



A Method for Directly Determining the Number of Dendritic Cells and for Evaluation of Their Function in Small Amounts of Human Peripheral Blood

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Abstract. Bone marrow-derived dendritic cells (DC) are highly potent antigen-presenting cells (APC) capable of initiating primary responses of naive T lymphocytes to antigen. Studies on DC in disease have been impeded by the lack of a defined method for accurate DC counting and for evaluation of their function in a small amount of blood. In order to detect and enumerate DC in whole peripheral blood preparations, we applied a direct two-color immunofluorescence method. Blood from healthy donors was stained with a mixture of fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (mAbs) recognizing lineage-associated molecules (CD3, CD14, CD16, CD20, CD57) and phycoerythrin (PE)-conjugated anti-HLA-DR mAb. DC were identified as lineage marker negative (lin⁻), HLA-DR highly positive cells. The mean percentage of these cells present in peripheral blood leukocytes (PBL) was 0.54%, and the mean absolute DC count was $31.4 \times 10^6/l$ of blood. DC stained directly in whole blood were heterogeneous with regard to their expression of CD2 and CD4 molecules, and did not express CD80 and CD83 molecules. Expression of CD80 and CD83 on DC was induced following a multistep isolation procedure, including overnight culture. We demonstrated a significant primary proliferative response to keyhole limpet hemocyanin (KLH) in cultures of peripheral blood mononuclear cells (PBMNC). Since primary proliferative response to neoantigens is entirely dependent on DC as APC, the cultures of unseparated PBMNC stimulated with KLH can be used to evaluate DC function in a relatively simple test. This test does not require previous isolation of DC and T lymphocytes and, therefore, can be performed on a small amount of blood. The elaborated flow cytometric method of DC counting in blood and the proliferative test of DC-dependent primary response to neoantigen are currently being applied in an ongoing study on the effect of chemotherapy on DC number and function in cancer patients.

Key words: dendritic cells; dendritic cell phenotyping; primary proliferative response.

Abbreviations used: DC – dendritic cells, FITC – fluorescein isothiocyanate, PE – phycoerythrin, PBL – peripheral blood leukocytes, KLH – keyhole limpet hemocyanin, PBMNC – peripheral blood mononuclear cells, APC – antigen-presenting cells, PBS – phosphate-buffered saline, lin – lineage.

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Introduction

Bone marrow-derived dendritic cells (DC) are highly efficient antigen-presenting cells (APC) distributed in low numbers in various tissues (ref.¹⁰). DC induce both primary and secondary response to antigen. The capability to prime naive T cells with an antigen is a unique feature of DC^{11, 13}, whereas the response of memory T cells to recall antigen can be triggered also by other professional APC, i.e. macrophages or B cells.

Human DC are not readily accessible for study, since DC circulating in the blood constitute only a minor subpopulation of leukocytes. Peripheral blood DC content estimated by extrapolation from the yield of DC obtained after a multistep purification procedure was below 1% of PBMNC. Peripheral blood DC, characterized following a complex isolation procedure, strongly express HLA-DR class II molecules and do not express markers of the monocyte, lymphocyte T and B, NK cell or granulocyte lineages^{9, 14, 25}, and no marker uniquely expressed on human peripheral blood DC has been described to date. DC isolated from peripheral blood are functionally mature, as they elicit strong allogeneic and autologous MLR^{4, 14, 25} and sensitize naive T cells to nominal antigens *in vitro*^{16, 17}. DC pulsed with tumor-associated antigen and reinfused stimulate anti-tumor immunity *in vivo*^{12, 18}.

A relatively large amount of blood has to be processed to obtain a sufficient number of isolated DC for any cytofluorographic analysis and for functional studies. Since complex methods of DC separation are not applicable to study DC in patients, there is little information on DC deficiencies in disease. Such information may be especially valuable for prospective use of DC as a natural adjuvant in attempts to develop anti-tumor immunity.

To elaborate a quantitative method to identify and count DC in whole peripheral blood, we have used a direct two-color immunofluorescence method based on the strong HLA-DR expression and the lack of lineage-associated molecules on the surface of these cells. In addition, we have verified the possibility of using the evaluation of proliferative responses to keyhole limpet hemocyanin (KLH) for functional assessment of circulating DC. The addition of IL-2 and IL-4 at small concentrations enhances the response of T lymphocytes to KLH¹⁷, but does not induce proliferation of DC-deprived T lymphocytes in the absence of antigen (unpublished data).

Materials and Methods

Cell preparations. Peripheral blood was obtained from healthy adult donors. Freshly drawn blood sam-

ples (7 ml) were collected into Lithium heparin Vacutainer tubes (Becton-Dickinson). Buffy coats processed from blood collected in ACD were purchased from a blood bank.

PBMNC were isolated by Ficoll-Uropline gradient centrifugation¹. Cell fractions enriched in DC, monocytes and T lymphocytes were isolated by gradient centrifugation steps including Percoll (Pharmacia LKB, Sweden) and Nycoprep (Nycomed Pharma AS, Oslo, Norway) gradient as previously described^{14, 16}. This isolation procedure included overnight culture in teflon vessels.

Monoclonal antibodies and immuno-labeling. Fluorochrome-conjugated mAbs: anti-CD3 FITC, anti-CD14 FITC, anti-Leu-11a (CD16) FITC, anti-CD20 FITC, anti-Leu-7 (CD57) FITC, anti-CD2 FITC, anti-Leu-3a (CD4) FITC, anti-HLA-DR FITC, anti-HLA-DQ FITC, anti-HLA-DR PE, anti-CD80 PE and anti-HPCA-2 (CD34) PE were purchased from Becton-Dickinson. Anti-CD83 PE mAb was purchased from Immunotech. Mouse IgG₁ and IgG_{2a} controls FITC and PE-conjugated were purchased from Becton-Dickinson.

To identify DC, whole blood or diluted buffy coat samples were stained with a mixture of FITC-conjugated mAbs recognizing CD3, CD14, CD16, CD20 and CD57 lineage-associated molecules (lin cocktail) and anti-HLA-DR PE mAb. Buffy coat samples were diluted to a concentration of 10×10^6 PBL/ml with PBS w/o calcium and magnesium before immuno-labeling. Controls consisted of 1) unlabeled sample (autofluorescence control), 2) sample incubated with FITC- and PE-conjugated irrelevant IgG (isotype control) and 3) sample labeled with FITC-conjugated lin cocktail and control IgG_{2a} PE. Direct two-color immunofluorescence staining was in general performed according to standard protocols recommended for Becton-Dickinson reagents. Blood was stained and lysed with FACS Lysing solution (Becton-Dickinson) at room temperature in the dark within 5 h after drawing. To achieve complete lysis of red blood cells in samples stained with the mAb mixture, the lysing step was repeated twice. 20 000 or 30 000 events were acquired from every sample. Isolated PBMNC fractions were stained at 4°C.

Stained samples were examined in a flow cytometer FACS'tar (Becton-Dickinson) equipped with Consort 30 version F software. Compensation was applied to correct for FITC emission entering the FL2 channel and for PE emission entering the FL1 channel. Gain settings were adjusted daily using standard labeled FITC and PE calibrite beads (Becton-Dickinson).

Proliferation assays. 50×10^3 or 100×10^3 PBMNC were cultured in U-bottom microtiter wells (Nunc, Den-

mark) in 0.2 ml of RPMI-1640 medium with L-glutamine (Gibco), supplemented with 10% heat-inactivated human AB serum (Sigma) and gentamycin at 50 µg/ml (Sigma). KLH (Calbiochem, CA) was added to the cultures at concentrations ranging 25–200 µg/ml. IL-2 (Genzyme, MA) was added at a final concentration of 1 U/ml, IL-4 (Genzyme, MA) at a final concentration of 1000 U/ml. Cells were cultured for 3, 5, 6 and 7 days. Cell proliferation was assessed by [³H]-TdR incorporation. One µCi per well [³H]-TdR (spec. act. 5.0 Ci/mmol, Amersham, England) was added to the cultures for 16 h prior to harvest. Results represent the mean counts per 1 min ± SD of 5 replicate cultures.

[³H]-TdR incorporation data were analyzed by Friedman ANOVA followed by Wilcoxon matched pairs test; $p < 0.05$ was considered significant.

Results

In order to identify and count DC, whole blood was stained with a mixture of FITC-conjugated mAbs recognizing lineage-associated molecules CD3, CD14, CD16, CD20 and CD57 (lin cocktail) and PE-conjugated anti-HLA-DR mAb. The procedure of flow cytometry analysis applied to detect and enumerate DC is illustrated in Fig. 1. The control sample stained with lin cocktail and control IgG_{2a} PE was included in the assay in order to discriminate between lin⁺HLA-DR^{bright} and lin⁺HLA-DR^{dim} cells in a sample stained with lin cocktail and mAb anti-HLA-DR. Since cells with the DC surface phenotype were not found in a gate encompassing granulocytes (data not shown), flow cytometry analysis was performed for events collected in a PBMNC gate. A better discrimination between lin⁺ and lin^{dim} cells was possible in a gate set for PBMNC than in a gate set for PBL because of the relatively high fluorescence intensity of granulocytes stained with FITC-conjugated control IgG. DC percentage in the entire PBL population was back-calculated from the percentage of DC in PBMNC and the proportion of PBMNC in PBL.

Dendritic cells enumerated in whole peripheral blood from 11 healthy donors (M/F: 4/7, age: range 22–56 years, median 31 years) comprised $0.54 \pm 0.16\%$ (mean ± SD) of PBL (Table 1). The mean absolute DC count was $31.4 \times 10^6/l$ of blood, SD $\pm 9.6 \times 10^6/l$. DC content in PBL determined in 4 buffy coat preparations fell in the range of 0.29–0.86% determined in measurements performed on whole peripheral blood.

Dendritic cells stained directly in whole peripheral

Table 1. Dendritic cell (DC) number in peripheral blood of normal individuals expressed as percentage of peripheral blood leukocytes (PBL) and DC absolute count per 1 µl of blood. DC were identified as lin⁺HLA-DR^{bright} cells by flow cytometry

Donor	% of PBL	DC count/µl
1	0.29	14.6
2	0.56	32.7
3	0.47	20.4
4	0.57	30.9
5	0.86	40.2
6	0.63	45.7
7	0.50	31.4
8	0.42	43.5
9	0.39	18.1
10	0.82	33.8
11	0.47	33.8
Mean ± SD	0.54 ± 0.16	31.4 ± 9.6

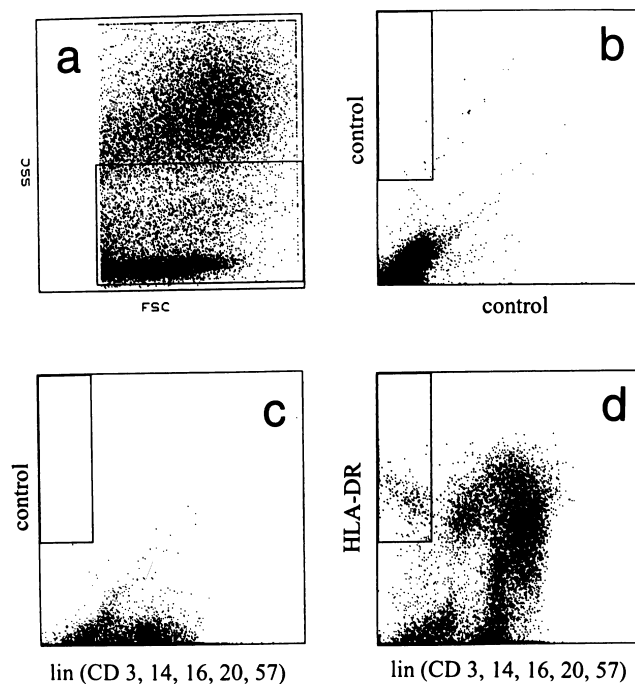


Fig. 1. Detection of DC (lin⁺HLA-DR^{bright}) in peripheral blood by flow cytometry. 20 000 events in PBL gate were acquired from an unlabeled sample and the PBMNC gate was set to determine a proportion of PBMNC in PBL (a). Events which fell within the gate encompassing PBMNC were collected from stained samples and displayed in plots of fluorescence intensity (log₁₀ scale from 1 to 10⁴). PBMNC stained with FITC- and PE-conjugated irrelevant IgG (abscissa and ordinate, respectively) are shown in plot (b), PBMNC stained with the FITC-conjugated lin cocktail and IgG_{2a} PE control in plot (c), and PBMNC stained with the FITC-conjugated lin cocktail and anti-HLA-DR PE mAb in plot (d). DC identified as cells which fell within the gate set for lin⁺HLA-DR^{bright} cells in plot (d) in this example comprised 0.84% of PBMNC population, and 0.39% of entire PBL population. DC percentage in PBL was back-calculated from their percentage in PBMNC and the proportion of PBMNC in PBL.

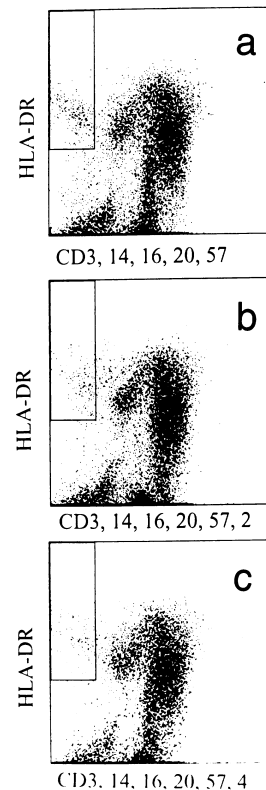


Fig. 2. DC stained in whole blood vary in their expression of CD2 and CD4 molecules. The gate was set for HLA-DR^{bright} cells, which did not stain with a mixture FITC-conjugated mAbs in plots displaying fluorescence intensity (log₁₀ scale from 1 to 10⁴) of events collected in PBMNC gate. In this example DC identified as lin⁻ HLA-DR^{bright} cells comprised 0.84% of the PBMNC population (plot a). Lin⁻ HLA-DR^{bright} cells, which did not stain with anti-CD2 mAb, included in the mixture of mAbs used for staining, comprised 0.56% of PBMNC (plot b). Lin⁻ HLA-DR^{bright} cells, which did not stain with anti-CD4 mAb, comprised 0.47% of PBMNC (plot c)

blood were not homogeneous in terms of expression of CD2 and CD4 molecules (Fig. 2). No cells with CD80 and CD83 molecules were found in whole blood preparations (data not shown). Expression of CD80 and CD83 on DC became, however, apparent following the isolation procedure including centrifugation on Percoll gradient, overnight culture and centrifugation on Nyco-prep gradient (Fig. 3). This procedure did not induce the expression of CD80 or CD83 molecules on CD14⁺ monocytes (Fig. 4).

The content of CD34⁺ cells and lin⁻ HLA-DR^{bright} cells was determined in buffy coat preparations and in DC-enriched fraction obtained by centrifugation on

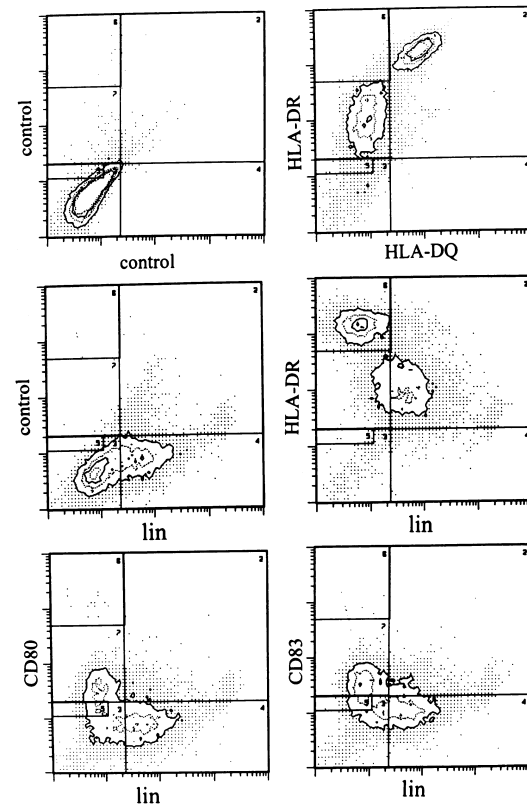


Fig. 3. Flow cytometry analysis of the Percoll high-density, Nyco-prep low-density cell fraction enriched with dendritic cells. The DC-enriched fraction was obtained by centrifugation on Percoll gradient followed by overnight culture and centrifugation on Nyco-prep gradient. HLA-DQ, CD80 and CD83 expression was found mostly on lin⁺ HLA-DR^{bright} cells

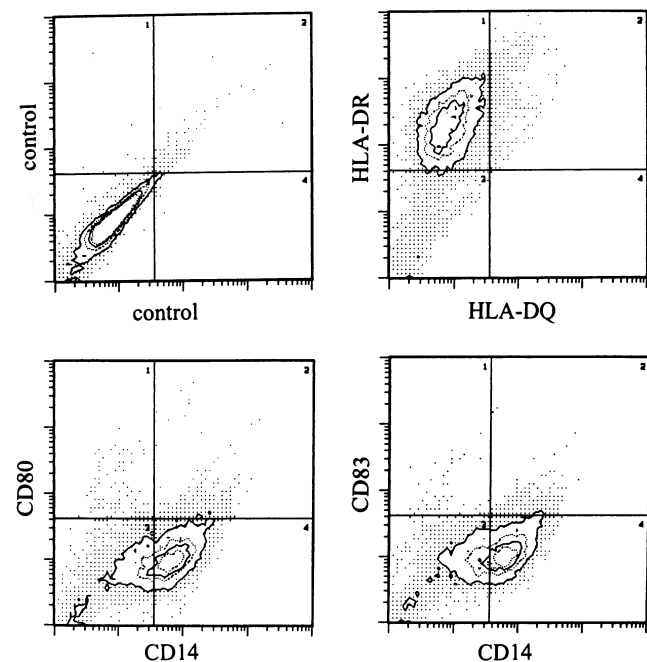


Fig. 4. Flow cytometry analysis of Percoll primary low-density cells enriched within CD14⁺ monocytes. Monocytes isolated by centrifugation on Percoll gradient and cultured overnight did not express CD80 and CD83 molecules

Percoll and Nycoprep gradients. CD34⁺ cells co-purified with DC during the isolation procedure. However, the content of CD34⁺ cells both in buffy coat preparation and in the DC-enriched cell fraction was at least one order of magnitude lower than the content of lin⁻HLA-DR^{bright} cells (data not shown).

KLH is a protein antigen to which healthy individuals are not sensitized and, therefore, is routinely used to detect primary responses of naive T cells. The primary proliferative response of CD4⁺ T cells to KLH is entirely dependent on DC, as previously shown in co-cultures of isolated cell populations. In this study we investigated whether the primary proliferative response to KLH can also be detected in cultures of unseparated PBMNC obtained from 16 healthy adult donors (Table 2). Mean values of [³H]-TdR incorporation in PBMNC cultures stimulated with KLH at the optimal concentration of 100 µg/ml for 7 days differed significantly from mean values in cultures without antigen, when analyzed by Friedman ANOVA followed by Wilcoxon matched pairs test ($p < 0.05$). The proliferative response of PBMNC stimulated with KLH in the presence of IL-2 and IL-4 differed significantly from the proliferative response of PBMNC cultured with IL-2 and IL-4 in the absence of antigen.

Discussion

Identification of dendritic cells in human peripheral blood by flow cytometry is hindered by the lack of markers applicable for detection of circulating DC. Dendritic cells isolated from human peripheral blood do not express markers defining other leukocyte subsets and strongly express HLA-DR class II molecules, and therefore the lin⁻HLA-DR^{bright} phenotype was a basis to discriminate DC from other leukocytes and to evaluate the purity of DC-enriched cell fractions^{9, 14, 25}. A better purification of DC was achieved by a negative panning procedure, utilizing a mixture of mAbs recognizing lineage-associated markers (lin cocktail)^{14, 19}. A highly purified DC population was obtained by labeling PBMNC with lin cocktail and separation of the positive and negative population on FACS⁴. DC identified on the basis of their lin⁻HLA-DR^{bright} phenotype are well characterized as antigen-presenting cells, capable of initiating primary response of naive T cells to antigen^{16, 17}. GM-CSF promotes survival of peripheral blood DC in culture and induces their morphological differentiation *in vitro* to cells with long-branched projections¹⁴.

DC are a small subset of PBMNC. Complex purification procedures, which require preparation of rela-

tively large volumes of blood, cannot be applied in studies of DC in disease. Therefore, our study was aimed at developing a method for the enumeration of DC directly in whole blood. We applied direct two-color immunofluorescence staining to detect cells of lin⁻HLA-DR^{bright} phenotype, followed by lysis of erythrocytes. An alternative method of DC counting in whole blood, based on their lin⁻HLA-DR⁺ phenotype, was performed with the use of nuclear dye LDS-751 to distinguish nucleated cells in three-color flow cytometry analysis¹⁵.

Identification of DC based on their lin⁻HLA-DR^{bright} phenotype is not strictly specific, because cells of this phenotype may also include HLA-DR⁺CD34⁺ hematopoietic progenitors circulating in the blood. It has been reported that the mean absolute count of CD34⁺ hematopoietic progenitors enumerated directly in peripheral blood obtained from healthy donors was 1.68×10^3 per 1 ml of blood²³. The absolute number of cells of lin⁻HLA-DR^{bright} phenotype in our study was 31.4×10^3 per 1 ml. Our preliminary experiments confirmed that CD34⁺ cells were at least one order of magnitude less numerous than the lin⁻HLA-DR^{bright} cells, both in the entire PBL population and in the DC-enriched cell fractions obtained by a multi-step purification procedure. CD34⁺ cells vary in the expression of HLA-DR molecules and may not be encompassed by the gate set for HLA-DR^{bright} cells. In our study, lin⁻HLA-DR^{dim} cells were not counted as DC. However, it can be anticipated that the content of lin⁻HLA-DR^{bright} cells which are not DC may increase in some diseases, especially when hematopoietic precursors are mobilized to peripheral blood. Since mature DC are CD34⁻, the enumeration of CD34⁺ cells may be helpful for discrimination between DC and hematopoietic progenitors. Hematopoietic progenitors circulating in human peripheral blood, common for monocytes and DC, are a potential source of DC²⁰. The CD34⁺ hematopoietic progenitors, isolated from bone marrow^{21, 26} or umbilical cord blood^{2, 22}, grow and differentiate in the presence of GM-CSF and TNF- α to mature DC with branched projections, identical in morphology with DC from the peripheral blood of adults induced to differentiate in the presence of GM-CSF¹⁴.

It has been reported, that human blood contains a small population of lin⁻HLA-DR⁺ fibrocytes, which are CD34⁺ and, after isolation, express surface components required for antigen presentation³. Blood-borne fibrocytes share many features with DC and the relation between these two cell types has to be further elucidated.

An alternative method for DC enumeration in blood preparations is based on the use of CMRF44 or CD83

Table 2. Primary proliferative responses of 5×10^4 PBMNC to KLH (100 $\mu\text{g/ml}$) and to KLH in the presence of IL-2 (1 U/ml) and IL-4 (1000 U/ml)

Donor	no KLH, no ILs	KLH	IL-2, IL-4	KLH, IL-2, IL-4
1	303 $\pm$ 54	9211 $\pm$ 2924	n.d.*	n.d.
2	427 $\pm$ 45	7555 $\pm$ 2238	3345 $\pm$ 518	13272 $\pm$ 2807
3	195 $\pm$ 29	3022 $\pm$ 782	1144 $\pm$ 367	6863 $\pm$ 1244
4	373 $\pm$ 23	1736 $\pm$ 716	n.d.	n.d.
5	549 $\pm$ 125	1563 $\pm$ 813	n.d.	n.d.
6	291 $\pm$ 48	1148 $\pm$ 582	1673 $\pm$ 563	4752 $\pm$ 351
7	174 $\pm$ 23	257 $\pm$ 99	1123 $\pm$ 256	1371 $\pm$ 292
8	319 $\pm$ 64	2141 $\pm$ 438	1852 $\pm$ 251	3381 $\pm$ 281
9	315 $\pm$ 139	776 $\pm$ 333	1623 $\pm$ 187	2748 $\pm$ 520
10	306 $\pm$ 38	1870 $\pm$ 623	1216 $\pm$ 254	3843 $\pm$ 1174
11	417 $\pm$ 58	1369 $\pm$ 397	605 $\pm$ 77	3964 $\pm$ 467
12	359 $\pm$ 75	2451 $\pm$ 465	1208 $\pm$ 95	1675 $\pm$ 715
13	408 $\pm$ 69	859 $\pm$ 366	1251 $\pm$ 242	1751 $\pm$ 257
14	299 $\pm$ 42	2709 $\pm$ 725	3267 $\pm$ 450	4188 $\pm$ 734
15	313 $\pm$ 85	474 $\pm$ 167	296 $\pm$ 91	367 $\pm$ 123
16	255 $\pm$ 55	3550 $\pm$ 1348	1554 $\pm$ 273	5825 $\pm$ 815

* n.d. – not done.

The proliferative response was measured by [^3H]-TdR incorporation. Results represent the mean counts per 1 min $\pm$ SD of 5 replicate cultures.

PBMNC from healthy donors were cultured with KLH for 7 days.

activation antigens, which are induced on DC isolated on Ficoll gradient and briefly cultured^{6, 7}. This method is, however, limited to the evaluation of DC content in the isolated PBMNC population. The cell separation procedure as well as PBMNC culture *in vitro*, with or without cytokines, may result in selective loss of different PBMNC subsets.

In this study we verified the suitability of anti-CD83 mAb for identification of DC in fractions obtained after the isolation procedure. However, in experiments performed on cultured cells, CD83 molecule cannot be used as a DC marker, since CD83 expression was inducible also on macrophages(ref.¹⁰).

We confirmed previous reports^{5, 24} indicating that the CD80 molecule is expressed on DC, but not on monocytes, after isolation and a brief culture *in vitro*. Monocytes express CD80 molecule when activated with IFN- γ ^{5, 8}. In our study, direct staining of whole blood preparation did not reveal any CD80⁺ cells. Therefore, CD80 expression cannot be used for DC identification in whole blood preparations. Enrichment procedures and culture in general induce upregulation of various adhesion molecules on DC surfaces^{15, 24}.

Heterogeneous expression of CD2 and CD4 was previously reported²⁴ on DC stained after the isolation procedure. In our study we found heterogeneous expression of CD2 and CD4 molecules also on DC stained directly in whole blood.

It has been previously shown that DC isolated from human peripheral blood primed naive CD8⁺ and CD4⁺

T cells to soluble antigens, whereas monocytes failed to induce such primary responses^{16, 17}. In this study we demonstrated that the primary proliferative response to KLH can be detected in cultures of unseparated PBMNC obtained from healthy donors. Therefore, PBMNC cultures stimulated with KLH can be used in a relatively simple test, performed on a small volume of blood to monitor DC-dependent priming of naive T cells. The test can be suitable in monitoring DC function in cancer patients undergoing chemotherapy, however, as it measures DC and T cell cooperation, the impaired responses may result either from DC or naive T cell dysfunction, or both.

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