Flow cytometric detection of circulating dendritic cells in healthy subjects

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Dendritic cells (DCs) are the key antigen-presenting cells controlling the initiation of the T cell- dependent immune response. Currently, two peripheral blood DC subsets have been identified on the basis of their CD11c expression. The CD11c-negative (CD11c⁻) DCs (expressing high levels of CD123) are designated as lymphoid-derived DCs (DC2), whereas the CD11c⁺/CD123⁻ cells, do identify the myeloid-derived DCs (DC1). A growing number of studies have been conducted in recent years on both the quantitative and functional alterations of DCs and their subsets in different pathological conditions. In the present study we assessed, using two different flow cytometric (FCM) techniques, the normal profile of blood DCs in 50 italian adult healthy subjects (M/F: 25/25, median age 42.5 years, range 20-65). The percentage and the absolute number of DCs and their subsets, were obtained starting from whole blood samples in two ways: 1) by calculating the number of DCs when gated as lineage-negative/ HLA-DR⁺ and identifing the two subsets as CD11c⁺ (DC1) and CD123⁺ (DC2) and 2) by using three specific markers: BDCA.1 (CD11c⁺ high/CD123⁺ low, myeloid DCs); BDCA.2 (CD11c-/ CD123⁺high, lymphoid DCs); BDCA.3 (CD11c⁺low /CD123⁻, myeloid DCs). Six parameters, 4-color FCM analysis were perfomed with a BD FACSCanto equipment. The mean values of the percentage and of the absolute number were: 0.5±0.2% and 30±11 cells/μL for DCs; 0.2±0.1% and 15±6 cells/μL for DC1; 0.2±0.1% and 15±7 cells/μL for DC2. The same values were: 0.2±0.1% and 16±7 cells/μL for BDCA.1; 0.2±0.1% and 12±7 cells/μL for BDCA.2; 0.02±0.01% and 2±1 cells/μL for BDCA.3, respectively. Our study confirmes that the two types of FCM analysis are able to identify the DC population. We also provides the first reference values on normal rates and counts of blood DCs in italian adult healthy subjects.

Key words: Circulating dendritic cells, flow Cytometry, normal reference values.

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Dendritic cells (DCs), are specialized and co-stimulatory cells which play an important role in the induction of the cellular immune response (Hart, 1997). These cells can be found in many tissues of the human body, as an heterogeneous population of specialized presenting cells, circulating via the blood to most of the tissue (Steinmann, 1973). Many studies revealed that DCs play a pivotal role in the induction of T cell responses resulting in cell-mediated immunity and that they are also able to initiate and regulate T- and B- lymphocyte responses, to activate these cells, and to tolerize T cells to self-antigens (Ho, 2001; Lukacs-Kornek, 2008; Shurin, 2007; Sozzani, 2007; Thurnher, 2007; Wilczynski, 2008).

Two peripheral blood DC subsets (DC1 and DC2) have been described (Liu, 2001, a; MacDonald, 2002). The CD11c-negative (CD11c⁻) DCs, which usually express high levels of CD123 (CD123⁺), are designated lymphoid-derived DCs, whereas the CD11c-positive (CD11c⁺) DC subset generally expresses low levels of CD123 (CD123⁻) and represents the myeloid-derived DCs (Shortman, 2002). Both subsets express high level of HLA-DR (HLA-DR⁺) and lack the lineage markers CD3, CD14, CD16, CD19, CD20 and CD56, several functional differences between CD11c⁺/CD123⁻ and CD11c⁻/ CD123⁺ DCs have been described (Ardavin, 2001). While both the DC1 and DC2 subset induce strong proliferation of naïve CD4⁺ cells, upon interaction with T cells, the DC1 subset predominantly prime a T helper type 1 (Th1) cell antimicrobial response, the DC2 subset seems to support the generation of a T helper type 2 cell (Th2) response (Rissoan, 1999; Grouard, 1997).

Flow cytometric analysis of circulating DCs and their subsets has proven a useful tool in both experimental and clinical studies on several pathological conditions such as: autoimmunity, immunodeficiency, infections, transplantation and, more recently, haematological and solid tumors.

Recently, we have demonstrated that high-dose cyclophosphamide followed by G-CSF (utilized as a mobilization regimen for CD34$^+$ cells into the PB for autologous transplantation procedures) induces a reversed DC1/DC2 ratio without a significant increase of blood DCs (Ferrari, 2003). We also did not observe any significant changes in the circulating DC pool in advanced breast cancer patients after different schedules of standard-dose chemotherapy as well as during immunotherapy with trastuzumab in HER2$^+$-patient subset. We have also shown that both DCs and their subset distribution, are comparable in both early stage and advanced breast cancer patients before starting CT, while, in this latter group, we observed a significant decrease of the DC1 subset after the completion of the CT program (Ferrari, 2005). In this field, an important issue for future studies comes from the fact that, due to both the different of potential markers of DCs and the technological advances in FCM equipments, there is not any universally accepted standard method to determine their number and immunophenotype; furthermore, there is still a limited knowledge of the number and distribution of blood DCs in the healthy populations (Narbutt, 2004).

The aims of the present study are: 1) to compare two FCM approaches for the analysis of blood DCs, and 2) to provide reference data on normal rates and counts of these cells in a representative italian healthy population.

Materials and Methods

Study population

A total of 50 adult healthy subjects (25 M, 25 F), with a median age of 42.5 years (range: 20-65 years), were examined. Each volunteer gave written informed consent before entry into the study and the experimental trial was conducted after the approval of our local Ethical Committee. The characteristics of the study population are showed in Table 1.

Selection criteria were based on a prospective written survey to disqualify donors associated with a range of known risk categories and on a retrospective serological testing to exclude donors with prior exposure to a range of known patogens. The principal selection criteria of the study population are listed in Table 2.

Monoclonal antibodies (MoAbs) for DCs analysis

The following mouse anti-human MoAbs directly conjugated were used: fluorescein isothiocyanate (FITC)-labelled anti-CD3 (SK7), -CD16 (B73.1), -CD19 (SJ25C1), -CD20 (L27); -CD14 (M5E2), -CD34 (8G12) , -CD56 (NCAM 16.2), -HLA-DR (L243) (BD Bioscences, San Jose, California, USA) and -CD11b (Bear 1, Coulter Immunotech); allophycocyanin (APC)-labelled anti-HLA-DR, (BD Bioscences); phycoerthrin (PE)-labelled anti-CD19 (SJ25C1), anti-CD11c, -CD123 (BD Bioscences); peridinim chlorophyll (PerCP)-labelled anti-CD45 (2D1) (BD Bioscences); FITC-labelled anti CD1c (BDCA.1, AD58E7), -BDCA.2 (AC144) and PE-labelled anti-BDCA.3 (AD5-14H12), (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), (Table 3a and Table 3b).

Multi-color staining and flow cytometric analysis for DCs

Dendritic cells are known to be detected as MCH class II-expressing cells (HLA-DR$^+$) and lacking of T cell (CD3,CD11b), B cell (CD19, CD20), NK (CD16, CD56), macrophage/monocyte (CD14), and hematopoietic progenitor cell (CD34) markers, so they were defined as lineage-negative (lin-neg)

Table 1. Characteristics of the studied population.

Number of studies healthy subjects	50
Gender (F/M)	25/25
Median age/range (years)	42,5/20-65
Median number of WBC (cells/µL)	6447
Median number of lymphocytes (cells/µL)	2236

Table 2. Summary of main selection criteria of the studied population.

Minimun body weight of 50 Kg
Human Immunodeficiency Virus- and Hepatitis B, C - negative
Not pregnant for 12 months prior to blood donation
No medical or dental treatment for 72 hours prior to blood donation
No fever >38°C for 4 weeks h prior to blood donation
No use of medication for 4 weeks prior to blood donation
No needle-stick injury, acupuncture, tattooing, body-piercing or for 12 months prior to blood donation
No blood transfusion for 12 months h prior to blood donation
No history of high blood pressure, heart attack, shortness of breath, stroke, or unconsciousness
No history of atopic disorders
No family member with diagnosed Creutzfeldt-Jacobs disease
No residence in the UK or >6 months during the period 1980-1986
No travel to malaria endemic regions for 6 months prior to blood donation
No history of Malaria, Leishmanias, Borreliosis, Brucellosis or Toxoplasmosis
No contact with persons exhibiting symptoms of infectious disease for 4 weeks prior to blood donation

cells. We identified dendritic cell subsets as lin-neg /CD11c+ (DC1) and lin-neg/CD123+ (DC2) or using three specific markers: BDCA.1 (CD11c-high/ CD123-low myeloid DCs in the blood); BDCA.2 (CD11c-neg/CD123-high lymphoid blood DCs) and BDCA.3 (CD11c-low/CD123− myeloid DCs) (Dzionek, 2000; Demedts, 2005; Patterson, 2005; Pilichowska, 2007; Tsoumakidou, 2006; Zaba, 2007; Pinzon-Charry, 2005).

For both the techniques, peripheral blood was collected into a 4 mL Vacutainer (Becton Dickinson, Basel, Switzerland) tube containing liquid tri-potassium ethylene diamine tetra-acetic acid (K_3EDTA) as an anticoagulant and processed within 4 h of collection. All the procedures utilized in this study are conformed to the Helsinki Declaration of 1975 (Anonymous, 2002). Anticoagulated venous whole blood was aliquoted in 100 µL amounts into 12x75 mm polypropilene tubes (Becton Dickinson Labware, Franklin Lake, NJ, USA) and after gently mixed, incubated with the appropriate fluorochrome-conjugated MoAbs at the manifacture recommended concentration for 15 min at room temperature (RT) in the dark. Stained whole blood samples were subjected to red blood cells lysis with 2 mL lysing solution (Auto Lyse BD Biosource, Europe SA.) vortexed and incubated for 15 min at RT in the dark. Preparated samples were stored at RT in the dark and analysed within 1 h.

Table 3a. Monoclonal antibodies employed for the *lineage-negative* analytical procedure.

Antigen	Fluorochrome	Clone	Isotype	Vendor	Volume (µL)
CD3	FITC	SK7	IgG1	BD Biosciences	20
CD11b	FITC	Bear 1	IgM	Coulter Immunotech	10
CD11c	PE	B-ly6	IgG1	BD Biosciences	20
CD14	FITC	M5E2	IgG2a	BD Biosciences	20
CD16	FITC	B73.1	IgG1	BD Biosciences	20
CD19	FITC	SJ25C1	IgG1	BD Biosciences	20
CD20	FITC	L27	IgG1	BD Biosciences	20
CD34	FITC	8G12	IgG1	BD Biosciences	20
CD45	PerCP	2D1	IgG1	BD Biosciences	20
CD56	FITC	NCAM 16.2	IgG2b	BD Biosciences	20
CD123	PE	9F5	IgG1	BD Biosciences	20
HLA-DR	APC	L243	IgG2a	BD Biosciences	5

Table 3b. Monoclonal antibodies employed for the analytical approach based on specific markers.

Antigen	Fluorochrome	Clone	Isotype	Vendor	Volume (µL)
CD19	PE	SJ25C1	IgG1	BD Biosciences	20
CD45	PerCP	2D1	IgG1	BD Biosciences	20
CD123	PE	9F5	IgG1	BD Biosciences	20
HLA-DR	FITC	L243	IgG2a	BD Biosciences	20
HLA-DR	APC	L243	IgG2a	BD Biosciences	5
BDCA.1	FITC	AD58E7	IgG2a	Myltenyi Biotec	5
BDCA.2	FITC	AC144	IgG1	Myltenyi Biotec	5
BDCA.3	PE	AD5-14H12	IgG1	Myltenyi Biotec	5

Evaluation of nucleated cells from whole blood specimens was performed using a BD FACSCanto flow cytometer (BD Biosciences, San Jose, CA, USA) instrument, with identical set up parameters between samples. Flow cytometry data was analysed using BD FACSDiva software.

Cells labelled with PE-, FITC-, Per-CP- and APC-conjugated isotype MoAbs that were non-reactive to human cells were used as a control to determine the fluorescence background.

A range of internal quality assurance procedures were employed, including daily calibration of the optical alignment and fluidic stability of the instrumentation (by using 7-color SetUp Beads, BD Biosciences). The sensitivity and the stability of cell count was tested using international quality controls purchased from United Kingdom National External Quality Assessment Scheme (UK NEQAS LI, Sheffield, UK) (Whitby, 2002) and daily monitoring of whole-blood preparation procedures and MoAbs reactivity using Immuno-Trol (Beckman Coulter, Fullerton, CA, USA) control cells.

Statistical analysis

Statistical analysis was performed using Microsoft Excel software. The mean ± standard deviation of the percentage and of the absolute number of DCs and their subsets were calculated, togheter with their range distribution. Student's t-test was used to compare the DC population identified by the two described FCM approaches. Correlations were considered significant with *p*-value <0.05.

Results

Gating strategy for DCs assay

Gating strategies and whole blood sample analysis were as follow: the threshold was set on PerCP fluorescence (FL3) to reduce debris; a gate for leukocyte analysis region was defined in a dot plot of CD45 *versus* side scatter (SSC). Dendritic cell subpopulations were easily distinguished from other cells. For rare events analysis of DC, at least

100000 cellular events were acquired from 0.1 mL of whole blood.

These events were displayed in a lin-neg-FITC *versus* HLA-DR-APC dot plot in which a second gate was created to identify *lineage-negative* cells (DCs). In the *lineage-negative* procedure DC1 and DC2 subsets were identified as lin-neg / HLA-DR$^+$/ CD11c$^+$ and lin-neg / HLA-DR$^+$/CD123$^+$, respectively, after gating on *lineage-negative* cells. The gating strategy utilized for definition of CD11c or CD123 on lin-neg/HLA-DR$^+$ cells was determined as shown in Figure 1 (panel a).

In the second approach based on specific markers, the DC subsets were identified directly from three specific markers: BDCA.1-FITC, BDCA.2-FITC and BDCA.3-PE. The gating strategy utilized for definition of BDCA subsets was determined as shown in Figure 2 (panel a).

Dendritic cells and DC subsets identified by the lineage-negative procedure

The absolute DC number was derived from the total white blood cells count as calculated on an haematological analyser (Coulter, Miami, USA) and the percentage of DC subsets as determined by FCM.

Dot plot panels utilized for definition of CD11c or CD123 on lin-neg/HLA-DR$^+$ cells are shown in Figure 1 (panels b,c,d) .

In our healthy population, the mean values of the percentage and of the absolute number were: 0.5±0.2% and 30±11 cells/µL for DCs; 0.2± 0.1% and 15±6 cells/µL for DC1; 0.2±0.1% and 15±7 cells/µL for DC2. We also calculated the DC1/DC2 ratio obtaining as mean value of the percentage 1.1±0.5 (Table 4).

Dendritic cell subsets identified by specific markers

The expression of BDCA.1, BDCA.2 and BDCA.3 subsets was determined, as shown in Figure 2 (panels b,c,d). The mean values of the percentage and of the absolute number were: 0.2±0.1% and 16±7 cells/µL for BDCA.1; 0.2±0.1% and 12±7 cells/µL for BDCA.2; 0.02±0.01% and 2±1 cells/µL for BDCA.3 (Table 4b).

Comparison between DC populations identified by the two different approaches

No statistically significant difference was found between the sum of the DC1+DC2 cell populations versus the sum of the BDCA.1+BDCA.2+BDCA.3 cell populations.

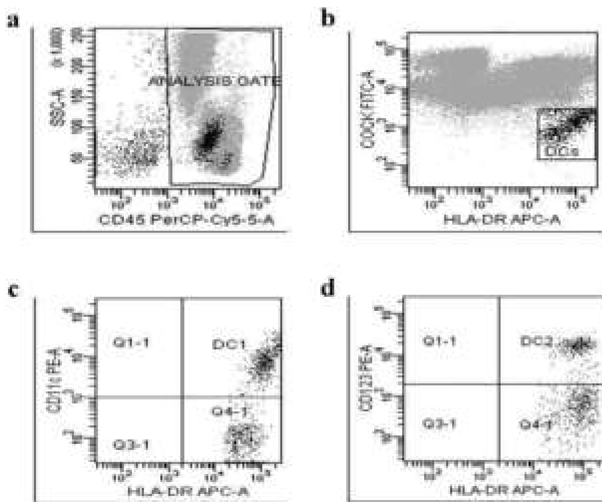

Figure 1. Flow cytometric dot plot panels show representative data relative to the method utilized for the identification of circulating dendritic cells (DC) and DC subsets, with the lineage-negative analytical procedure. Panel a shows leukocyte analysis region applied to PerCP- labelled anti-CD45 versus side scatter (SS) data acquired for exsclusion of debris. Panel b shows the same identified cell population after labelling with a cocktail of FITC-conjugated MoAbs recognizing the lin-neg associated antigens (see Table 3a) and APC-labelled anti-HLA-DR MoAb. Panels c and d show the DC subsets identified from HLA-DR-APC and CD11c-PE for DC1 and HLA-DR-APC and CD123-PE for DC2.

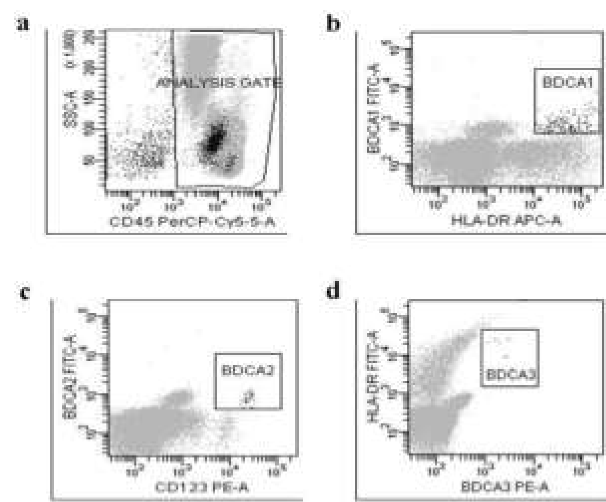

Figure 2. Flow cytometric dot plot panels showing representative data relative to the analytical method for identification of DC subsets by specific markers starting from peripheral blood nucleated cells. Panel a shows the leukocyte analysis region applied to PerCP- labelled anti-CD45 versus side scatter (SS) data acquired for exsclusion of debris. Panel b shows BDCA.1 (CD11c-high/CD123-low myeloid DCs) expression. Panel c and Panel d show BDCA.2 (CD11c-neg/CD123-high lymphoid blood DCs) and BDCA.3 (CD11c-low/CD123 neg myeloid DCs) expression, respectively.

Table 4a. Mean values of percentage and absolute number of dendritic cells (DCs), DC subsets and their ratio (DC1/DC2) in healthy subjects.

DCs	*DC1*	*DC2*	*DC1/DC2 (ratio)*
0.5±0.2%	0.2±0.1%	0.2±0.1%	1.1±0.5%
30±11 cells/μL	15±6 cells/μL	15±7 cells/μL	-

Table 4b. Mean values of percentage and absolute number of DC subsets (identified by BDCA markers) in healthy subjects.

BDCA.1	*BDCA.2*	*BDCA.3*
0.2±0.1%	0.2±0.1%	0.002±0.002%
16±7 cells/μL	13±7 cells/μL	2±1 cells/μL

The sum of the mean values of the BDCA.1+ BDCA.2+BDCA.3-positive cells ranged from 0.2 to 0.62% and, in absolute numbers from 16 to 46 cells/μL, while the sum of the DC1 + DC2 cell populations ranged from 0.2 to 0.61% and from 17 to 43 cell/μL.

The corresponding mean values of the percentage and of the absolute number were 0.42±0.22% and 31±15 cells/μL for the BDCA.1+BDCA.2+ BDCA.3, identified subsets. The same values were 0.40±0.20% and 30±13 cells/μL for the DC1+ DC2 subsets.

Discussion

The importance of DCs in the initiation and control of innate and adaptative immune response is well documented. Dendritic cells are specialized for uptake, processing and presentation of antigens to T cells, thus activating and modulating key immune responses. Even if the continuous replenishement of any DC pool by blood monocytes (which are established circulating precursors for DCs) in the steady state remains to be established (Tacke, 2006), it is well known that, in the peripheral blood, DCs cells are present in the immature state and have the capability to internalize different protein antigens through various endocytic mechanisms and subsequently, subject them to endosomal processing. (Banchereau, 2000; Liu, 2001, b; Thurnher, 2007). Upon activation in the presence of inflammatory mediators, DCs undergo to a process of maturation and acquire the capacity to migrate into secondary draining lymph nodes and tissues, where they become efficient in presenting antigens to naïve T lymphocytes. This process is associated with the upregulation of co-stimolatory molecules and the secretion of cytokines that polarize the T cell-mediate immunity to a Th1 or Th2 response (Jung, 1993).

In recent years, there has been an increased interest in the study of peripheral blood DCs and their role (still not completely clarified) in the dynamics of T-cell priming that could be utilized also for practical approaches, for example in vaccine design, cancer therapy and treatment of inflammatory diseases (Henrickson, 2007).

These studies have been facilitated by important technical improvements in the blood DC enumeration methods available (Vuckovic, 2004; Servet, 2002).

By using FCM-based techniques, circulating dendritic cells were first shown to be decreased in HIV-infection (Grassi, 1999; Donaghy, 2001; Finke, 2004), and more recently, in other viral infectious diseases (Pichyangkul, 2003), whereas increased DC counts were found in acute influenza infection (Coates, 2003). Functional and quantitative variations were also described as a function of age, in surgical and physical stress (Ho, 2001) as well as in advanced breast cancer (Ferrari, 2003; Ferrari, 2005), in B-lineage acute lymphoblastic leukaemia and in other oncological conditions (Mami, 2004; Della Porta, 2005).

In our experience, the ability to enumerate DCs and their subsets, togheter with the study of their function, directly in the PB mononucleated cells of cancer patients, has provided broader understanding of the role of these cells in tumor immunology as well as of their potential applications in the clinical setting (Ferrari, 2005).

No single specific marker, that could be used to exactly identify all the DCs was available until recently. Therefore, a combination of MoAbs, the so called *lineage cocktail* has been used to easilty identify lin-neg/HLA-DR+ DCs by FCM in PB.

Lin-neg/HLA-DR+ DCs account for 0.1 to 2% of the peripheral blood nucleated cells in healthy individuals and most of these cells express either CD11c or CD123 antigens. A panel of MoAbs that identify three presumably novel blood DC antigens has been recently, tested: BDCA.1, which identifies CD11c-high/CD123-low myeloid DCs; BDCA.2 for CD11c-neg/CD123-high lymphoid DCs and BDCA.3 for CD11c-low/CD123-neg myeloid DCs).

As recently performed on patients with breast cancer (Ferrari, 2005) and on patients affected by coeliac disease (Ciccocioppo, 2007), our present study on healthy subjects was performed using multicolor FCM after staining the whole PB with: 1) a cocktail of lineage-specific MoAbs and 2) with the above mentioned new specific MoAbs.

It is known that BDCA.1(also known as CD1c)-positive cells show CD11c-high and CD123-low expression, that BDCA.2-positive cells show CD123-high expression and that BDCA.3-positive cells show CD11c-low expression. With this expression profile, each one of the BDCA markers (1, 2 or 3) clearly do not identify exactly and exclusively DC1 or DC2 cell populations, because they react with only one part of the DC1 or of the DC2 cell populations.

Taking into account the above mentioned point, we confirmed that the two analytical methods are able to identify the DC population. Both the FCM approaches we utilized are rapid and sensitive. They allow the determination of circulating DCs and their subsets in a relatively small blood volume so that they can be used in the clinical setting as an additional indicator of the patients' immunocompetence in different pathological conditions.

We also provided the first data about the normal values of blood DCs in adult healthy italian population. This may be helpful in the early detection of underlying immune alterations and it is crucial for creating a range of reference value for comparative clinical studies.

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