

Identification of Circulating Endothelial Cells in Peripheral Blood

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Abstract: The Circulating Endothelial Cells (CECs) and their activated and resting subsets (aCECs and rCECs) represent extremely rare cell population that play important roles in vascular pathophysiology. Their number and function are modulated in several diseases involving vascular injury such as human tumors. Although, consensus on the phenotypic definition of endothelial cells and on the optimal enumeration technique is lacking, the number of clinical studies that are based on the assessment of endothelial cells the types of analytical methods that are employed are rapidly expanding. The goal of the current study was to develop a rapid and sensitive flow cytometric method to quantify and characterize CECs (their subsets and the apoptotic fraction of cells. In total, 75 peripheral blood samples were collected from normal donors and were analyzed with a six-color flow cytometric technique for the simultaneous analysis of the cell phenotypes of CECs and circulating progenitors cells using the following monoclonal antibodies: CD146, CD34, CD45, CD106 and CD133. In addition, the samples were analyzed with using the gating strategy. Apoptotic CECs and dead cells were detected using Annexin V and 7-amino-actinomycin D staining, respectively. The results show that the described technique is a reliable tool to increase the knowledge of endothelial cell biology and can be easily applied to the study of many pathological conditions.

Key words: Circulating endothelial cells, flow cytometric technique, monoclonal antibodies, apoptosis, reliable tool, Saudi Arabia

INTRODUCTION

The Endothelial cells participate in numerous functions of vascular physiology (Esmon, 1987; Brenner *et al.*, 1989; Pober and Cotran, 1990). Blood Circulating Endothelial Cells (CECs) including their resting and activated subsets (rCECs and aCECs) and Circulating Endothelial Progenitors cells (CEPs) represent two cell populations that play important roles in tissue visualization (Esmon, 1987). Many factors such as cytokines alter the surface of endothelial cells and thereby modulate the role of the endothelium in coagulation inflammation, vaso regulation and adhesion (Rodgers, 1988; Pober, 1988; Favalaro, 1993; Hebbel and Vercellotti, 1997).

CECs probably originate from vessels walls and have limited growth capability whereas CEPs are thought to originate from the bone marrow and to have a proliferative potential (Strijbos *et al.*, 2008). CECs and CEPs are have been recently currently studied as alternative biomarkers in patients with several pathological conditions including sickle cell anemia (Sowemimo-Coker *et al.*, 1989; George *et al.*, 1992) and other conditions associated with

vascular injury such as those caused by cytomegalovirus infection (Grefte *et al.*, 1993; Percivalle *et al.*, 1993), rickettsial infection (George *et al.*, 1993), myocardial infarction (Hladovec *et al.*, 1978) intravascular instrumentation (Sbarbati *et al.*, 1991) and cancer (Esmon, 1987).

CECs and CEPs exist at a low frequency and their phenotypes have not been standardized. However, the number of clinical studies that assessing them is rapidly expanding (Duda *et al.*, 2007). Different approaches have been used to detect CECs such as the use of *in vitro* cultures, magnetic bead separation and fluorescence microscopy as well as immunocytochemistry and flow cytometry (Goon *et al.*, 2006; Mancuso *et al.*, 2001; Bull *et al.*, 2003; Del Papa *et al.*, 2004; Nakatani *et al.*, 2003). Each approach has specific capabilities and limitations. Flow Cytometry (FCM) is an accepted tool for the immunophenotyping of CECs and CEPs (Khan *et al.*, 2005) and this technique offers several advantages (e.g., endothelial microparticles and platelets can be excluded from evaluation) using a multiparameter approach. Nevertheless, FCM-based techniques that are performed using whole blood risk overestimating the number of

CECs by enumerating false-positive cells. The possibility of false-positive cells necessitates the use of blocking serum to inhibit non-specific binding or specific binding via Fc receptors. Furthermore, dead cells another major source of non-specific staining by Monoclonal Antibodies (MoAbs). For this reason, the use of real-time vitality stains such as propidium iodide or 7-amino-actinomycin D is crucial for the identification of dead cells and for their exclusion from the gating strategy (Khan *et al.*, 2005). Similarly, an increased fraction of apoptotic endothelial cells has been reported *in vitro* and *ex vivo* in patients with ischemic heart disease (El-Solh *et al.*, 2007) and in cancer patients undergoing antiangiogenic treatments (Bertolini *et al.*, 2007; Bertolini, 2008). The aim of the current study was as to develop a reliable six-color FCM method that could be used *in vivo* to simultaneously characterize the cell surface phenotypes and to quantitate the apoptotics of CECs in the peripheral blood.

MATERIALS AND METHODS

Study population: The current study was conducted using 75 adult healthy subjects (35 males, 40 females), aged 18-35 years (median age of 23 years). Each donor provided written informed consent before entering the study and the experimental trial was performed after donor approval.

Reagents: The following mouse anti-human MoAbs that were directly conjugated were used: Fluorescein othiocyanate (FITC)-labeled anti-CD106, (BD PharmingenTM, USA), R-Phycoerythrin (R-PE)-labeled anti-CD146 (BD PharmingenTM, USA) phycoerythrin-Cyanin 7 (PE/Cy7)-labeled anti-CD34, (BD Biosciences, USA); allophycocyanin (APC)-labeled anti-CD133, (Miltenyi Biotec GmbH, Germany) and allophycocyanin-Cyanin 7 (APC/Cy7)-labeled anti-CD45 (BD Biosciences, USA).

The Annexin V Kit (Bender MedSystems, Boehringer Ingelheim) was used to quantify apoptotic CECs and contained human Annexin V FITC-conjugated and a binding buffer solution. The viability of the cells was detected by adding 7-amino-actinomycin D (7-AAD Viability Dye; Beckman Coulter, USA). The emission of 7-AAD was detected at 655 nm filter (Table 1). The samples were lysed Auto Lyse PLUS (BioSource Europe SA, Belgium) and washed with Dulbecco's phosphate-buffered saline (D-PBS) (EuroClone, Italy). Fc-receptor blocking reagent and mouse serum (Sigma-Aldrich, USA) were used to block non-specific antibody binding.

Table 1: Reagents employed for the analysis of the cell surface phenotypes and the apoptosis of CECs with their subsets and CEPs

Parameters	Fluorochrome	Clone	Isotype	Vendor	Volume
CD34	PE/Cy7	8G12	IgG 1	BD Biosciences	5
CD45	APC/Cy7	2d1	IgG 1	BD Biosciences	5
CD133	APC	AC133	IgG 1	BD Biosciences	5
CD106	FITC	51-10C9	IgG 1	BD Biosciences	5
CD146	PE	P1H12	IgG 1	BD Biosciences	5
Annexin V Kit	FITC			Bender	5
7-AAD	-			MedSystems	
				Beckman Coulter	20

Staining procedure: Peripheral blood was collected into 4 mL Vacutainer tubes (Becton Dickinson, Switzerland) containing liquid tripotassium Ethylene Diamine Tetra-Acetic Acid (K3EDTA) and processed immediately.

Non-specific antibody binding was blocked using 20 μ L of Fc-Receptor blocking reagent and 200 μ L of mouse serum for 20 min at Room Temperature (RT) before incubating the sample with conjugated antibodies. Anticoagulated venous whole blood was aliquoted in 100 μ L amounts using two 12 \times 75 mm polypropylene tubes (Becton Dickinson Labware, Franklin Lake, NJ, USA). After gentle mixing, the samples were incubated with the appropriate fluorochrome-conjugated MoAbs at the manufacturers' recommended concentrations for 15 min at RT in the dark.

We used the following MoAbs panel: FITC-labeled anti-CD106, R-PE-labeled anti-CD146, PE/Cy7-labelled anti-CD34, APC-labeled anti-CD133 and APC/Cy7-labeled anti-CD45 for the first tube and R-PE-labeled anti-CD146, PE/Cy7-labeled anti-CD34, APC-labeled anti-CD133 and APC/Cy7-labeled anti-CD45 for the second tube.

Stained whole blood samples were subjected to red blood cells lysis with 2 mL of lysis buffer in each tube for 10 min at RT in the dark. A total of 20 μ L of 7-AAD were added into the first tube which was then and incubated for 15 min at RT in the dark.

The sample was measured within 15 min the flow cytometer. The second tube was centrifuged at 500 \times g for 10 min using a refrigerated centrifuge (Minifuge RF, Heraeus Sepatech) at 20°C to re-pellet the cells. The cells were then washed with PBS and the tube was centrifuged again.

The pellet was resuspended in 400 μ L of binding buffer containing 5 μ L of FITC-conjugated Annexin V. The sample was incubated for 10 min at RT in the dark and washed again with the binding buffer. Finally, 20 μ L of 7-AAD was added for 15 min at RT in the dark and the sample was analyzed within 15 min (Table 2).

Table 2: A summary of the panel that was used to analyze cell surface phenotypes and apoptosis of CEPs with their subsets and CEPs

Parameters	FITC	PE	PerCP	PE/Cy7	APC	APC/Cy7
Tube 1	CD106	CD146	7-AAD	CD34	CD133	CD45
Tube 2	ANNEX V	CD146	7-AAD	CD34	CD133	CD45

Flow cytometric analysis: Six-color flow cytometric analyses were performed using a BD FACSCanto™ (BD Biosciences, San Jose, California, USA) that was equipped with: blue 488 nm solid state laser and a red 633 nm HeNe laser.

The fluorescence was detected using six photomultiplier tube detectors. Four wavelength ranges were detected using the 488 nm laser: 750-810 nm (PE/Cy7), >670 nm (PerCP/Cy5-5), 564-606 nm (PE) and 515-545 nm (FITC). Two wavelength ranges were detected using the 633 nm laser: 750-810 nm (APC/Cy7) and 650-670 nm (APC). Forward scatter was detected a photodiode with a 488/10 band pass filter and side scatter was detected using a photomultiplier tube with a 488/10 band pass filter. Because CECs and their progenitors are rare events in peripheral blood, the instrument was prepared by an extensive cleaning process (>60 min) of the sequential tubes using different solutions before data acquisition (Khan *et al.*, 2005).

The analyses were standardized by performing the following quality control checks: Flow-Check microbeads (Beckman Coulter, Fullerton, CA, USA) which were used to check the stability of the optical and fluidic systems and BD 7-color Setup Beads which were used to standardize the light scatter and fluorescence intensity. The cytosetting of the instrument was performed using anti-CD8-FITC, -PE, -PerCP/Cy5-5, -PE/Cy7, -APC and -APC/Cy7 (BD Biosciences, San Jose, California, USA) yielding the following results: Forward Scatter (FS) at 71 V, Side Scatter (SS) at 397 V, FITC at 432 V, PE at 455 V, PerCP/Cy5-5 at 681 V, PPE/Cy7 at 545 V, APC at 589 V and APC/Cy7 at 615 V.

The color compensation values were obtained using the automatic compensation control system of the cytometer. The threshold was applied to forward scatter and the fluorescence data were displayed using a four-decade log scale. Flow cytometric data were analyzed using BD FACSDiva software. The sensitivity and the stability of the cell counts were tested using the international quality controls that were purchased from the United Kingdom National External Quality Assessment Scheme (UK NEQAS LI, Sheffield, UK) (Whitby *et al.*, 2002).

In addition, the whole blood preparation procedures were monitored daily and the MoAb reactivity was tested

using Immuno-Trol (Beckman Coulter, Fullerton, CA, USA) control cells. Gating strategies and whole blood analysis were as follows: adequate background exclusion criteria were defined, the electronic gate of the nucleated cell population was set to exclude platelets, dead cells and debris and an electronic gate was applied in the CD45 versus Side Scatter (SS) dot plot using CD45-negative events to exclude hematopoietic cells (Fig. 1a and b; Fig. 2a and b).

Mature CECs were defined as CD45-, CD146+, CD34+ and CD133-. The rCECs were identified as CD45-, CD146+, CD34+, CD106- and CD133-.

The CECs were defined as CD45-, CD146+, CD34+, CD106+ and CD133-. Circulating endothelial progenitor cells were identified as CD45-, CD34+, CD146+ and CD133+. Finally, apoptotic CECs (CECsAnnV+) were defined as CD45-, CD146+, CD34+, CD133- and Annexin V+. As shown in Fig. 1 and 2 (g and e), dot plot windows were generated to identify of mature CECs (g) and CEPs (e).

The levels of rCECs and aCECs (Fig. 1h) as well as apoptotic CECs (Fig. 2h) were determined. For the detection of extremely rare events such as CECs and CEPs, 5×10^5 - 1×10^6 cellular events were acquired from 0.1-0.2 mL of whole blood. The acquisition rate did not exceed 2000 events sec^{-1} .

The sample analysis was considered informative if an adequate number of events (at least 1500-2000 cells) were collected in the CEC and CEP enumeration gates (31). Cells that were labeled with FITC-, PE-, Per-CP/Cy5-5-, PE/Cy7-, APC- and APC/Cy7-conjugated MoAbs which were non-reactive to human cells were used as a control to determine the background fluorescence.

Statistical analysis: Statistical analysis was performed using the statistical package Microsoft Office Excel 2003. The median value of the percentage and of the absolute number of CECs, CEPs and CECsAnnV+ were calculated with their range distribution.

To test the assay reproducibility, 75 parallel samples from one collection of whole blood samples were measured in one run after separate staining. These replicates were processed and acquired by the same technician.

A good reproducibility was obtained in all populations with a Coefficient of Variation (CV) of approximately 15%. Furthermore, to assess the reliability, seven replicates from peripheral whole blood samples from a healthy individual were processed and acquired at four different times (0, 2, 6 and 24 h after the initial

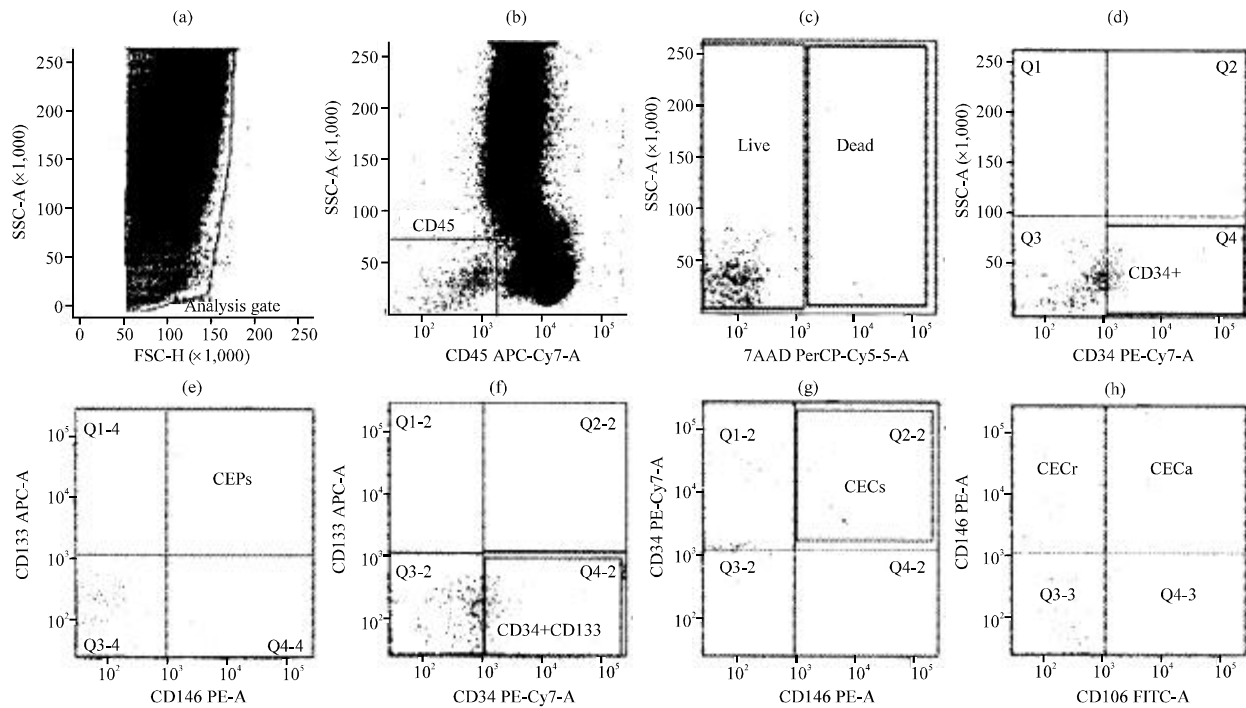


Fig. 1: Flow cytometric dot plot panels show representative pictures according to the method that was utilized for the identification of CECs their subsets (rCECs and aCECs) and CEPs. Panel (a) shows the gating analysis that was used to exclude platelets and debris. Panel (b) shows the gating analysis that was used to exclude hematopoietic cells expressing the CD45 antigen which were detected using APC/Cy7-labeled anti-CD45 versus Side Scatter (SS). Panel (c) shows the gating analysis that was used to distinguish live from dead cells. Panel (g) indicates the expression of the antigens that were used to evaluate CECs using PE-labeled anti-CD146 versus PE/Cy7-labeled anti-CD34. This population was derived from the gating analysis that was restricted to the CD133-negative population using PE/Cy7-labeled anti-CD34 versus APC-labeled anti-CD133 (Panel f). Panel (e) shows CEPs that were detected using APC-labeled anti-CD133 versus PE-labeled anti-CD146. This population was derived from the gating analysis that was restricted to a CD34-positive population which was detected using PE/Cy7-labeled anti-CD34 versus SS (Panel d). 254×190 mm (96×96 DPI)

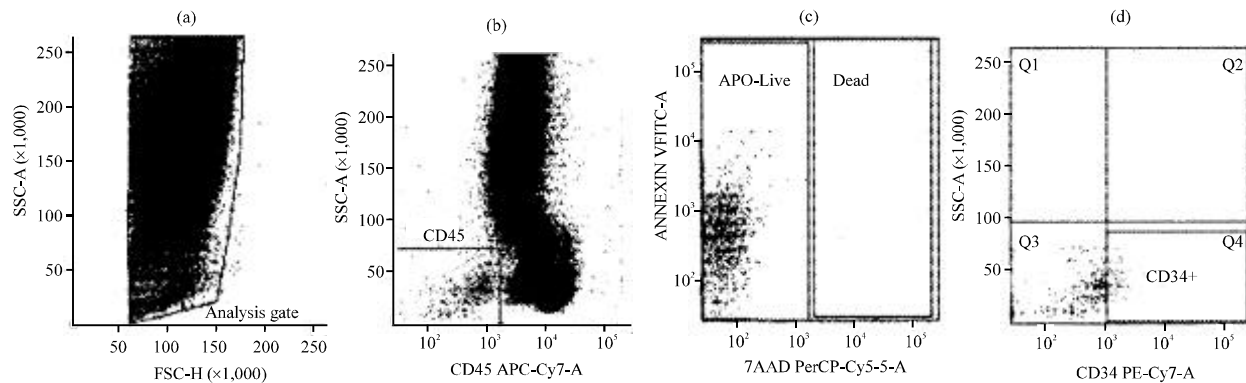


Fig. 2: Continue

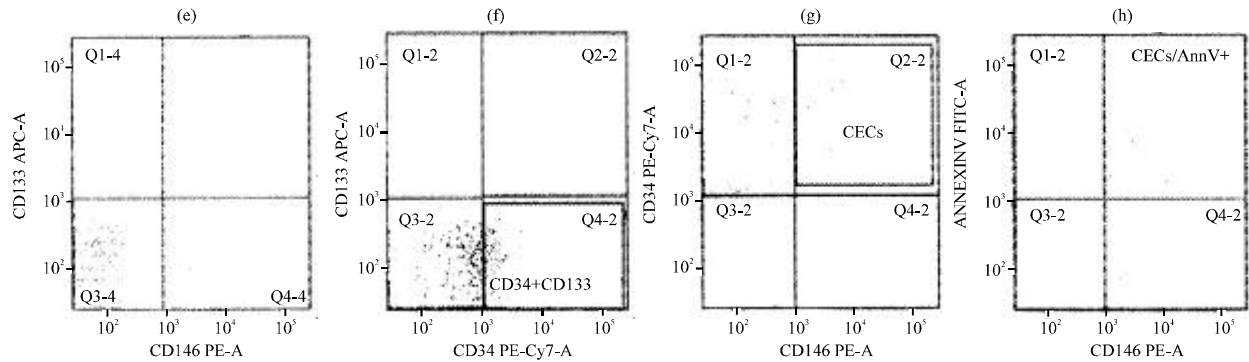


Fig. 2: Flow cytometric dot plot panels showing representative pictures relative to the method that was utilized for the identification of CECs with their apoptotic subset and CEPs. a shows the gating analysis that was used to exclude platelets and debris. b shows the gating analysis that was used to exclude hematopoietic cells expressing the CD45 antigen which were detected using APC/Cy7-labeled anti-CD45 versus Side Scatter (SS). c indicates the gating analysis that was used to distinguish apoptotic or live cells from dead cells. g shows the expression of antigens that were used to evaluate CECs which were detected using PE-labeled anti-CD146 versus PE/Cy7-labeled anti-CD34. This population was derived from the gating analysis that was restricted to the CD133-negative population which was detected using PE/Cy7-labeled anti-CD34 versus APC-labeled anti-CD133. f. h shows CECsAnnV+ cells which were detected using FITC-labeled Annexin V versus PE-labeled anti-CD146. This population was derived from the gating analysis that was restricted to CEC population g. e shows CEPs that were detected using the PE-labeled anti-CD146 versus APC-labeled anti-CD133. This population was derived from the gating analysis that was restricted to a CD34-positive population which was detected using PE/Cy7-labeled anti-CD34 versus SS. d. 254×190 mm (96×96 DPI)

assessment) by a staff member who was trained in flow cytometry. The samples required evaluation within 2 h to prevent cell death.

RESULTS AND DISCUSSION

The results obtained using these methods are shown in Fig. 1 and 2. As shown in Fig. 1, the data obtained by staining the sample with the following reagents: anti-CD146-PE, anti-CD34-PE/Cy7, anti-CD45-APC/Cy7, anti-CD106-FITC, anti-CD133-APC and 7-AAD. We designed the gating analysis to exclude platelets and debris (Fig. 1a), to select CD45-negative cells to exclude hematopoietic cells (Fig. 1b), to distinguish live from dead cells (Fig. 1c) and to view CECs their subsets (Fig. 1f-h) and CEPs (Fig. 1d, e). As shown in Fig. 2, the data were obtained by staining the sample with the following reagents: anti-CD146-PE, anti-CD34-PE/Cy7, anti-CD45-APC/Cy7, anti-CD133-APC, Annexin V-FITC and 7-AAD. Dedicated gating analyses were employed to exclude platelets and debris (Fig. 1a), to select CD45-negative cells for the exclusion of hematopoietic cells (Fig. 2b), to distinguish apoptotic or live cells from dead cells (Fig. 2c) and to view CECs the apoptotic fraction of CECs (Fig. 2f-h) and CEPs (Fig. 2d and e). The current

study obtained the following median percentage values from the analysis of the healthy population: 0.00021% (range, of 0-0.00075%) for mature CECs; 0.00020% (range of, 0-0.00045%) for rCECs; 0.00011% (range, of 0-0.00049%) for aCECs; 0.0000026% (range, of 0-0.000012%) for CEPs and 0.00028% (range, of 0-0.00059%) for CECs^{AnnV+}.

We determined the CECs and CEPs from the absolute number of white blood cells which were provided by the hematological analyzer (Coulter, Miami, USA). In addition, we calculated the percentages of CECs, CEC subsets and CEPs using FCM and the absolute number of the CECsAnnV+ cells. Therefore, the median values of the absolute numbers were the following: 20 cells mL⁻¹ (range of, 0-40 cells) for mature CECs; 12 cells mL⁻¹ (range of, 0-30 cells) for rCECs; 9 cells mL⁻¹ (range of, 0-30 cells) for aCECs; 1 cell mL⁻¹ (range of, 0-2 cells) for CEPs and 10 cells mL⁻¹ (range of, 0-30 cells) for CECs^{AnnV+}.

CONCLUSION

A critical issue for the study of CECs and CEPs is to provide a clear definition of these cells coupled with a reliable and reproducible method of detection. Flow cytometry offers the advantage of being a rapid and

accessible technique with the availability of multiple markers and the possibility of distinguishing various CEC subsets (e.g., activated or apoptotic CECs) (Korbling *et al.*, 2006; Mariucci *et al.*, 2010) and/or CEPs using a small blood volume (Jacques *et al.*, 2008; Redondo *et al.*, 2008). Because CECs and CEPs have very low frequencies, their detection, enumeration and functional study are subject to technical difficulties. Therefore, the analysis of this rare event must compensate for the background noise which may cause false-positive results (Van Craenenbroeck *et al.*, 2008). The conventional approach to this problem was adequately discussed by Khan *et al.* (2005).

Briefly, the following objects require attention: preparing the flow cytometer using an intensive cleaning process (≥ 60 min) using several markers to positively define the rare event and minimizing the potential for non specific binding of antibodies to non-relevant cells by pre-treating samples with blocking serum before antibody incubation. Contextually, dead cells may act as a potential source of non-specific antibody binding which suggests that a viability stain in addition to the blocking serum should be used to distinguish between live and dead cells (Barnett and Reilly, 2001).

The collection of a large number of events is necessary to identify adequate numbers of rare events (Khan *et al.*, 2005). We determined that the optimal results were obtained using a number between 5×10^5 - 1×10^6 cells and at least 1500-2000 events which were collected from the gated regions of CECs or CEPs. The protocol described herein required a small volume of whole blood without any enrichment procedures and was performed using a six-color flow cytometer. The analysis took approximately 120 min. This method simultaneously allowed us to determine the phenotype of CECs (with their resting and activated subsets) and CEPs and characterize the apoptotic fraction of CECs (and eventually CEPs) appropriate conditions for the gating analysis. Due to the extremely low CEP concentration in healthy subjects, we did not perform the analysis of the apoptotic fraction of CEPs in the samples from the healthy subjects.

Taking into account the recommendations listed above, the simultaneous analysis of the cell surface phenotypes of CECs (with their subsets and the apoptotic fraction) and CEPs using the multiparametric approach may be utilized in a clinical setting, to further the understanding of the role of these cells in many pathological conditions (Wirezbwska *et al.*, 2008; Go *et al.*, 2008).

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