells) (all MultiSET reagents; BD Biosciences). Controls for determining concentrations were performed with TruCOUNT beads (BD Biosciences). Evaluation was performed with CellQuestPro or Multiset V1.1.2 software, which automatically analyzes three- and four-color samples using CD45 vs. SSC or CD3 vs. SSC gating. Monocytes were detected using $CD14^+/CD45^+$ SimulTEST LeucoGATE (BD Biosciences).

For the identification and total count of $CD34^+$ peripheral blood hematopoietic stem and progenitor cells (HSC/HPC) either the ProCOUNT Progenitor Cell Enumeration Kit (BD Biosciences, Heidelberg, Germany) was used or, alternatively, a manual staining and gating strategy was applied (see below). The ProCOUNT Kit was used in combination with the ProCOUNT phenotyping software on a FACSCalibur flow cytometer according to the manufacturer's instructions. In duplicate experiments, one test tube was mixed with CD34 staining reagent and another tube with control reagent. The staining reagent specifically stained hematopoietic stem and progenitor cells and contained a nucleic acid staining dye, a monoclonal anti-human CD34 antibody coupled to PE and a PerCP-coupled monoclonal anti-human CD45 antibody. The control reagent contained anti-human CD45 antibody, a nucleic acid specific dye and an IgG1 isotype control for the anti-CD34 antibody to exclude non-specific labeling. TruCOUNT beads (BD Biosciences) were used as an internal standard to assess correct cell counting.

Alternatively, $CD34^+$ hematopoietic stem and progenitor cells were manually stained with anti-CD34 PE and anti-CD45 FITC monoclonal antibodies (BD Biosciences) and gated following the guidelines of the International Society of Hemotherapy and Graft Engineering (ISHAGE). Nonviable cells were excluded by labeling with the nucleic acid dye 7-AAD (BD Biosciences) and subsequently hematopoietic stem and progenitor cells were quantified by the following criteria: high expression of the CD34 antigen and low expression of the CD45 antigen as well as specific light scatter characteristics (low side scatter and low/medium forward scatter). To determine nonspecific staining of the anti-CD34 PE antibody, an IgG1 PE isotype control antibody (BD Biosciences) was used.

2.6. Preparation of PBMC from Filter Buffy Coats and from standard buffy coats

After preparation of Filter Buffy Coats, these were further processed by density gradient centrifugation to obtain mononuclear cells (MNCs). These MNCs obtained from FBCs were termed Filter-PBMCs. Three slightly different protocols were tested, with comparable yields for MNCs.

1. Filter Buffy Coats were centrifuged at $445\times g$ for 35 min at 21 °C without break. The white blood cell layers, diluted threefold in PBS, pH 7.4, containing 5 mM EDTA (PBS/EDTA), were overlaid carefully on Ficoll Paque Plus (Pharmacia, Uppsala, Sweden) for density gradient centrifugation ($445\times g$ for 35 min at 21 °C without break). Filter-PBMCs

were collected from the interphase and washed twice with PBS/EDTA.

2. Filter Buffy Coats were overlaid directly on Ficoll Paque Plus and were centrifuged at 445×*g* for 35 min at 21 °C without break. Filter-PBMCs were collected from the interphase and washed twice with PBS/EDTA.
3. Filter Buffy Coats were transferred onto a Ficoll Paque Plus Leucosep Tube (Greiner Bio-One) and were centrifuged at 1000×*g* for 11 min at 21 °C without break. Filter-PBMCs were collected from the interphase and washed twice with PBS/EDTA.

PBMCs from standard buffy coats (termed standard PBMCs) were prepared as follows. The buffy coat layer containing the leukocytes was diluted with PBS to obtain a volume of about 200 ml per 500 ml whole blood and overlaid carefully on Ficoll Paque Plus for density gradient centrifugation (445×*g* for 35 min at 21 °C without break). The standard PBMCs were collected from the interphase and washed twice with PBS/EDTA.

2.7. Isolation of leukocyte subsets from Filter-PBMC by MACS

Various leukocyte populations were isolated from Filter-PBMCs by immunomagnetic cell sorting (MACS) using the Mini- or the MidiMACS system. $CD34^+$ and $CD133^+$ hematopoietic stem and progenitor cells were isolated using the Direct CD34 Progenitor Cell Isolation Kit and the CD133 Cell Isolation Kit, respectively. $CD304^+$ (BDCA-4^+) plasmacytoid dendritic cells were isolated using the CD304 (BDCA-4/Neuropilin-1) MicroBead Kit. $CD3^+$ T cells, $CD4^+$ T helper lymphocytes and $CD8^+$ cytotoxic T lymphocytes were isolated using CD3 MicroBeads, the Pan T Cell Isolation Kit II, CD4 MicroBeads, the $CD4^+$ T Cell Isolation Kit II, CD8 MicroBeads and the $CD8^+$ T Cell Isolation Kit II, respectively. B cells were isolated using CD19 MicroBeads and the B Cell Isolation Kit II. $CD14^+$ monocytes were isolated using CD14 MicroBeads and the Monocyte Isolation Kit II. Granulocytes were depleted using CD15 MicroBeads or CD16 MicroBeads. $CD56^+$ NK cells were isolated using CD56 MicroBeads. All separation reagents and separation systems (MiniMACS or MidiMACS) were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). All separations were performed according to the manufacturer's instructions, except for the depletion of granulocytes, where CD15 MicroBeads or CD16 MicroBeads with a titer of 1/10 instead of 1/5 and 1/2, respectively, were used.

Samples before and after separation were analyzed by flow cytometry to determine purity and yield as follows. Flow cytometric analysis was performed using a FACSCalibur or a FACScan (BD Biosciences) flow cytometer. Data were analyzed using CellQuest or CellQuestPro software (BD Biosciences). Dead cells and debris were excluded from the analysis based on scatter properties and propidium iodide staining.

The following monoclonal antibodies (mAbs) were used in this study for flow cytometry: anti-BDCA-2 (clone AC144), CD3 (BW264/56), CD4 (MT321), CD8 (BW135/80), CD14 (TÜK4), CD15 (VIMC6), CD16 (VEP13), CD19 (LT19), CD34 (AC136), CD45 (5B1), CD45RA (L48), CD56 (AF12-7H3), CD123 (AC145) and CD133/2 (293C3), all from Miltenyi Biotec. The mAbs were used as FITC-, PE-, or APC-conjugates.

2.8. Colony assays to determine colony-forming units (CFUs)

Colony assays were performed to assess the functionality of $CD34^+$ cells in Filter-PBMCs or standard PBMCs as well as MACS isolated $CD34^+$ cells prepared from Filter-PBMCs. Colony assays were set up according to standard protocols using Methocult GF H4434 (Stem Cell Technologies, Vancouver, Canada) in 35 mm dishes and counted microscopically after 14 days incubation in a humified incubator at 37 °C, 5% CO_2, and 5% O_2. We identified and counted granulocyte/macrophage colonies (CFU-GM; colony forming unit-granulocyte/macrophage), erythroid colonies (BFU-E; burst forming unit-erythrocyte) and mixed colonies (CFU-GEMM; colony forming unit-granulocyte/erythrocyte/macrophage/megakaryocyte) in accordance with standard protocols. Usually, clusters containing 50 or more cells were counted as colonies.

2.9. Activation of PBMCs

Freshly prepared Filter-PBMCs or standard PBMCs were analyzed for expression of the activation markers CD69 and CD25 by flow cytometry to assess the activation state of the mononuclear cells. In addition, the PBMCs were cultured for 16 h at a cell concentration of 1×10^7/ml in RPMI1640 (Gibco) containing 5% human AB serum (heat inactivated; Sigma-Aldrich) and 1 μg/ml Staphylococcal Enterotoxin B (SEB; Sigma-Aldrich) to determine their activation potential, essentially as described (Caruso et al., 1997; McLeod et al., 1998). Cells were harvested and analyzed by flow cytometry as described above, using the following monoclonal antibodies: CD3 (clone BW264/56), CD8

(BW135/80), CD25 (4E3) and CD69 (FN50) from Miltenyi Biotec, and CD4 (SK3), CD14 (M5E2), CD19 (4G7) and CD56 (NCAM16.2) from BD Biosciences. These antibodies were used in combination with 7-AAD (BD Biosciences) to determine activation of total viable T cells ($CD3^+$/7-AAD^-), viable T helper cells ($CD3^+$/$CD4^+$/7-AAD^-), viable cytotoxic T cells ($CD3^+$/$CD8^+$/7-AAD^-), viable B cells ($CD19^+$/7-AAD^-), viable monocytes ($CD14^+$/7-AAD^-) and viable NK cells ($CD56^+$/7-AAD^-).

For detection of cytokine secreting lymphocytes we used the IFN-γ Secretion Assay Detection Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. For this purpose, Filter-PBMCs were prepared and stimulated with SEB as described above.

2.10. Mixed lymphocyte reaction

$CD304^+$ ($BDCA\text{-}4^+$) PDCs were enriched from Filter-PBMCs and standard PBMCs using the CD304 (BDCA-4/Neuropilin-1) MicroBead Kit. Naive $CD4^+$/$CD45RA^+$ T helper cells were isolated from Filter-PBMCs and standard PBMCs using the $CD4^+$ T cell Isolation Kit II and CD45RO MicroBeads. PDCs were stimulated for 18 h with the CpG oligonucleotide M362 (5′-tcg tcg tcg ttc gaa cga cgt tga t- 3′), 1 μM, from Biosource in the presence of 10 ng/ml IL-3 (Peprotech). After stimulation, PDCs were washed once and 1×10^4 PDCs were co-cultured with 5×10^4 (1/5), 1×10^5 (1/10) and 2×10^5 (1/20) allogeneic $CD4^+$/$CD45RA^+$ T cells for 5 days in a 96-well plate in 150 μl RPMI1640 (Gibco), supplemented with 10% FCS (PAA Laboratories), 1 mM Sodiumpyruvat (Gibco), 100 units/ml Penicillin G (Gibco), 100 μg/ml Streptomycin (Gibco), 2 mM L-glutamine (Gibco) and 50 μM 2-mercaptoethanol (Gibco). For the last 8 h of culturing 0.5 μC methyl-[^{3}H]Thymidine (Amersham Bioscience) was added to measure T cell proliferation by Tritium incorporation.

3. Results

3.1. Filter elution medium

The first step for generating Filter Buffy Coats was to develop an elution medium providing optimal conditions concerning leukocyte yield in Filter Buffy Coats. PBS (without Mg^{2+} or Ca^{2+}) containing EDTA–Na_2 (5 mM) and sucrose 2.5% [w/v] turned out to be a simple and efficient medium to back-flush leukocyte depletion filters. Various experiments were conducted using different concentrations of both compounds with no significant difference in leukocyte recovery. Buffers containing different types of sugars or polysaccharides did not enhance cell yield or change the cellular composition in the Filter Buffy Coats significantly. Polymers added to the elution medium were also not able to release more leukocytes adsorbed to the filters. Also, biochemicals known to interfere with cellular adhesion molecules (e.g. *N*-acetylglucosamine) did not increase leukocyte yields (data not shown). These results prompted us to turn our attention to basic physical properties of the filter elution medium such as volume or pH. The volume of the leukocyte elution medium was optimized by performing filter back-flushing in four subsequent steps of 30 ml or 50 ml each. Best results were achieved using three flushing steps of 50 ml each. Increasing the number or the volume of the elution steps led to a dilution of cell concentration with no significant increase in leukocyte yield (data not shown).

Preliminary results for filter back-flushing using filter elution media with the pH adjusted to different values indicated that an optimal pH range exists for filter back-flushing to further increase leukocyte recoveries. In our experiments, the pH of the buffer solution was monitored, but not adjusted, resulting in a pH of usually between 6.5 and 7.0.

3.2. Leukocyte recovery and cellular composition of Filter Buffy Coats (FBCs)

After optimizing the leukocyte elution medium, we compared four different leukocyte depletion filters by analyzing total leukocyte recoveries and distribution of major leukocyte subsets (granulocytes, lymphocytes and monocytes) detected in the Filter Buffy Coats. The leukocyte depletion filters were: Compoflex T3908 (Fresenius Hemocare, Friedberg, Germany), Leukoflex LST-1 (MacoPharma, Tourcoing, France), Leukotrap WBF-3 (Pall Medical, Ascoli, Italy) and Optipure RZ 2000 (Baxter, Unterschleißheim, Germany) (Fig. 1). Each filter type shows a specific profile of cell elution patterns, probably because of the different composition and structure of the filter layers. FBCs prepared from Leukoflex LST-1 filters are most similar to standard buffy coats in their relative proportions of leukocyte subpopulations. The Leukotrap WBF-3 FBCs show a higher relative proportion of granulocytes compared to lymphocytes. Compoflex T3908 and the Optipure RZ 2000 FBCs show similar leukocyte elution patterns with higher proportions for lymphocytes and lower proportions for granulocytes (Fig. 1).

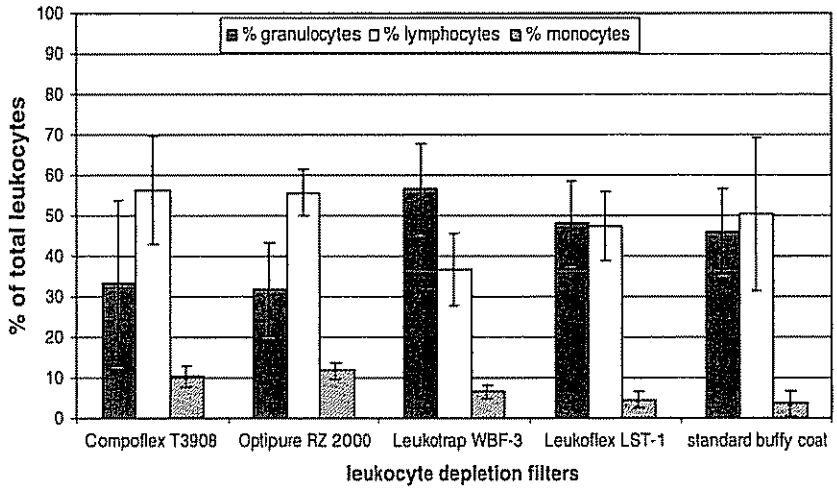

Fig. 1. Distribution of leukocyte subpopulations in Filter Buffy Coats. The relative distributions of granulocytes, lymphocytes and monocytes in either standard buffy coats (far right) or Filter Buffy Coats prepared from four different leukocyte depletion filters (as specified) are shown. Total leukocyte suspensions were analyzed using a CELL-DYN hematology blood analyzer. The bars indicate averages for $n=70$ (Compoflex T3908), $n=15$ (Optipure RZ 2000), $n=110$ (Leukotrap WBF-3), $n=35$ (Leukoflex LST-1) and $n=16$ (standard buffy coats). Error bars indicate standard deviations.

In addition to the relative leukocyte distributions that are displayed in Fig. 1, Table 1 shows total leukocyte recoveries per Filter Buffy Coat compared to standard buffy coats and to whole blood from normal healthy donors. Based on these data generated with a blood hematology analyzer, we obtained similar recoveries for total leukocytes in FBCs prepared from three of the four filter types tested, namely Compoflex T3908, Optipure RZ 2000 and Leukoflex LST-1 (Table 1). FBCs eluted from Leukotrap WBF-3 filters contained higher amounts of total leukocytes as well as of granulocytes but not of lymphocytes (Table 1). Compared to standard buffy coats the total leukocytes as well as the lymphocyte and granulocyte subpopulations recovered from the leukocyte depletion filters were lower (Table 1). The recovery of monocytes, however, seemed to be at least as good in FBCs as in standard buffy coats.

Flow cytometric analysis of FBCs ($n=66$) revealed an average viability of 97.4% (7-AAD negative cells; data not shown). Additional characterization (using the MultiSET system and SimulTEST antibodies) is shown in Fig. 2. Both, Leukoflex LST-1 and Compoflex T3908 Filter Buffy Coats contain all major lymphocyte subsets including B cells, T cells ($CD4^+$ T cells and $CD8^+$ T cells), NK cells and monocytes. In addition, the relative distributions of the leukocyte populations were rather similar for these two Filter Buffy Coats (Fig. 2).

3.3. Kinetics of FBC preparation

Next, we investigated whether there was a correlation between leukocyte content in FBCs and the time between leukocyte depletion and filter back-flushing.

Table 1
Recoveries of leukocyte subpopulations in Filter Buffy Coats

Cell population	Whole blood	Standard buffy coat	Compoflex T3908	Optipure RZ 2000	Leukotrap WBF-3	Leukoflex LST-1
Volume (ml)	500	40	165	165	165	165
Total leukocytes ($\times 10^8$)	20–45	18.4	10.1	9.7	15.8	10.1
Granulocytes ($\times 10^8$)	11.4–33.8	8.5	3.4	3.1	9.0	5.6
Lymphocytes ($\times 10^8$)	5.0–18	9.3	5.7	5.4	5.8	3.6
Monocytes ($\times 10^8$)	0.4–2.7	0.7	1.0	1.1	1.0	1.0
n	Ref.	16	70	15	110	35

The absolute amounts of leukocytes and leukocyte subpopulations (granulocytes, lymphocytes and monocytes) per Filter Buffy Coat prepared from four different leukocyte depletion filters are shown as averages. Total leukocyte suspensions were analyzed using a CELL-DYN hematology blood analyzer. As a comparison, the amounts of leukocytes found in standard buffy coats (average) as well as the range found in whole blood from normal healthy donors (Ref.: Begemann, 1999) are displayed on the left. All leukocyte amounts are calculated for 500 ml whole blood.

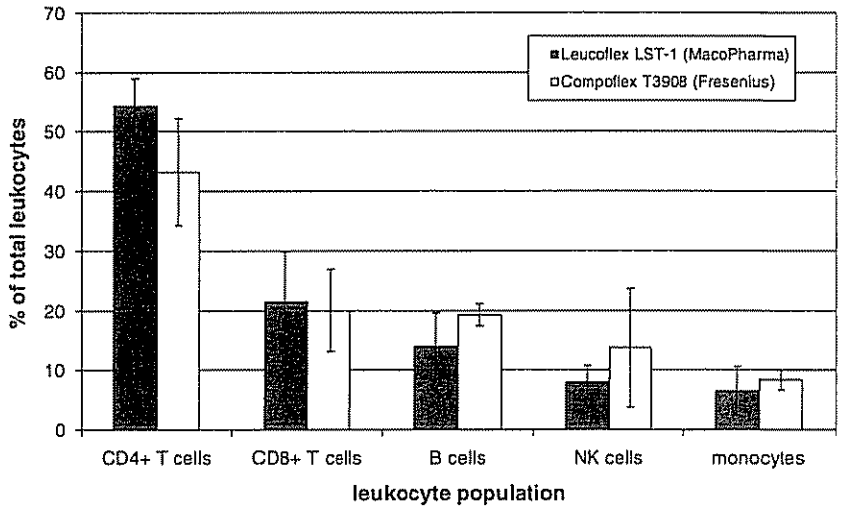

Fig. 2 Flow cytometric analysis of Filter Buffy Coats Flow cytometric analysis of Filter Buffy Coats prepared from Compoflex T3908 ($n=4$) or Leukoflex LST-1 ($n=4$) leukocyte depletion filters The following cell surface markers were used for identification of leukocyte populations in FBCs (without prior preparation of mononuclear cells): $CD45^+/CD3^+/CD4^+$ (T helper cells), $CD45^+/CD3^+/CD8^+$ (cytotoxic T cells), $CD45^+/CD19^+$ (B cells), $CD45^+/CD16^+/CD56^+$ (NK cells) and $CD45^+/CD14^+$ (monocytes). Error bars indicate standard deviations.

Compoflex T3908 and Leukoflex LST-1 filters were used to determine the optimal time for the generation of Filter Buffy Coats. Results shown in Fig. 3 represent elution experiments carried out between 2.8 and 8.2 h after leukodepletion of whole blood. A major drop in cell yield can be observed about 5 h after whole blood leukodepletion, followed by a continuous decrease of leukocyte concentration in Filter Buffy Coats (Fig. 3). As a consequence, FBCs were routinely prepared within 4 h of leukodepletion.

3.4 Characterization of PBMCs

To characterize PBMCs, mononuclear cell fractions were prepared from FBCs (termed Filter-PBMCs) and from standard buffy coats (termed standard PBMCs) ($n=21$). We observed an average yield of $3.8 \pm 1.4 \times 10^8$ leukocytes (average $\pm$ S.D.; range: 1.8–6.0) for Filter-PBMCs and $8.5 \pm 3.0 \times 10^8$ leukocytes (average $\pm$ S.D.; range: 1.3–13.6) for standard PBMCs as measured with a blood cell analyzer. Viabilities deter-

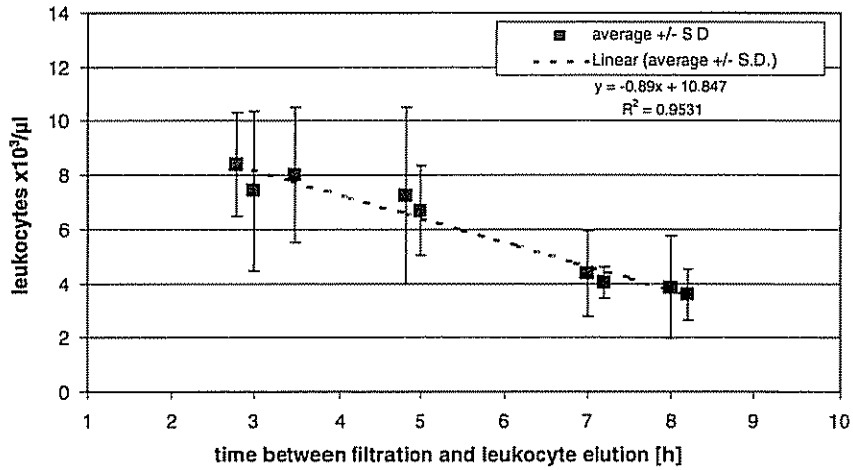

Fig. 3 Leukocyte recovery from leukocyte depletion filters at various times after filtration The concentration of total leukocytes in Filter Buffy Coats is displayed as a function of time between whole blood filtration and leukocyte elution. Squares indicate average leukocyte concentrations ($\times 10^3/\mu l$; $n=5$–10), error bars indicate standard deviation). All Filter Buffy Coats were prepared from Compoflex T3908 leukocyte depletion filters, except 2.8 h, 7.0 h and 8.2 h (Leukoflex LST-1). The average FBC volume is 165 ml.

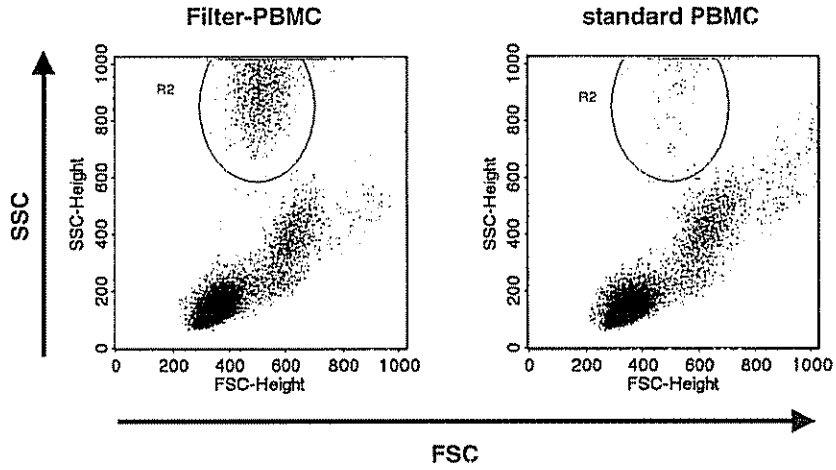

Fig. 4. Scatter characteristics of Filter-PBMCs and standard PBMCs. Forward Scatter (FSC) and Side Scatter (SSC) dot plots of Filter-PBMCs and standard PBMCs of representative samples are shown. The regions drawn in the plots were used to calculate granulocytes.

mined by flow cytometry were above 97%. In addition, 3 slightly different protocols (see Materials and methods) were compared for Filter-PBMC preparation yielding similar results for total leukocytes as well as for removal of granulocytes ($n=10$; data not shown).

Compared to standard PBMCs, Filter-PBMCs usually contain more residual granulocytes. The light scattering characteristics of PBMC preparations from FBC and standard buffy coats can be seen in Fig. 4, which displays a representative set of flow cytometric FSC/SSC dot plots. Analysis of 36 mononuclear cell preparations revealed an average of $7.1 \pm 5.7\%$ granulocytes (range: 0.3–22.3%) for Filter-PBMC and $2.1 \pm 2.1\%$ (range: 0.3–11.2%) for standard PBMCs.

To determine the activation state of Filter-PBMCs and to exclude the possibility that the procedure of leukocyte binding to and back-flushing from the filters leads to leukocyte activation we isolated Filter-PBMCs and standard PBMCs, stained them for expression of the activation markers CD69 and CD25 and analyzed them by flow cytometry. The results of one representative experiment ($n=4$) are shown in Table 2. As displayed in the lines labeled "no activation", only a minor fraction of the T cells, B cells, monocytes and NK cells express CD69 or CD25. Notably, most leukocyte populations of Filter-PBMCs express CD69 and CD25 at a similar or lower level compared to standard PBMCs, demonstrating that Filter-PBMCs are not pre-stimulated. Rather in contrast, 2–3 fold higher CD69 expression for T and B cell populations can be observed in standard PBMCs (Table 2; "no activation"). Dot plots for the expression of CD69 and CD25 by $CD3^+$ T cells in Filter-PBMCs and standard

Table 2
Stimulation of Filter-PBMCs and standard PBMCs

		T cells	T helper cells	Cytotoxic T cells	B cells	Monocytes	NK cells
CD69 expression							
Filter-PBMC	No activation	7.6	6.4	15.1	4.8	1.4	4.3
	SEB	33.2	27.5	65.3	97.8	97.0	83.6
Standard PBMC	No activation	18.4	16.9	21.1	13.6	1.3	6.5
	SEB	49.2	42.4	67.8	97.8	99.3	77.5
CD25 expression							
Filter-PBMC	No activation	19.8	25.3	2.9	26.1	2.5	4.6
	SEB	21.6	24.0	13.4	85.0	56.9	33.5
Standard PBMC	No activation	22.8	31.5	3.7	26.1	1.6	5.9
	SEB	24.5	32.3	9.2	84.9	69.3	21.4

Expression of the activation markers CD69 (top) and CD25 (bottom) in Filter-PBMCs and standard PBMCs without activation (freshly isolated) or after stimulation with SEB is shown as % expression of the indicated populations (representative experiment of $n=4$). For the flow cytometric characterization of T cells, B cells, monocytes and NK cells, see Materials and methods.

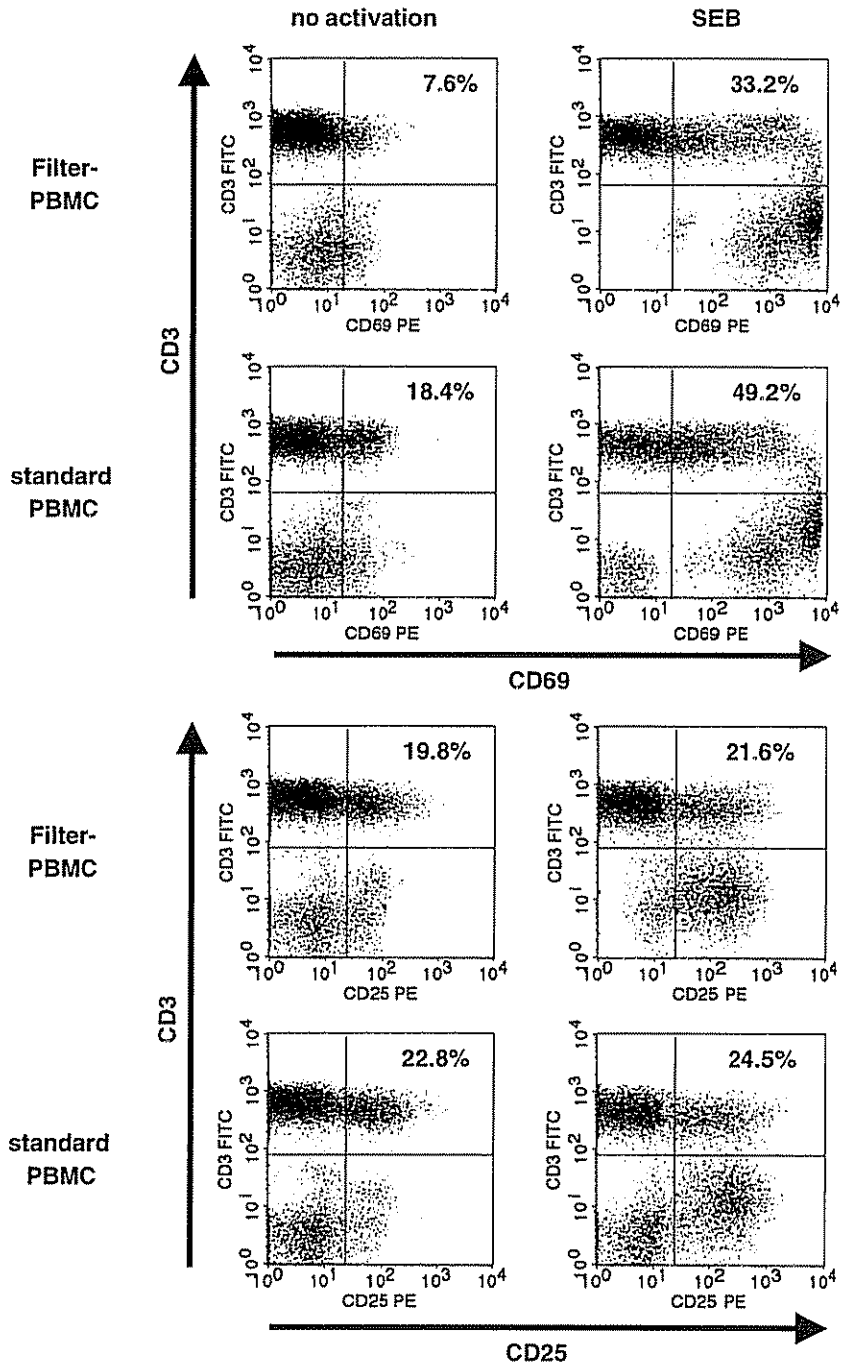

Fig. 5. Expression of CD69 and CD25 by T cells in Filter-PBMCs and standard PBMCs. Representative flow cytometric dot plots are shown for expression of CD69 (top) and CD25 (bottom) by CD3 positive T cells. Filter-PBMCs and standard PBMCs were isolated and analyzed for CD69/CD25 expression prior to activation (left) or after stimulation with SEB (right). Percentages given are % of total $CD3^+$ cells; $n=4$.

PBMCs of a representative experiment are shown in Fig. 5.

We also investigated whether Filter-PBMCs and standard PBMCs increase expression of CD69 and CD25 as a response to stimulation. After overnight incubation with SEB ("SEB"), the levels of CD69 and CD25 expression were determined and compared to the baseline levels ("no activation"). As shown in Table 2, a

higher percentage of lymphocytes and monocytes from both Filter-PBMCs and standard PBMCs express CD69 and to a lesser extent also CD25, demonstrating that Filter-PBMCs can be stimulated comparably to standard PBMCs.

3.5. $CD34^+$ hematopoietic progenitor cells (HPCs)

Subsequently, we determined the concentrations and total amounts of $CD34^+$ hematopoietic progenitor cells (HPCs) in Filter Buffy Coats. The results of flow cytometric analysis (using the ProCount kit, BD Biosciences) of FBCs generated from three different filters are shown in Table 3. Filter Buffy Coats prepared from Compoflex T3908 and Leukoflex LST-1 filters contain similar amounts of HPCs, on average 6.6×10^5 and 4.7×10^5 $CD34^+$ cells per FBC, respectively (Table 3). More HPCs were detected after back-flushing Leukotrap WBF-3 filters, on average 10.8×10^5 $CD34^+$ cells, which is in accordance to the higher concentration of $CD45^+$ cells in these FBCs (Table 3).

3.6. Immunomagnetic cell sorting (MACS)

We analyzed whether FBCs, especially Filter-PBMCs, can be used for the isolation of leukocyte subpopulations by immunomagnetic cell sorting (MACS).

Various leukocyte subpopulations were isolated from Filter-PBMCs by MACS. As shown in Table 4, we performed MACS for three rare cell populations. Isolation of $CD34^+$ HPCs ($n=10$) using the CD34 Progenitor Cell Isolation Kit resulted in purities from 30.6% to 83.0% and yields from 30.5% to 81.9%. Two experiments were performed to isolate $CD133^+$ HPC resulting in purities of 79.0% and 88.5% and yields of 44.1% and 29.4%. The third rare cell marker we used was CD304 (BDCA-4) for the isolation of plasmacytoid dendritic cells (PDC) ($n=9$). We found average purities of 91.2% (range 85.3–95.8%) and average yields of 58.9% (range 45.7–79.5%).

Cell populations with start frequencies above 10%, such as $CD3^+$ T cells, $CD4^+$ T helper cells, $CD8^+$ cytotoxic T cells and $CD19^+$ B cells, were isolated with purities and recoveries well above 90% when separated using the direct magnetic cell labelling strategy with Antibody MicroBeads (for details, see Table 4). Two $CD56^+$ NK cell isolations, performed with CD56 MicroBeads resulted in purities of 96.7% and 97.6% and yields of 64.7% and 71.3%. For the isolation of unmarked T cells, T helper cells and B cells we used the indirect magnetic cell labelling strategy. Using the Pan T Cell Isolation Kit II, the $CD4^+$ T Cell Isolation Kit II, and the B Cell Isolation Kit II, we obtained purities of 95.5% and 97.6% and yields of almost 90% for unmarked $CD3^+$ T cells ($n=2$), more than 90% pure unmarked $CD4^+$ T helper cells ($n=2$) with more than 92% recovery and unmarked B cells ($n=2$) with purities above 85% and recoveries above 95% (Table 4). Representative flow cytometric data for the immunomagnetic isolation of $CD34^+$ HPCs and $CD3^+$ T cells are shown in Fig. 6.

Monocytes were isolated using either CD14 MicroBeads ($n=6$) or the Monocyte Isolation Kit II ($n=2$; for isolation of unmarked monocytes). The start frequencies of monocytes were heterogeneous, ranging from 1.7% to 14.6%.

Two isolations of unmarked monocytes resulted in purities of 70.6% and 74.8% and yields of 80.5% and 85.4%. Using CD14 MicroBeads, we obtained purities from 24.1% to 80.8% and recoveries above 94%. The purity of the isolated monocytes could not be substan-

Table 3
$CD34^+$ hematopoietic stem and progenitor cells in FBCs

Filter type		$CD45^+$/μl	$CD34^+$/μl	Total $CD34^+$ ($\times 10^5$)
Compoflex T3908 ($n=43$)	Average	5284	4.0	6.6
	S.D.	1598	2.3	3.9
	Range	3200–11 000	0.8–10.2	1.3–16.8
Leukoflex LST-1 ($n=26$)	Average	4705	2.9	4.7
	S.D.	1533	1.7	2.7
	Range	2620–10 300	0.2–6.7	0.3–11.1
Leukotrap WBF-3 ($n=21$)	Average	8482	6.5	10.8
	S.D.	2016	3.8	6.3
	Range	4960–12 550	0.6–12.7	1.0–20.9

Results from flow cytometric quantification of $CD45^+$ leukocytes and $CD34^+$ hematopoietic stem and progenitor cells in Filter Buffy Coats, shown as average, standard deviation (S.D.) and range. Filter Buffy Coats derived from three different leukocyte depletion filters were analyzed without prior preparation of mononuclear cells. The average FBC volume is 165 ml. The frequency of $CD34^+$ in normal whole blood was determined as 2.4 ± 1.3 $CD34^+$ cells/μl (average ± S.D.; range 0.8–5.9; $n=28$).

Table 4
MACS isolations of leukocyte populations from Filter-PBMCs

Experiment: isolation of	MACS product	n	Average start frequency (%)	Range start frequency (%)	Average purity (%)	Range purity (%)	Average yield (%)	Range yield (%)
$CD34^+$ HPCs	CD34 Progenitor Cell Isolation Kit; MS Columns	10	0.15	0.08–0.22	59.9	30.6–83.0	52.9	30.5–81.9
$CD133^+$ HPCs	CD133 Cell Isolation Kit MS; Columns	2		0.06 0.31		79.0 88.5		44.1 29.4
Plasmacytoid dendritic cells	CD304 (BDCA-4, Neuropilin) MicroBead Kit; MS Columns	9	0.36	0.24–0.51	91.2	85.3–95.8	58.9	45.7–79.5
$CD3^+$ T cells	CD3 MicroBeads; MS Columns	5	58.9	54.7–64.3	99.6	99.5–99.7	93.4	92.4–94.6
$CD4^+$ T helper cells	CD4 MicroBeads; MS Columns	4	36.6	31.6–41.1	98.4	97.8–98.9	95.0	92.8–96.5
$CD8^+$ cytotoxic T cells	CD8 MicroBeads; MS Columns	4	13.1	58.7–18.8	91.9	86.7–96.9	98.0	97.2–98.8
$CD19^+$ B cells	CD19 MicroBeads; MS Columns	4	11.4	7.0–15.5	97.2	96.0–98.8	93.9	92.4–95.6
$CD56^+$ NK cells	CD56 MicroBeads; MS Columns	2		16.0 18.6		96.7 97.6		64.7 71.3
$CD14^+$ monocytes	CD14 MicroBeads; MS Columns	6	4.7	1.7–7.6	56.6	24.1–80.8	97.8	94.9–100
$CD14^+$ monocytes after $CD15^+$ granulocytes depletion	CD15 MicroBeads, CD14 MicroBeads; LD Columns, MS Columns	2		8.9 7.1		89.9 92.7		70.0 82.8
$CD14^+$ monocytes after $CD16^+$ granulocytes depletion	CD16 MicroBeads, CD14 MicroBeads; LD Columns, MS Columns	1		10.9		92.8		96.1
Untouched $CD3^+$ T cells	Pan T Cell Isolation Kit II; LS Columns	2		62.8 58.0		95.5 97.6		85.6 89.7
Untouched $CD4^+$ T helper cells	$CD4^+$ T Cell Isolation Kit II; LS Columns	2		39.5 33.1		91.8 92.6		92.0 95.9
Untouched B cells	B cell Isolation Kit II; LS Columns	2		11.4 11.8		85.9 87.3		95.8 97.5
Untouched monocytes	Monocyte Isolation Kit II; LS Columns	2		4.9 14.6		70.6 74.8		80.5 85.4

The results of various MACS isolations of leukocyte populations from Filter Buffy Coats after preparation of mononuclear cells are shown. The start frequency of the target cell population as well as the purity and yield after purification are given as average and range except for $n=2$, where both values are given.

tially improved by separation over a second MS Column. As determined by flow cytometry, major contaminations in the positive fraction (isolated monocytes) were $CD16^+$ granulocytes (data not shown). To eliminate contaminating granulocytes, we depleted granulocytes prior to the $CD14^+$ monocyte isolation procedure using CD15 MicroBeads or CD16 MicroBeads and LD columns. When CD15 MicroBeads had been used for

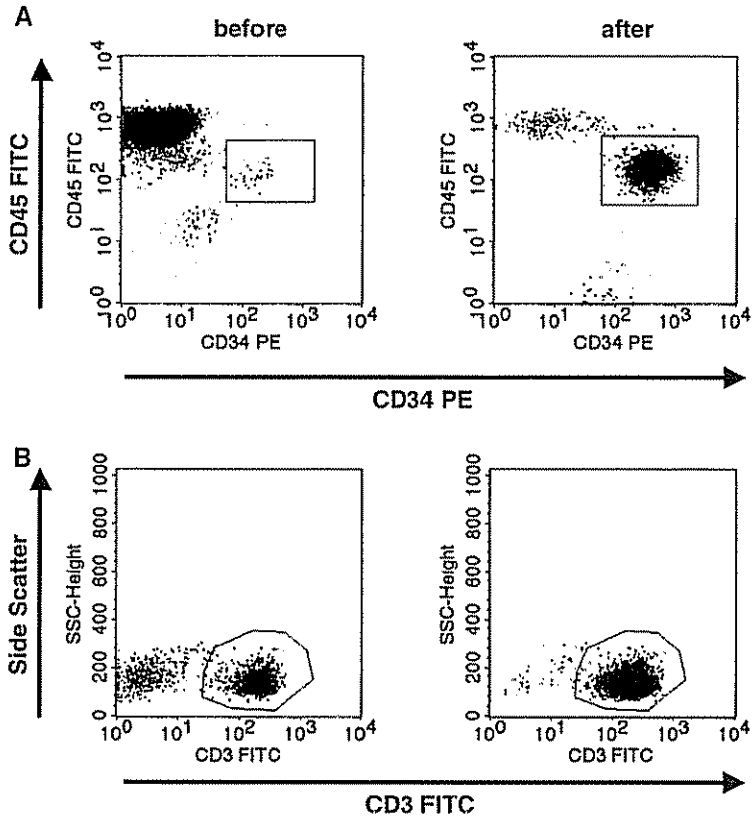

Fig. 6. Isolation of $CD34^+$ and $CD3^+$ cells from Filter-PBMCs. Flow cytometry dot plots are displayed for MACS isolations of $CD34^+$ hematopoietic stem and progenitor cells (A) and $CD3^+$ T lymphocytes (B). Two dot plots are displayed for each isolation, showing Filter-PBMCs (labeled "before") and the purified cell fraction (eluted from separation columns; labeled "after"). Percentages reflect the frequency of the target cell population (% of all viable cells).

depletion of granulocytes the subsequent isolations of monocytes started with frequencies of 8.9% and 7.1%, respectively ($n=2$), and resulted in purities of 89.9% and 92.7% and recoveries of 70.0% and 82.8%. When CD16 MicroBeads had been used for depletion of granulocytes the subsequent isolation of monocytes started with a frequency of 10.9%, and a purity of 92.8% and a recovery of 96.1% was observed (Table 4). However, the depletion step caused loss of monocytes from 14.2% and 25.4%, respectively, when CD15 MicroBeads were used, and 19.9% when CD16 MicroBeads were used (data not shown).

3.7. Functional assays

The functionality of the $CD34^+$ cells detected in FBCs was assessed using standard colony assays. When directly comparing Filter-PBMCs and standard PBMCs from the same donors in colony assays we obtained very comparable results. In Filter-PBMCs about 72% of $CD34^+$ cells gave rise to colonies (717 ± 172 total CFU per 1000 $CD34^+$ cells) compared to 66% (660 ± 172) in standard PBMCs (Table 5A). In addition, colony forming unit-granulocyte/macrophage (CFU-GM), burst forming unit-erythrocyte (BFU-E) and colony forming unit-granulocyte/erythrocyte/macrophage/megakaryocyte (CFU-GEMM) were detected in similar proportions in those two PBMC preparations (for details, see Table 5A).

We also performed colony assays to determine whether $CD34^+$ hematopoietic progenitor cells (HPCs) can still differentiate into hematopoietic lineages after MACS isolation. As shown in Table 5B, about 60% of purified $CD34^+$ cells gave rise to colonies (603 ± 177 total CFU per 1000 $CD34^+$ cells), thus demonstrating that MACS isolation did not significantly reduce the functionality of $CD34^+$ cells present in Filter-PBMCs. The distribution of CFU-GM, BFU-E and CFU-GEMM was similar to the results obtained with $CD34^+$ cells from Filter-PBMCs prior to MACS isolation (Table 5B).

To analyze whether T cells in Filter-PBMCs can be stimulated to produce IFN-γ by the Staphylococcal

Table 5
Functional analysis of $CD34^+$ cells from Filter-PBMCs and standard PBMCs

A

	Filter-PBMC			Standard PBMC		
	Average	S.D.	Range	Average	S.D.	Range
Total CFU/1000 $CD34^+$	717	172	552–973	660	172	427–860
CFU-GM (%)	19	6	10–82	21	9	10–34
BFU-E (%)	72	8	61–82	69	8	59–83
CFU-GEMM (%)	9	5	3–19	10	6	0–18

B

	Filter-PBMC			MACS isolated $CD34^+$ cells		
	Average	S.D.	Range	Average	S.D.	Range
Total CFU/1000 $CD34^+$	702	42	640–744	603	177	466–873
CFU-GM (%)	21	7	17–34	28	5	24–35
BFU-E (%)	71	8	59–80	62	7	55–70
CFU-GEMM (%)	8	4	3–13	10	6	5–18

In standard colony assays, colony forming unit-granulocyte/macrophage (CFU-GM), burst forming unit-erythrocyte (BFU-E) and colony forming unit-granulocyte/erythrocyte/macrophage/megakaryocyte (CFU-GEMM) were counted and the results either displayed as total CFU (the sum of these three colony types) or as % of the total colonies. (A) Data for $CD34^+$ cells in Filter-PBMCs (left) and standard PBMCs (right) are shown ($n=7$) (B) Data for $CD34^+$ cells in MNCs prepared from FBCs (Filter-PBMC) prior to isolation (left) and of MACS isolated $CD34^+$ cells (right) are shown ($n=5$)

Enterotoxin B (SEB), we stimulated with or without 1 μg/ml SEB for 14 h and enumerated IFN-γ-producing $CD4^+$ and $CD8^+$ T cells using a IFN-γ Cytokine Secretion Assay Detection Kit (PE). In the sample without SEB, we found 0.01% IFN-γ-secreting cells among $CD4^+$ lymphocytes and 0.01% IFN-γ-secreting cells among $CD8^+$ lymphocytes. The SEB-stimulated sample contained 0.45% $CD4^+$ IFN-γ^+ cells and 0.37% $CD8^+$ IFN-γ^+ cells. Analysis of a second Filter-PBMC resulted in 0.01% IFN-γ-secreting cells among $CD4^+$ lymphocytes and 0.03% IFN-γ-secreting cells among $CD8^+$ lymphocytes in the nonstimulated sample and 1.59% $CD4^+$ IFN-γ^+ cells and 1.66% $CD8^+$ IFN-γ^+ cells in the SEB-stimulated sample (data not shown).

Finally, we investigated whether plasmacytoid dendritic cells (PDCs) are functional in a mixed lymphocyte reaction (MLR). Therefore, we isolated PDCs from Filter-PBMCs and standard PBMCs (samples Filter-PBMC A and standard PBMC 1; Fig. 7) as well as allogeneic naive $CD4^+$/$CD45RA^+$ T helper cells from a different set of samples (Filter-PBMC B and standard PBMC 2). CpG-oligonucleotide-stimulated PDCs were

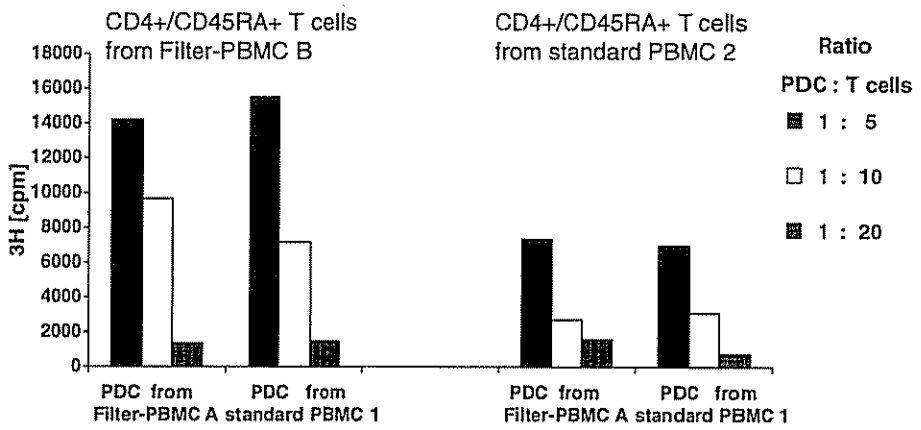

Fig. 7. Mixed Lymphocyte Reaction (MLR). Functionality of PDCs, isolated from Filter-PBMCs or standard PBMCs and $CD4^+$/$CD45RA^+$ T cells, isolated from Filter-PBMCs or standard PBMCs, shown in a mixed lymphocyte reaction. 1×10^4 CpG-oligonucleatide-stimulated PDCs were co-cultured with 5×10^4 (1/5), 1×10^5 (1/10) and 2×10^5 (1/20) allogeneic $CD4^+$/$CD45RA^+$ T cells for 5 days [^{3}H]Thymidine incorporation was measured.

co-cultured with allogeneic $CD4^+/CD45RA^+$ T cells for 5 days. Proliferation of T cells was determined by [^{3}H]Thymidine incorporation. As shown in Fig. 7, PDCs as well as naive $CD4^+/CD45RA^+$ T cells isolated by MACS from Filter-PBMC and from standard PBMC were still functional. T cell proliferation was similar, independent on whether PDCs were derived from Filter-PBMCs or standard PBMCs. T cell proliferation was even higher with $CD4^+/CD45RA^+$ T cells from Filter-PBMCs as compared to T cells derived from standard PBMCs (Fig. 7).

4. Discussion

4.1 Filter Buffy Coats

In this study, we have shown that elution of viable leukocytes of all major subtypes from leukocyte depletion filters is generally possible. Thus, a novel source of leukocytes, which we termed Filter Buffy Coats (FBCs), is available for scientific research and development. Depending on the leukocyte depletion filter used, some FBCs displayed a similar composition of main leukocyte subsets when compared to standard buffy coats. Other FBCs contained high percentages of lymphocytes combined with reduced fractions of granulocytes (see Fig. 1). We believe that the reduction of granulocytes in FBCs observed in those filters is due to disintegration of parts of the granulocytes after adhesion to the filters, which in consequence can no longer be isolated from the filters. In order to optimize elution of leukocytes from filters, adhesion effects of the cells to the filter fibers had to be overcome. The mechanisms of cellular adhesion on various polymer fibers are not very well understood (Bruil et al., 1995). One adhesion mechanism responsible for leukocyte retention was studied by Barbe et al. (2000), using leukocyte cell fractions from venous blood bound to filter surfaces. These investigators could identify certain adhesion molecules of the integrin family (e.g. the CD11b/CD18 and CD11c/CD18 integrin α/β dimers) as being responsible for firm contact to artificial surfaces such as filter fibers. Barbe et al. (2001) also discovered that leukocyte subtypes revealed different retention characteristics on different polymer surfaces.

Filter characteristics such as surface charge can influence leukocyte adhesion: some filters are chemically modified with amine, carbonyl or hydroxyl groups leading to differences in surface charge and cell adhesion (Bruil et al., 1995). We observed differences in leukocyte content and distribution of leukocyte subpopulations when we compared FBCs generated from four different filters. With Compoflex T3908 and Optipure RZ 2000 filters, the relative recoveries for lymphocytes and monocytes were somewhat higher (compared to standard buffy coats) than those for granulocytes. These relative proportions were reversed when using Leukotrap WBF-3 filters, and finally, when using Leukoflex LST-1 filters to generate FBCs, about equal recoveries for mononuclear cells and granulocytes (compared to standard buffy coats) were observed (see Fig. 1). The total amounts of leukocytes were very similar for FBCs prepared from T3908, RZ 2000 and LST-1 filters, representing about 55% of the total leukocytes found in standard buffy coats. FBCs prepared from WBF-3 filters contained higher amounts of total leukocytes and granulocytes, but not of lymphocytes (Table 1). Thus, we believe that the higher content of total leukocytes in FBCs prepared from WBF-3 filters was mainly due to granulocytes.

Despite the differences observed, Filter Buffy Coats prepared from basically all of these filters turned out to be a valuable leukocyte source which can replace standard buffy coats. Even though the total leukocyte recovery in Filter Buffy Coats appears to be rather low compared to whole blood and also lower than in standard buffy coats, the relative recoveries for the mononuclear leukocytes (lymphocytes and monocytes), which are mainly used for research, are higher than for total leukocytes (Table 1). Nevertheless, the total amounts of lymphocytes are higher in standard buffy coats than in FBCs.

By exploring the kinetics of filter elution, we determined that fast elution of leukocytes from the leukocyte depletion filters resulted in higher recoveries (Fig. 3). After 4–5 h post filtration leukocytes seem to be bound too strongly to the filter fibers to allow elution, thus leading to major cell loss. The requirement of fast back-flushing implies that elution should be carried out on the blood bank site as soon as possible in order to achieve optimal cell recovery and viability. Changes in the filter elution medium, e.g. replacing single compounds such as sugars or polymers, did not significantly influence the compositions of leukocyte subsets or total cell recoveries in the Filter Buffy Coats.

Although leukocytes can generally be retrieved from leukocyte depletion filters only very few laboratories have utilized this cell source to date. Weitkamp and Crowe (2001) have isolated human B lymphocytes by back-flushing RCM-1 filters (Pall) and collected approximately 1.5×10^8 mononuclear cells (MNCs) after density gradient separation of 90 ml of back-flushing

medium composed of PBS containing 5 mM EDTA. Filter elution was performed less than 8 h after filtration with intermediate storage of the filters at 4 °C. In comparison, we prepared our Filter Buffy Coats from various filters using 150 ml filter elution medium at room temperature within 4 h. In these FBCs, we detected from 10 to 16×10^8 total leukocytes containing an estimated $4.6–6.8 \times 10^8$ mononuclear cells (calculated from Table 1; without actually performing a density gradient mononuclear cell preparation). When preparing MNCs from Compoflex T3908 FBCs ($n=21$) we obtained $3.8 \pm 1.4 \times 10^8$ MNCs, an amount that exceeds the numbers obtained by Weitkamp and Crowe (2001) more than two times

Wu et al. and Vyas et al. utilized a whole blood Pall filter to generate $CD4^+$ enriched cell fractions. They used 300 ml of PBS to back-flush leukocyte depletion filters and compared the results with standard buffy coats. Average yields of MNCs from standard buffy coats were 8.7×10^8 and from filters 2.6×10^8 (Wu et al.; Vyas et al.). Similar amounts of MNCs ($2.7 \pm 1.2 \times 10^8$) were isolated from Leukotrap filters by Longley and Stewart (1989). We obtained an average yield of $8.5 \pm 3.0 \times 10^8$ leukocytes from standard buffy coats (standard PBMCs) and $3.8 \pm 1.4 \times 10^8$ from FBCs (Filter-PBMCs) ($n=21$). We believe that the reason we obtained higher cell yields from our FBCs was due to the composition of our filter elution medium. When we performed filter elution experiments using PBS without supplements as elution medium we observed significantly lower leukocyte yields as well (data not shown). Ebner et al. (2001) differentiated human dendritic cells from leukocyte suspensions generated by back-flushing Leukotrap WBF-2 leukocyte depletion filters (Pall), an older type than we used in our experiments. The total mononuclear cell yields in this study (1.1×10^9) were higher compared to our results (6.8×10^8) as calculated from Leukotrap WBF-3 data in Table 1. Comparing monocyte concentrations in Filter-PBMCs, Ebner et al. detected an average of 6.2% monocytes in the mononuclear cell fraction, which agrees well with our data (data not shown). In standard buffy coat preparations, however, Ebner et al. (2001) detected significantly lower amounts of leukocytes compared to the leukocyte suspensions eluted from filters whereas we usually detected more leukocytes in standard buffy coats than in FBCs.

4.2. $CD34^+$ hematopoietic progenitor cells (HPCs)

$CD34^+$ hematopoietic progenitor cells (HPCs) are not very abundant in normal (i.e. non-mobilized) peripheral blood, so we did not expect to find significant numbers of those cells in Filter Buffy Coats. Nevertheless, we were able to detect $CD34^+$ progenitor cells in Filter Buffy Coats in numbers that are sufficient for scientific purposes ($0.5–1.1 \times 10^6$, see Table 3). The differing amounts of $CD34^+$ cells detected in the different FBCs analyzed reflects donor variation as well as differences in mononuclear cell recoveries obtained with these filters (see Table 1). Leukotrap WBF-3 FBCs, in which the highest numbers of HPCs were detected also generally contained the highest amounts of MNCs, the opposite being the case for Leukoflex LST-1 FBCs with the lowest numbers of MNCs and HPCs (see Tables 1 and 3). We determined an average number of 2.4 ± 1.3 $CD34^+$ cells/μl in the peripheral blood of normal healthy donors, which is supported by Fukuda et al. (1998) and Lee et al. (2000). Assuming a volume of 500 ml, we calculated that about 1.2×10^6 $CD34^+$ cells are present per whole blood unit. Thus, we obtained rather high recoveries for HPCs in FBC (Table 3).

4.3. Immunomagnetic cell sorting (MACS)

Using different MACS immunomagnetic cell isolation kits, we successfully isolated a variety of leukocyte cell populations from Filter-PBMCs including lymphocytes (B cells, T cells and T cell subpopulations), NK cells, dendritic cells and hematopoietic progenitor cells ($CD34^+$, $CD133^+$). The purities as well as yields for these isolations from Filter-PBMCs were comparable to those obtained using standard PBMCs (Penna et al., 2002; Finney et al., 2004, Wang et al., 2004, Wagner et al., 2004), thus demonstrating that FBCs can serve as a valuable alternative to standard buffy coats as leukocyte source. The isolation of monocytes from Filter-PBMCs resulted in good recoveries, but rather poor purities compared to the isolation of monocytes from standard PBMCs. Modifications to the isolation protocol (e.g. using a successive second column) or isolation of unmarked monocytes using an indirect monocyte isolation kit did not significantly improve purities of isolated monocytes. Most likely, the rather low purities were due to lower starting frequencies of monocytes in Filter-PBMCs as compared to standard PBMCs. Furthermore, the preparation of Filter-PBMCs was not as efficient as the preparation of standard PBMCs regarding removal of granulocytes (Fig. 4). There is additional evidence that protein in the filter elution medium could improve the frequencies of monocytes in FBCs as well as in Filter-PBMCs. Immunomagnetic depletion of contaminating granulocytes effectively

removed granulocytes and resulted in improved purities of subsequent monocyte isolations.

4.4. Functional assays

Experiments using colony assays revealed that the $CD34^+$ cells that were detected in FBCs are functional, yielding comparable results for Filter-PBMCs and standard PBMCs in respect to total colonies and colony distribution (Table 5). In addition, immunomagnetic isolation of $CD34^+$ cells did not significantly impair the functionality of the $CD34^+$ cells in this assay (Table 5). Further experiments are under way to elicit more detailed functional properties of $CD34^+$ HPCs.

To determine the activation state as well as the stimulation potential of Filter-PBMCs in comparison to standard PBMCs, we determined the expression of the lymphocyte activation markers CD69 and CD25 before and after stimulation with SEB (Staphylococcal Enterotoxin B) employing a flow cytometry assay (Maino et al., 1995; Caruso et al., 1997; McLeod et al., 1998). Freshly isolated Filter-PBMCs express CD69 and CD25 at low levels, demonstrating that the filtration and elution steps during preparation of FBC do not lead to leukocyte activation (Table 2). We found differences between Filter-PBMCs and standard PBMCs concerning the level of CD69 expression in T- and B cells, without knowing the reason for this observation nor whether this is statistically significant. A possibly explanation could be that pre-activated T cells and B cells are bound irreversibly to the filters. After stimulation with the superantigen SEB, the lymphocyte populations as well as the monocytes responded well with a comparable increase of CD69 and CD29 expression in Filter-PBMCs and standard PBMCs (Table 2, Fig. 5), indicating that these populations remain functional during preparation of MNCs.

Functionality of PDCs from Filter-PBMCs was shown in an allogeneic MLR. No differences were found between PDCs derived from Filter-PBMCs and PDCs derived from standard PBMCs in their ability to stimulate T cells. Notably, in our experiment the proliferation of T cells derived from Filter-PBMCs was higher than the proliferation of T cells derived from standard PBMCs (Fig. 7).

In conclusion, our results revealed that Filter Buffy Coats are a valuable source for white blood cells, containing all major subsets of leukocytes in normal distributions. Successful immunomagnetic isolation of various leukocytes as well as the functional characterization of some cell types was demonstrated.

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References

Barbe, I. L., Boval, B. M., Wautier, M. P., Wautier, J. L., 2000. Human-promyelocytic cell line: a convenient tool for studying the molecular basis of WBC filtration. Transfusion 40, 1250.

Barbe, I. L., Boval, B. M., Wautier, M. P., Wautier, J. L., 2001. An in vitro system for testing leucocyte and leukaemic cell line adhesion to synthetic fibres. Br. J. Haematol. 115, 664.

Begemann, M., 1999. Praktische Hämatologie. Klinik–Therapie–Methodik. Georg Thieme, Stuttgart, Germany.

British Committee for Standards in Haematology, 1998. Blood Transfusion Task Force: guidelines on the clinical use of leucocyte-depleted blood components. Transfus. Med. 8, 59.

Bruil, A., Beugeling, T., Feijen, J., van Aken, G., 1995. The mechanisms of leukocyte removal by filtration. Transfus. Med. Rev. 9, 145.

Caruso, A., Licenziati, S., Corulli, M., Canaris, A. D., De Francesco, M. A., Fiorentini, S., Peroni, L., Fallacara, F., Dima, F., Balsari, A., Turano, A., 1997. Flow cytometric analysis of activation markers on stimulated T cells and their correlation with cell proliferation. Cytometry 27, 71.

Dzik, S., 1996. Leukoreduced blood components: laboratory and clinical aspects. Principles of Transfusion Medicine, (Second ed.)William & Wilkins, USA, p. 353.

Ebner, S., Neyer, S., Hofer, S., Nussbaumer, W., Romani, N., Heufler, C., 2001. Generation of large numbers of human dendritic cells from whole passaged through leukocyte removal filters: an alternative to standard buffy coats. J. Immunol. Methods 252, 93.

Finney, H. M., Akbar, A. N., Lawson, A. D. G., 2004. Activation of resting human primary T cells with chimeric receptors: costimulation from CD28, inducible costimulator, CD134, and CD137 in series with signals from the TCR_ζ chain. J. Immunol. 172, 104.

Fukuda, J., Kaneko, T., Egashira, M., Oshimi, K., 1998. Direct measurement for CD34+ blood stem cell absolute counts by flow cytometry. Stem Cells 16, 294.

Goldman, M., Delage, G., 1995. The role of leukodepletion in the control of transfusion-transmitted disease. Transfus. Med. Rev. 9, 9.

Lee, S., Im, S.-A., Yoo, E.-S., Nam, E.-M., Lee, M.-A., Ahn, J.-Y., Huh, J.-W., Kim, M.-J., Lee, S.-J., Chung, W.-S., Seong, C.-M., 2000. Mobilization kinetics of CD34+ cells in association with modulation of CD44 and CD31 expression during continuous intravenous administration of G-CSF in normal donors. Stem Cells 18, 281.

Longley, R. E., Stewart, D., 1989. Recovery of functional human lymphocytes from Leukotrap filters. J. Immunol. Methods 121, 33.

Maino, V. C., Suni, M. A., Ruitenberg, J. J., 1995. Rapid flow cytometric method for measuring lymphocyte subset activation. Cytometry 20, 127.

McLeod, J. D., Walker, L. S. K., Patel, Y. I., Boulougouris, G., Sansom, D. M., 1998. Activation of human T cells with superantigen

(Staphylococcal Enterotoxin B) and CD28 confers resistance to apoptosis via CD95. J. Immunol. Methods 160, 2072.

Mishell, B.B., Shiigi, S.M. (Eds.), 1980. Selected Methods in Cellular Immunology. Freeman, San Francisco.

Penna, G., Vulcano, M., Roncari, A., Facchetti, F., Sozziani, S., Adorini, L., 2002. Cutting edge: differential chemokine production by myeloid and plasmacytoid dendritic cells. J. Immunol. 169, 6673.

Seghatchian, J., 2003. Universal leucodepletion: an overview of some unresolved issues and the highlights of lessons learned. Transfus. Apher. Sci. 29, 105.

Vyas, G.N., Wu, C.R., Gandhi, M. Whole inactivated virus vaccine using pooled primary isolates of plasma-borne wildtype HIV-1 propagated in PBMN cell–substrate. Poster. Aids Research Institute, University of California, San Francisco. Internet-link: http://ari.ucsf.edu/pdf/posters/vyas_uarp.pdf.

Wagner, M., Poeck, H., Jahrsdoerfer, B., Rothenfusser, S., Prell, D., Bohle, B., Tuma, E., Giese, T., Ellwart, J.W., Endres, S., Hartmann, G., 2004. IL-12p70-dependent Th1 induction by human B cells requires combined activation with CD40 ligand and CpG DNA. J. Immunol. 172, 954.

Wang, X., Li, N., Liu, B., Sun, H., Chen, T., Li, H., Qui, J., Zhang, L., Wan, T., Cao, X., 2004. A novel human phosphatidylethanolamine-binding protein resists tumor necrosis factor α-induced apoptosis by inhibiting mitogen-activated protein kinase pathway activation and phosphatidylethanolamine externalization. J. Biol. Chem. 279, 45855.

Weitkamp, J.H., Crowe Jr., J.E., 2001. Blood donor leukocyte reduction filters as a source of human B lymphocytes. BioTechniques 31, 464.

Wu, C.R., Evans, C., Vyas, G.N. Enhanced production of primary isolates of HIV-1 in CD4-enriched cell substrates. Poster #192A, Aids Research Institute, University of California, San Francisco. Internet-link: http://ari.ucsf.edu/pdf/posters/wu.pdf.