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Multidimensional flow-cytometric analysis of dendritic cells in peripheral blood of normal donors and cancer patients

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Abstract We studied the potential of multidimensional flow cytometry to evaluate the frequency and maturation/activation status of dendritic cells in minimally manipulated peripheral blood mononuclear cell preparations (i.e., only separated on Ficoll-Hypaque) of normal donors and cancer patients. A rare subset of HLA-DR$^+$ leukocytes (less than 1% mononuclear cells) was detected in blood of normal donors that displayed all the features of dendritic cells: these cells had high forward-light-scatter characteristics and coexpressed CD4, CD86 and CD54 surface antigens, but lacked the lineage-associated surface markers of T cells, B cells, monocytes, granulocytes or NK i.e. they were CD3$^-$, CD19$^-$, CD20$^-$, CD14$^-$, CD11b$^-$, CD16$^-$, CD56$^-$). These physical and phenotypic properties were virtually identical to those of immunomagnetically sorted leukocytes characterized as dendritic-cells on the basis of morphology, phenotype and high stimulatory activity in allogeneic mixed-lymphocyte cultures. Using this flow-cytometric approach we observed that the frequency of dendritic cell-like cells in peripheral blood mononuclear cell specimens of cancer patients receiving chemotherapy alone or those recovering from stem cell transplantation was significantly lower than that of normal individuals (mean $\pm$ SE: 0.36 $\pm$ 0.05%, 0.14 $\pm$ 0.06%, and 0.75 $\pm$ 0.04% respectively). Multidimensional flow-cytometric analysis of dendritic cells might represent an important new tool for assessing immunocompetence, and for monitoring the effects of therapeutic regimens on the immune system.

Key words Dendritic cells · Flow cytometry · Stem cell transplantation · Breast cancer

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Introduction

Dendritic DC) are a subset of bone-marrow-derived leukocytes present in trace amounts in virtually all tissues, with the exception perhaps of brain [14]. Because they express MHC class I and class II molecules and the costimulatory structures required for optimal activation of naive and memory T lymphocytes, DC have been considered to be the most efficient of antigen-presenting cells (APC) [14]. The study of DC has been difficult, though, because of their very low numbers in peripheral blood and other tissues. Another difficulty has been the failure thus far to identify any unique surface marker that could be used to enumerate, isolate, and analyze human DC directly. Furthermore, evidence suggests that the phenotype of DC may vary with the stage of maturation or activation and tissue source [6, 12, 13]. As a result, DC are usually identified (a) *morphologically* by their numerous cytoplasmic processes [14], (b) *phenotypically* by their expression of HLA-DR and B7 costimulatory molecules, and lack of surface markers

associated with mature NK cells, B cells, T cells, macrophages/monocytes, or granulocytes [3, 9], and (c) *functionally* by their potent T-cell-stimulatory activity [3, 14].

Much of our information on human peripheral blood DC has come from analysis of DC that are highly enriched through rigorous and multiple fractionation procedures [3, 14, 18]. Since DC are estimated to represent fewer than 1% of mononuclear cells, such studies require a large amount of peripheral blood. Thus investigations using enriched DC are impractical for many studies involving patients, particularly those with leukopenia. Furthermore, the cell loss that occurs from the multiple manipulations involved in DC enrichment may decrease the accuracy of the results. Finally, DC isolation is too complex and time-consuming for routine analysis of patient samples. Therefore, we investigated the feasibility of using multidimensional flow cytometry to analyze DC directly in peripheral blood mononuclear cell (PBMC) samples obtained with only minimal manipulation, i.e. Ficoll-Hypaque gradient separation. We show that the phenotype and light-scatter properties of these cells are indistiguishable from those of DC isolated by immunomagnetic sorting. Finally, we show the usefulness of this technique in evaluating the DC-like subset in peripheral blood specimens from cancer patients.

Materials and methods

Preparation of mononuclear cells

Peripheral blood from normal donors and cancer patients was obtained by venipuncture and collected into heparinized tubes. Patients included seven with breast cancer who were undergoing standard chemotherapy treatments and six individuals recovering from autologous or allogeneic stem cell transplantation (one with acute myelogenous leukemia, one with lymphoma, and four with breast cancer). In some studies, buffy coat samples from normal donors were purchased from a local blood bank. Blood specimens were diluted with two parts Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS), layered onto Accu-Prep gradient solution (density = 1.077 g/ml; Accurate Chemical and Scientific Corp., Westbury, N.Y.), and centrifuged at 750 *g* for 20 min. The PBMC were collected from the interface and washed three times in PBS.

Flow cytometry

The cell surface phenotype was examined by two- and three-color immunofluorescence using monoclonal antibodies (mAb) that were directly conjugated to fluorescein isothiocyanate (FITC), R-phycoerythrin (PE), or Tri-color (TRI). These mAb included PE- and FITC-labeled anti-CD3, -CD11b, -CD14, -CD19 and -CD20, PE-anti-CD1a, -CD4, -CD16 and -CD54, and FITC- or TRI-anti-HLA-DR (Caltag, Burlingame, Calif.); PE-anti-CD56 and anti-CD80, and FITC-anti-CD16 (Becton-Dickinson Immunocytometry Systems, San Jose, Calif.); FITC-anti-CD56 (Antigenix America Inc., Franklin Square, N.Y.); and PE-Beckton-Dickinson). For two-color flow-cytometric detection of DC in PBMC preparations, 10^6 cells were incubated for 20 min at 4 °C with FITC-conjugated anti-HLA-DR, together with a cocktail of PE-conjugated mAb reactive against CD3, CD11b, CD14, CD16, CD19, CD20, and CD56. The latter lineage (lin) cocktail was titrated to label simultaneously all NK cells, T cells, B cells, monocyte/macrophages, and granulocytes. Cells labeled with PE- and FITC-conjugated isotype control mAb (Caltag and Becton-Dickinson) that were nonreactive to human cells were used as a control. The labeled cells were washed, fixed with 1% paraformaldehyde, and analyzed using a FACScan flow cytometer (Becton Dickinson) equipped with a single 488-nm argon laser and three fluorescence detectors with filter settings for FITC (530 nm), PE (585 nm) and >650 nm). A total of 30 000 to 50 000 events were collected in list mode and analyzed using FACScan Research Software (Becton Dickinson). Forward scatter (FSC) and side scatter (SSC) gates were set to exclude erythrocytes and debris, and markers set to exclude background fluorescence as established using appropriate isotype controls. In these experiments DC were identified as lin^-DR^+ leukocytes with high forward-light-scatter properties.

For three-color flow-cytometric analysis of DC, 10^6 cells were labeled simultaneously with a FITC-lin cocktail, TRI-conjugated anti-HLA-DR, and a PE-conjugated mAb recognizing one of the following cell surface determinants: CD1a, CD4, CD54, CD80, or CD86. The cells were washed and fixed with 1% paraformaldehyde. For flow-cytometric analysis, a live gate was set on the lin^-DR^+ DC population (i.e. $FITC^-$,TRI^+), and 1000–2000 gated events routinely collected. This population was then analyzed for co-expression of a third marker, identified as positive events (above background fluorescence) within the PE channel.

Immunomagnetic sorting of DC

Highly enriched preparations of DC were obtained by immunomagnetic separation using DC isolation kits obtained from Miltenyi Biotec Inc., Auburn, Calif. [7]. The isolation procedure included a negative selection step to remove NK cells, T cells, and monocytes, followed by positive selection of $CD4^+$ DC. Briefly, PBMC were suspended in cold PBS supplemented with 2 mM EDTA and 0.5% bovine serum albumin (fraction V, Sigma Chemical Co., St. Louis, Mo.) at a concentration of 3×10^8 cells/ml. The cells were incubated for 10 min at 4 °C with an FcR blocking reagent and a cocktail of mAb recognizing CD3, CD11b and CD16 antigens. The suspension was then washed, and incubated for an additional 15 min at 4 °C with paramagnetic microbeads recognizing the mAb cocktail. This suspension was passed through a type CS iron-fiber-depletion column placed within a strong magnetic field (SuperMACS, Miltenyi Biotec). Effluent cells that were depleted of macrophages, granulocytes, NK cells and T cells were collected, washed, incubated for 30 min at 4 °C with anti-CD4 microbeads, and passed through a type MS iron bead separation column placed within the magnetic field. After extensive washing the column was removed from the magnet and the $CD4^+$ adherent cells flushed from the column with cold buffer. These cells were then passed through a second positive-selection column to purify the DC further. The purity of the sorted population was analyzed by labeling the recovered cells with FITC-anti-HLA-DR, and a cocktail of PE-conjugated mAb directed against TCRαβ and TCRγδ (Immunotech, Westbrook, Maine), CD19, CD56, and CD14 to detect any residual NK cells, T cells, B cells, or monocytes.

Culture of DC

In some experiments, immunomagnetically sorted DC were suspended in RPMI-1640 medium (10^5 cells/ml) supplemented with 10% heat-inactivated fetal bovine serum (GibcoBRL, Grand Island, N.Y.), 10 mM HEPES buffer, 100 µg/ml streptomycin, 100 U/ml penicillin, 50 µg/ml gentamicin, 2 mM glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 20 µM 2-mercaptoethanol, 50 ng/ml human recombinant granulocyte/macrophage-colony-stimulating factor (GM-CSF; R&D Systems, Minneapolis, Minn.), and 10 ng/ml highly purified human recombinant tumor necrosis factor α (TNFα) from *Escherichia coli* (Cetus Corp., Emeryville, Calif.) [17]. The cells were cultured for 5 days at 37 °C in a 5% CO_2 humidified atmosphere.

Allogeneic mixed-lymphocyte response

The mixed-lymphocyte response was assayed as described previously [20]. DC stimulators were suspended in RPMI-1640 medium supplemented with 10% heat-inactivated human AB serum (Pel Freez, Brown

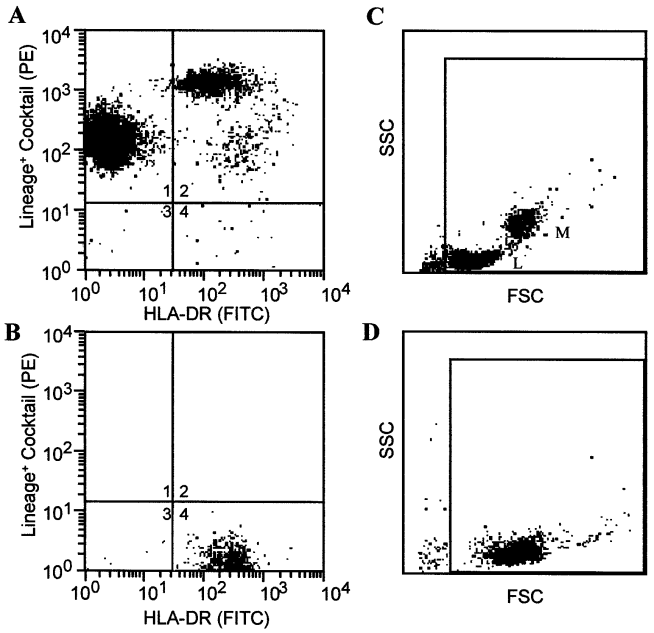

Fig. 1A–D Enrichment of lin$^-$DR$^+$ leukocytes by immunomagnetic cell sorting. Unsorted peripheral blood mononuclear cells (PBMC) (**A**, **C**) from a normal donor and cells sorted using a dendritic cell (DC) isolation kit as described in Materials and methods (**B**, **D**) were analyzed by flow cytometry. Markers were set using phycoerythrin(*PE*)- and fluorescein-isothiocyanate(*FITC*)-conjugated isotype control antibodies to exclude background events. DC-like cells were characterized as the lin$^-$DR$^+$ events in quadrant 4 of the FITC/PE dot plots in **A** and **B**. The lineage cocktail consisted of PE-conjugated mAb recognizing TCRαβ, TCRγδ, CD19, CD56 and CD14 antigens. The light-scatter characteristics of the leukocyte populations are shown in **C** and **D**. The two major leukocyte populations, i.e. lymphocytes (*L*) and monocyte/macrophages (*M*) are labeled in **C**. The experiment shown is a representative experiment from three sorts of PBMC from normal donors. *SSC* side-scattered light, *FSC* forward-scattered light

Deer, Wis.), HEPES buffer, antibiotics, nonessential amino acids, sodium pyruvate, glutamine and 2-mercaptoethanol as described above, and irradiated with 3000 cGy delivered from a cesium source. Serial dilutions of the DC were prepared in triplicate in 96-well round-bottomed plates (0.1 ml/well) and an equal volume of medium containing 1.5×10^5 allogeneic nylon-wool-nonadherent T lymphocytes was added to each well (stimulator:responder ratios ranged from 0.01:1 to 1:1). Controls included DC and T cells incubated alone. The plates were cultured for a total of 5 days, with 1 μCi ([^{3}H]thymidine [^{3}H]dT; NEN-DuPont, Boston, Mass.) added during the final 16 h of culture. Cells were harvested onto glass-fiber filters, and the radioactivity measured by β-scintillation counting. The results are expressed as counts per minute (cpm).

Digital imaging

Images of cells in cultures were captured as described previously [15] using a VI-470 change-coupled device (CCD) video camera system (Optronics Engineering, Goleta, Calif.) attached to a Nikon DIAPHOT-TMD inverted microscope (Nikon Inc., Melville, N.Y.) equipped with 10× or 20× objectives. The images were digitized using a QuickCapture frame grabber board (Data Translation Inc., Marlboro, Mass.).

Data analysis

Data were analyzed using Student's *t*-test. Results were considered significant when P was less than 0.05.

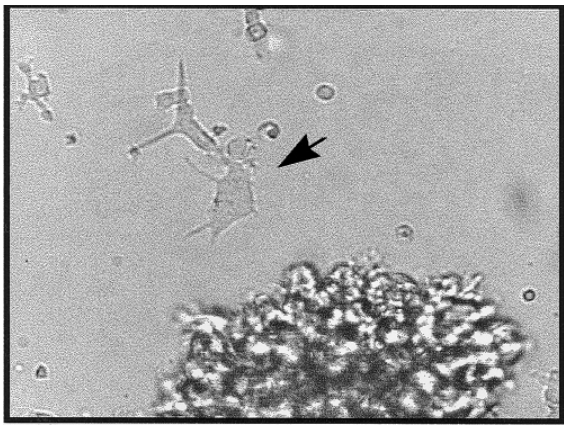

Fig. 2 Morphology of immunomagnetically sorted lin$^-$DR$^+$ leukocytes. Lin$^-$DR$^+$ leukocytes from PBMC of a normal donor were sorted as described in Fig. 1 and cultured for 5 days with granulocyte/macrophage-colony-stimulating factor and tumor necrosis factor α. DC-like cells were observed in clusters (*lower right*) or as individual cells with elongated cytoplasmic processes typical of DC (*arrow*)

Results

Phenotype of DC isolated from peripheral blood of normal donors

Freshly isolated peripheral blood DC are known to be of low buoyant density (<1.077 g/ml) and lack surface markers expressed by T cells, B cells, natural killer (NK) cells, monocytes/macrophages, or granulocytes, i.e. they are CD3$^-$CD11b$^-$CD14$^-$CD19$^-$CD16$^-$CD20$^-$CD56$^-$ [9, 14, 18]. However, DC express MHC class II [14]. Another distinguishing characteristic of freshly isolated peripheral blood DC is the expression of CD4 surface molecules [2, 9]. A DC-isolation kit, purchased from Miltenyi Biotec Inc. (Sunnyvale, Calif.), was used for immunomagnetic sorting of DC from peripheral blood specimens on the basis of the above-mentioned characteristics. This isolation was performed in two steps according to the manufacturer's recommendations: PBMC were first depleted of T cells, NK cells, and monocytes by a cocktail of mAb recognizing CD3, CD16, and CD11b respectively (negative selection step), and then CD4$^+$ leukocytes were obtained by positive selection [2, 3, 10]. Using this technique, we obtained highly enriched populations of up to 95% purity) displaying a phenotype consistent with DC, as analyzed by flow cytometry, i.e., TCRαβ$^-$TCRγδ$^-$CD19$^-$CD14$^-$CD56$^-$, but HLA-DR$^+$ (Fig. 1A, B). When the light scattering of these cells was examined, it was found that their size (as estimated by FSC) was between that of lymphocytes and monocytes (Fig. 1C, D).

Morphology and function of sorted DC

Immunomagnetically sorted lin$^-$DR$^+$ cells were placed in culture for 5 days with GM-CSF and TNFα, and then analyzed by light microscopy. As seen in Fig. 2, these

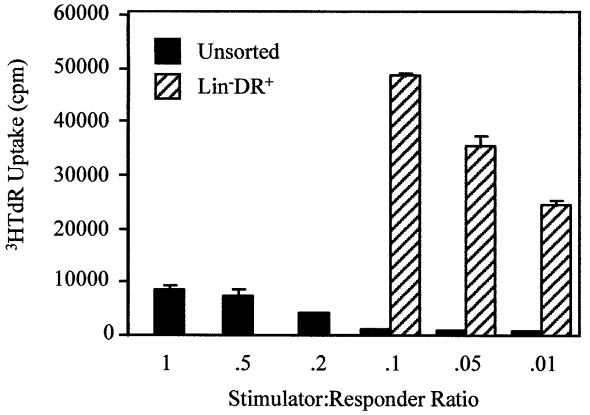

Fig. 3 Allostimulatory activity of immunomagnetically sorted lin$^-$ DR$^+$ cells. Unsorted PBMC and immunomagnetically sorted lin$^-$DR$^+$ leukocytes were tested for the ability to stimulate allogeneic lymphocytes in a standard mixed-leukocyte reaction. *Bars* means ± SE of replicate samples. Proliferation of responder cells was evaluated by [^{3}H]dT uptake measured after 5 days of culture. There was a significant difference between the stimulatory activity of unsorted cells and lin$^-$ DR$^+$ cells at stimulator:responder ratios of 0.01–0.1 ($P<0.001$). Because of the low recovery of lin$^-$DR$^+$ cells from peripheral blood, these were not tested at higher stimulator:responder ratios

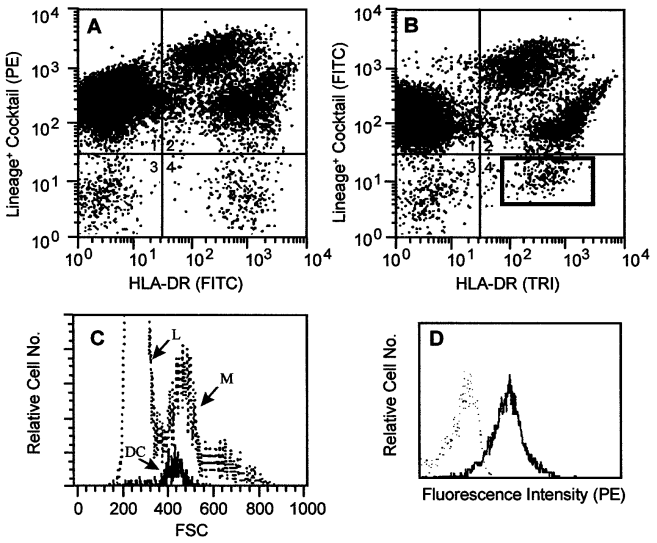

Fig. 4A–D Flow-cytometric analysis of lin$^-$DR$^+$ leukocytes within PBMC preparations. **A** PBMC of normal donors were labeled with a cocktail of PE-conjugated mAb recognizing CD3, CD19, CD20, CD16, CD56, CD11b and CD14, and FITC-conjugated anti-HLA-DR mAb. **B** The same PBMC population was labeled with a cocktail of FITC-conjugated mAb recognizing the lin-associated antigens listed above and Tri-color(TRI)-labeled anti-HLA-DR mAb. **C** Size characteristics of lin$^-$DR$^+$ cells. The forward-light-scatter (size) histogram of the lin$^-$ DR$^+$ cells (__), contained within the rectangular gate shown in quadrant 4 of the TRI/FITC dot plot in **B**, is shown in overlying with the FSC histogram of the whole PBMC population (···). *Arrows* the peaks corresponding to the lin$^-$DR$^+$ DC, lymphocytes (*L*) and monocyte/macrophages (*M*). **D** PBMC were labeled simultaneously with FITC-lin cocktail, TRI-anti-HLA-DR and PE-anti-CD86, or with the same FITC- and TRI-labeled antibodies and a PE-conjugated isotype control antibody. The histograms represent the proportion of lin$^-$DR$^+$ cells in the live gate of FITC$^-$,TRI$^+$ events that react with the isotype control (···) or anti-CD86 (__) mAb

cultures contained cells with elongated cytoplasmic processes typical of DC. In agreement with the observations of others [11–13], these DC-like lin$^-$DR$^+$ leukocytes were present either as individual cells or in loosely adherent clusters (Fig. 2). Furthermore, these cells were potent stimulators of a primary allogeneic mixed-lymphocyte response, as shown by their ability to stimulate significant proliferation of allogeneic T lymphocytes at stimulator-to-responder ratios as low as 0.01:1 (Fig. 3).

Detection of DC-like cells among PBMC

On the basis of the information obtained from the sorting experiments, we next determined whether multidimensional flow cytometry could be used to accurately detect lin$^-$DR$^+$ cells directly in minimally manipulated PBMC preparations (i.e. cells obtained from blood specimens subjected to Ficoll-Hypaque gradient separation only). PBMC from normal donors were labeled simultaneously with a PE-labeled cocktail of mAb recognizing the lin-associated markers CD3, CD11b, CD14, CD16, CD19, CD20, and CD56 (first color), and FITC-conjugated anti-HLA-DR (second color). Cells labeled with isotype control antibodies were included to determine background fluorescence. Because of the low frequency of lin$^-$DR$^+$ cells, we routinely collected 30 000–50 000 events to visualize and gate on this population more easily.

As seen in the representative experiment shown in Fig. 4A, a very small subset of lin$^-$DR$^+$ leukocytes could be detected using this technique. This population represented 0.75 ± 0.04% (mean ± SE; $n=12$) PBMC from normal individuals. Similar levels of lin$^-$DR$^+$ cells were observed by using instead a cocktail of FITC-conjugated lineage-associated mAb and TRI-anti-HLA-DR (Fig. 4B). By gating on the lin$^-$DR$^+$ events in quadrant 4 of the bivariate plot and viewing the FSC profile, we observed that this subset was between lymphocytes and monocytes in size, a characteristic that was similar to the immunomagnetically sorted DC (compare Figs. 1D and 4C). Although these cells were rare, this detection technique was highly reproducible, as seen by the consistency of lin$^-$DR$^+$ cells detected in the same sample with the different mAb combinations (Fig. 4A, B), as well by the relatively low

Table 1 Detection of lin$^-$DR$^+$ leukocytes in peripheral blood mononuclear cells (PBMC) of normal donors. Lin$^-$DR$^+$ cells in PBMC were detected by two-color flow cytometry. Replicate samples from donors 1–3 were tested within a 24-h period; blood specimens of donors 4 and 5 were retested (test 2) 1 month after the first test (test 1)

Donor	Lin$^-$DR$^+$ cells (%)		
no.	Test 1	Test 2	Test 3
1	1.0	1.1	1.0
2	0.9	0.9	0.9
3	0.7	0.7	–
4	1.1	0.9	–
5	0.5	0.5	–

Table 2 Three color flow-cytometric analysis of lin$^-$DR$^+$ leukocytes in unsorted and immunomagnetically sorted peripheral blood specimens. Lin$^-$DR$^+$ cells in unsorted PBMC or immunomagnetically sorted populations were analyzed for coexpression of several other surface markers using three-color flow-cytometric analysis. The sorted cells had been positively selected by an anti-CD4 mAb and were not reanalyzed for expression of this antigen

Expt.	Lin$^-$DR$^+$ cells coexpressing the particular antigen (%)				
	CD4	CD54	CD86	CD80	CD1a
PBMC					
1	94.6	98.1	93.2	5.3	6.0
2	87.2	93.6	88.4	0.6	0.3
3	88.2	93.8	93.1	6.0	–
4	92.2	97.4	99.3	1.6	1.3
Sorted					
1	–	98.9	79.4	1.4	–
2	–	97.6	91.4	2.2	4.9
3	–	98.8	86.5	3.2	6.7

variability among replicate samples from five other donors (Table 1).

To characterize this lin$^-$DR$^+$ subset further and to compare it more closely with immunomagnetically sorted DC, we used three-color flow cytometry to analyze the coexpression of surface molecules that have been associated with DC maturation/activation [9, 10]. PBMC were labeled simultaneously with a cocktail of FITC-lin-associated mAb, TRI-conjugated anti-HLA-DR, and PE-conjugated mAb recognizing CD1a, CD54, CD80, or CD86 surface antigens. A live gate was set on the lin$^-$DR$^+$ cells as shown in Fig. 4B, and these cells were analyzed for coexpression of a third surface marker detected by the PE-conjugated mAb. An example of CD86 coexpression by lin$^-$DR$^+$ cells in a PBMC specimen is shown in Fig. 4D. Using this approach it was observed that the phenotype of the majority of lin$^-$DR$^+$ cells was consistent with that described recently for DC freshly obtained from peripheral blood by other isolation techniques, i.e., CD4$^+$CD54$^+$CD86$^+$CD80$^-$CD1a$^-$ (Table 2) [9]. Furthermore, this phenotype was similar to that of the highly enriched DC obtained by immunomagnetic cell sorting (Table 2).

As further support that the lin$^-$DR$^+$ cells detected in PBMC were comparable to sorted DC, we spiked a sample of PBMC labeled with a PE-lin cocktail alone with purified FITC-DR$^+$ DC obtained from the same individual. The sorted DC displayed the same fluorescence and size characteristics as the DC-like cells gated within the whole PBMC population (data not shown).

Analysis of DC in PBMC of cancer patients

Having determined that the above-described two-color flow-cytometric technique could be used confidently to detect DC-like cells in peripheral blood of normal donors, we applied the same approach to determine the levels of DC in cancer patients. As seen in Fig. 5, the proportion of lin$^-$DR$^+$ leukocytes in PBMC specimens of breast cancer

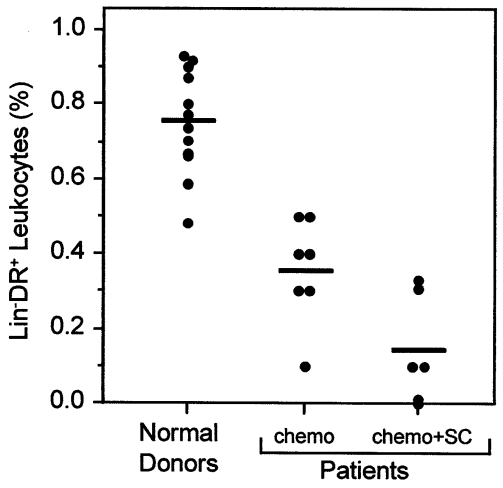

Fig. 5 Frequency of lin$^-$DR$^+$ cells in PBMC of normal donors and cancer patients. The percentage of lin$^-$DR$^+$ leukocytes was determined by two-color flow cytometry as described in the legend of Fig. 4. Patients included seven breast cancer patients who received prior chemotherapy (*chemo*) and six patients who had received stem cell transplants (*chemo+SC*) 1–5 months prior to testing. *Horizontal lines* the mean

Table 3 Phenotype of lin$^-$DR$^+$ leukocytes of breast cancer patients. PBMC were analyzed by three-color flow cytometry as described in the legend of Fig. 4

Patient	Lin$^-$DR$^+$ cells (%) coexpressing:				
no.	CD4	CD54	CD86	CD80	CD1a
1	83	92	4	1	3
2	87	99	91	30	n.t.
3	43	7	55	0	0
4	68	51	22	0	0
5	65	55	11	5	0
6	85	97	98	0	n.t.
7	54	67	77	0	0

patients who had undergone prior chemotherapy was only 0.36 $\pm$ 0.05% (mean $\pm$ SE), which was significantly less than that of normal individuals ($P<0.0005$). With the exception of patient 3, the majority of lin$^-$DR$^+$ cells detected in these patients, like those of normal donors, coexpressed CD4 and CD54 (Table 3). Also, with one exception (patient 2), few or none of the lin$^-$DR$^+$ cells detected in PBMC of these patients coexpressed CD1a or CD80.

Even lower levels of lin$^-$DR$^+$ leukocytes were detected in PBMC samples of cancer patients recovering from stem cell transplantation (Fig. 5). The frequency of DC-like cells in these patients, who had undergone stem cell transplantation 1–5 months earlier, was only 0.14 $\pm$ 0.06% (mean $\pm$ SE), and was significantly lower than in both normal donors and non-transplanted cancer patients ($P<0.0005$ and <0.025 respectively). In fact, lin$^-$DR$^+$ cells were undetectable in one transplanted patient.

Discussion

We have evaluated the feasibility of using a simple two-color flow-cytometric technique to detect DC-like cells in mononuclear cell preparations of peripheral blood of normal donors and cancer patients. These cells represented approximately 1% or less of the PBMC fraction, a frequency that is in agreement to that estimated for DC analyzed in a variety of other types of assay conditions [9, 14, 18]. Furthermore, they expressed a phenotype and size that were virtually identical to those of immunomagnetically sorted DC, and of freshly isolated peripheral blood DC described in previous reports [3]. However, because our approach allows for detection of DC directly in blood samples that have received only minimal manipulation (Ficoll-Hypaque), it allows for a faster, and perhaps more accurate assessment of this important APC population than is achieved by other DC-enrichment procedures. While other investigators have shown that DC-like cells can be detected in PBMC populations [2, 3], our study is the first to use multidimensional flow cytometry to analyze in more depth the frequency and maturation/activation status of these cells in blood specimens of cancer patients, including those who are judged as immunodeficient by other criteria (e.g. low numbers of circulating $CD4^+$ T lymphocytes). Our data suggest that DC testing could become a part of the routine clinical evaluation of immunocompetence.

We have shown for the first time that, while lin^-DR^+ leukocytes in PBMC specimens of breast cancer patients who have received prior chemotherapy phenotypically resemble those detected in normal PBMC (i.e. $CD4^+CD86^+CD54^+CD80^-CD1a^-$), this population is reduced by approximately 50% in the patients. Studies are planned to determine whether this effect is related to the disease-state or to the chemotherapy. The functional capabilities of the small numbers of DC-like cells detected in these patients remain to be determined, but it was recently reported that DC antigen presentation is compromised in cancer patients [4]. In any case, the lower levels of APC might contribute to the inability of patients to mount an effective anticancer response. Similarly, the low levels of lin^-DR^+ DC-like cells in stem-cell-transplanted patients could contribute to the increased susceptibility of these patients to opportunistic infections. Our observations suggest that the multidimensional flow-cytometric technique described here could be used to monitor DC recovery after transplantation and to evaluate the effect of various treatment regimens or environmental conditions on DC frequency or maturation. Such a tool might also aid in identifying therapies that spare or boost immunity.

We found that the two-color flow analysis of DC could be performed with only 2×10^6 PBMC, a number of cells that was easily obtained from normal donors but, more importantly, also from immunocompromised patients who often have low leukocyte counts. Additional cells were required for the more extensive three-color analyses. However, as the technology for multicolor analysis is improving and expanding, it should be possible to analyze DC simultaneously within a single sample for co-expression of multiple surface and cytoplasmic markers.

Analysis of human DC will also be significantly facilitated if mAbs specific for these populations can be identified. Recently it was shown that some subsets of human peripheral blood DC express CD83 [18–20]. This surface antigen is not specific for DC, and may not detect less mature DC subsets [20]. However, the availability of antibodies to CD83 has assisted the analysis of cytokine- and chemokine-gene expression in peripheral blood DC, and the discrimination of DC subsets that are susceptible to HIV infection [18–20]. The mAb CMRF-44 also identifies only a small subset of freshly isolated human DC, but appears to recognize an activation-associated surface antigen that is expressed by cultured DC [6]. On the other hand, it was recently reported that most peripheral blood DC constitutively express an intracytoplasmic p55-kDa actin-bundling protein not found in monocytes, granulocytes, T cells, or B cells [8]. Addition of such markers to the multidimensional flow analysis of lin^-DR^+ cells could allow further dissection and quantification of individual DC subsets that might be altered as a consequence of disease or therapy.

Until more direct analysis of DC is possible, the exact composition of the lin^-DR^+ population, and the proportion of cells within this subset that are truly committed DC cannot be precisely determined. It is possible, for example, that this population may contain DR^+ progenitor cells that are not yet committed to a specific lineage [16]. Whether such cells could be driven towards the DC lineage, however, is an intriguing question with relevance perhaps for the treatment of immunodeficient patients. Our analysis also does not discriminate between DC that might be of lymphoid rather than myeloid lineage [1, 5, 11]. For example, it has been shown that $CD14^+$ monocytes can differentiate into $CD14^-$ DC when exposed to appropriate cytokines [11, 21], and some DC appear to arise from progenitors common to T cells, B cells and NK cells [5]. However, as all of these populations are lin^-DR^+, they should be detected within the population identified by multidimensional flow cytometry, provided that they enter the peripheral blood compartment.

It remains to be determined whether the frequency and function of DC in the peripheral blood will reflect the activity of these cells within various tissues. Nevertheless, the co-expression of MHC class II and costimulatory molecules by the lin^-DR^+ cells, detected by the multidimensional flow-cytometric technique described here, indicate that they might possess potent antigen-presenting activity. Consequently, the loss of such cells, as observed in cancer patients, is likely to weaken the immune response to infectious disease and malignancy.

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