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Human blood contains two subsets of dendritic cells, one immunologically mature and the other immature

U. O'DOHERTY, M. PENG, S. GEZELTER, W. J. SWIGGARD, M. BETJES, N. BHARDWAJ & R. M. STEINMAN *Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York, U.S.A.*

SUMMARY

Two subsets of dendritic cells, differing in T-cell stimulatory function, have been purified directly from human blood. Both subsets are positive for major histocompatibility complex (MHC) class II expression and negative for lineage-specific antigens (e.g. CD3, CD14, CD16, CD19 negative), but are separated by exploiting differences in expression of the β_2-integrin, CD11c. The CD11c-negative subset is functionally immature, requiring monocyte-derived cytokines to develop into typical dendritic cells. The CD11c-positive subset has potent T-cell stimulating activity and expresses the activation antigen CD45RO, unlike its immature counterpart. However, these mature cells only develop typical dendritic morphology and high levels of MHC proteins and adhesins after a period of culture independent of exogenous cytokines. Although the freshly isolated mature dendritic cells resemble monocytes in cytospin preparations, the former lack CD14 and have a much stronger primary T-cell stimulatory capacity. We hypothesize that the CD11c-negative immature cells are marrow-derived precursors to tissue dendritic cells, such as epidermal Langerhans' cells, while the CD11c-positive cells are derived from tissues where they have been activated by antigen, and are *en route* to the spleen or lymph nodes to stimulate T-cell responses there.

INTRODUCTION

Dendritic cells (DC) are antigen-presenting cells (APC) distinguished by their potency and ability to stimulate primary T-cell responses *in vitro*[1] and *in vivo*.[2–6] As such, the origin and development of DC are topics of considerable relevance to the onset of cell-mediated immunity. For studies of human immune responses, blood remains the most accessible tissue. However, most purifications of DC from human blood are carried out after 1–3 days of culture. This culture period reduces the adhesiveness[7] and the buoyant density[8] of DC, simplifying their enrichment from monocytes and lymphocytes, respectively.

Recently, DC have been isolated from fresh blood. Thomas *et al.*[9] concluded that the cells have the typical stimulatory activity of cultured DC, while O'Doherty *et al.*[10] found the cells to be poorly stimulatory or immature, requiring culture in a monocyte-conditioned medium to become typical DC. These immature DC had the appearance of medium-sized lymphocytes, and had lower levels of major histocompatibility complex (MHC) products and accessory molecules than typical DC. In the presence of monocyte-conditioned medium, the cells acquired high levels of MHC proteins and adhesins, developed dendritic morphology, and became potent stimulators.

Here, we have explored additional methods for enriching DC from fresh blood. We first noted that the standard FcR panning method for depleting monocytes[8] resulted in a loss of about half of the fresh DC. (Panning is normally performed after 36 hr of culture, at which time cultured DC are not lost).[8,11] When this step was bypassed, not only did the yield of DC increase, but we observed an additional DC subset. Here, we compare the morphology and phenotype of the two DC subsets with $CD14^+$ blood monocytes, each enriched by cell sorting. We describe their immunostimulatory function and phenotype before and after a short period of culture.

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Abbreviations: APC, antigen-presenting cell; DC, dendritic cell.

Correspondence: Dr R. M. Steinman, Laboratory of Cellular Physiology and Immunology, The Rockefeller University, 1230 York Avenue, New York, NY 10021–6399, U.S.A.

MATERIALS AND METHODS

Culture medium

H10 contains 10% heat-inactivated human serum prepared from laboratory donors, 20 μg/ml gentamicin (Gibco Laboratories, Grand Island, NY), and 10 mM HEPES in RPMI-1640 (Gibco). The pooled commercial serum used in a previous paper[10] did not support cell viability as well as laboratory-prepared, unpooled serum (data not shown). Monocyte-conditioned medium was prepared by adhering fresh blood mononuclear cells to γ-globulin (Calbiochem, La Jolla, CA)-coated Petri dishes for half an hour, washing away non-adherent cells, then incubating adherent cells in H10 for

24 hr. The supernatant was harvested, centrifuged, divided into aliquots, and frozen at −20° until needed.

DC purification

This method was modified from a previous paper[10]. The most important change was the elimination of the γ-globulin adherence step from the previous procedure. Buffy coats from units of blood were purchased from the New York Blood Center. Mononuclear cells were isolated by flotation on Ficoll–Hypaque (Pharmacia, Uppsala, Sweden). T cells were depleted by rosetting with neuraminidase (Calbiochem-Behring Corp., La Jolla, CA)-treated sheep erythrocytes (Cocalico, Reamstown, PA). For convenience, the T-depleted mononuclear cells (Er^-) were often left overnight on ice, a step that was previously shown not to change function or phenotype[10]. Er^- cells were mixed with a 'cocktail' of monoclonal antibodies (mAb) against CD3 [Becton Dickinson Immunocytometry Systems Inc. (BDIS), Mountain View, CA), CD11b (ATCC, Rockville, MD; CRL8026), CD16 (3G8; gift of J. Unkeless)[12] and CD19 (AMAC, Westbrook, MA), to label T cells, monocytes, natural killer (NK) cells and B cells, respectively. CD11b stains a subset of DC only weakly, but monocytes and NK cells strongly, and so can be used for DC purification. After a 30-min incubation on ice, unbound mAb were washed away with Ca^{2+} and Mg^{2+} chelating medium [1 × Hanks, (Gibco) + 1% bovine serum albumin (BSA) + 1 mM EDTA], and the cells were panned by adherence to goat anti-mouse IgG (Cappel Laboratories, West Chester, PA)-coated Petri dishes (Falcon Labware, Oxnard CA). Non-adherent cells were stained with FITC–goat anti-mouse IgG and IgM (Boehringer Mannheim Biochemicals, Indianapolis, IN). Cells were quenched with 100 μg/ml mouse IgG (Jackson Immuno-Research, Westgrove, PA), then stained with PerCP–anti-HLA-DR of IgG2a isotype (BDIS) and washed in Ca^{2+} and Mg^{2+} chelating medium. Cells that were FITC-cocktail negative and also PerCP–HLA-DR positive were then purified on a FACStar Plus® cell sorter (BDIS). When sorting with PerCP, the power of laser excitation (488 nm Innova 90–5 argon laser; Coherent Inc., Palo Alto, CA) was reduced from the standard 200 mW to 100 mW. For phenotyping studies with indirect antibodies (below), we omitted the PerCP-anti-HLA-DR. Data analysis was performed using WINLIST software (Verity Software House, Topsham, ME).

Separation of two subsets of dendritic cells

To purify the two subsets of DC, the purification described above was modified to include a three-colour staining step, by adding phycoerythrin (PE)–anti-CD11c of IgG2b isotype to the prior staining mixture. By sorting, two cell populations were isolated. One was $CD11c^+$, $cocktail^-$, $HLA\text{-}DR^+$, the other was $CD11c^-$, $cocktail^-$, $HLA\text{-}DR^+$.

Cytospin preparations

These were made in a cytocentrifuge (Shandon Southern Instruments Inc., Sewickley, PA), loading 2×10^4 cells/slide. Slides were stored with desiccant at −20° before fixation in acetone and staining with an anti-HLA-DR (ATCC, HB96) of IgG1 isotype, followed by biotin–goat anti-mouse IgG1 (Accurate Chemical and Scientific Corporation, Westbury, NY), avidin–biotin/horseradish peroxidase (PK4000; Vector Laboratories, Burlingame, CA), and diaminobenzidine (04008; Polysciences, Warrington, PA)–hydrogen peroxide. The secondary anti-IgG1 reagent was chosen because it would not bind to the mAb already bound to the sorted populations, which were of IgG2a (anti-HLA-DR) and IgG2b (anti-CD11c) isotypes, respectively.

Phenotyping with mAb

In order to characterize fresh $CD11c^+$ and $CD11c^-$ DC with unconjugated primary antibodies (Fig. 2), it was necessary to modify the procedure shown in Fig. 1a by omitting PerCP–anti-HLA-DR, so that the sorted DC would not be labelled with anti-HLA-DR antibodies. In the modified procedure, anti-CD14 (ATCC; TIB228) was substituted for anti-CD11b. 'Cocktail non-adherents' were then stained with FITC–goat anti-mouse IgG/IgM (Boehringer Mannheim). The larger, $cocktail^-$ cells (CD3, CD14, CD16, CD19-negative) were selected using a cell sorter. Aliquots of these cells were then stained with primary mouse mAb, followed by FITC–goat anti-mouse IgG/IgM. Cells were quenched with 100 μg/ml mouse IgG, and then stained with PerCP–anti-HLA-DR and PE–anti-CD11c. DR^+ cells accounted for more than 90% of the sorted population. By gating for DR^+ cells and the presence or absence of CD11c, the level of fluorescein staining on the $CD11c^+$ and $CD11c^-$ cells could be analysed on a FACScan® (BDIS). Most of the antibodies for phenotyping have been described elsewhere.[10] All of the mAb used for phenotyping were unconjugated. The following mAb were gifts or were purchased: CD13 (M812; Dako, Carpinteria, CA), CD34 (Oncogene Science, Manhasset, NY), and CD33 (gift of Dr David Scheinberg, New York, NY).[13]

Mixed leucocyte reactions

DC were irradiated with 1500 rads of ^{137}Cs γ radiation, and were mixed at graded doses with 2×10^5 purified allogeneic T cells in 96-well, flat-bottomed microwell plates. T cells were enriched by rosetting mononuclear cells with neuraminidase-treated sheep erythrocytes. Contaminating non-T cells were removed by γ-globulin panning, followed by panning with anti-MHC class II antibodies. Tritiated thymidine ([^{3}H]Tdr) incorporation was measured at 120–132 hrs (6 Ci/mmol; 1 μCi/well final).

RESULTS

Purification of two subsets of DC from human blood

In this paper, we have used the previously established criteria for identifying DC,[11] i.e. class II MHC^+ cells that are negative for lineage-specific antigens. The T-cell stimulatory activity of fresh blood[9] and bone marrow[14] mononuclear cells can be enriched by depleting cells that express lineage-specific antigens (CD3, CD14, CD16, CD19). Approximately 1% of peripheral blood mononuclear cells (PBMC) are negative for lineage markers, but are $HLA\text{-}DR^+$ (data not shown). In a previous study,[10] mononuclear cells were first depleted of T cells by E-rosetting, and monocytes were removed by γ-globulin panning. A mixture of antibodies to B, T and NK cells was then used to pan other lineages. Finally, the non-adherent cells were stained with an independent cocktail of mAb to monocytes, B and NK cells, and purified by two-colour sorting as $cocktail^-$, $HLA\text{-}DR^+$. By monitoring the enrichment obtained at every step of the purification, it was realized that almost half of the candidate DC ($cocktail^-$, DR^+ cells) were

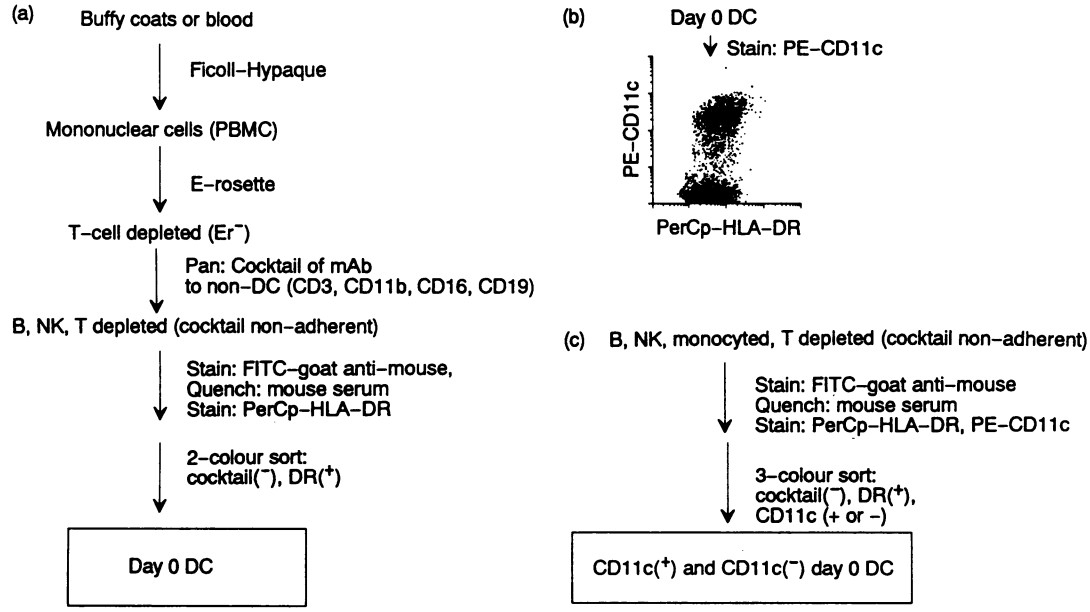

Figure 1. (a) Purification of DC from fresh blood. (b) Staining of fresh DC with PE–CD11c reveals two subsets of DC present in blood. Fresh DC, purified as in Fig. 1a, are stained with PE–CD11c. (c) Purification of two subsets of DC from fresh blood.

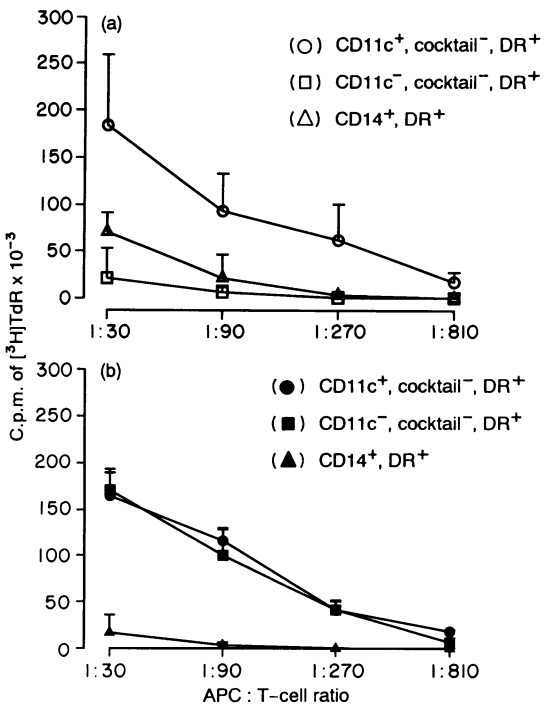

Figure 2. The MLR stimulatory capacity of CD11c$^-$ and CD11c$^+$ DC and CD14$^+$ monocytes, with and without culture in monocyte-conditioned medium. Graded doses of APC were used to stimulate 2×10^5 allogeneic T cells. Cells were pulsed with [^{3}H]TdR for 12 hr after 120 hr of culture. (a) APC were added freshly to allogeneic T cells. (b) APC were cultured for 36 hr in monocyte-conditioned medium, then washed three times before adding them to allogeneic T cells. This experiment is representative of three.

lost during γ-globulin panning. The purification procedure was therefore modified (Fig. 1a), by eliminating the γ-globulin adherence step and by adding a monocyte marker to the cocktail of mAb used for panning non-DC. The cocktail non-adherents were then purified as before by two-colour sorting.

When we observed the candidate DC under the microscope, it was clear that there were at least two populations of cells. Roughly half the cells had the appearance of medium-sized lymphocytes, as described previously.[10] The rest had a more ruffled appearance, and looked more like monocytes. Approximately half of the candidate DC stained with CD11c (Fig. 1b), unlike the cells purified in the prior procedure, which were mostly CD11c$^-$.[10] To characterize the CD11c$^+$ and CD11c$^-$ cells further, we used three-colour sorting (FITC–goat anti-mouse IgG and M, PerCP–HLA-DR, and PE–CD11c; Fig. 1b,c).

T cell-stimulating function of DC subsets and monocytes

The fresh CD11c$^-$ DC were poor stimulators of allogeneic T cells (Fig. 2a), but become potent APC after 36 hr culture in monocyte-conditioned medium (Fig. 2b), as previously shown.[10] If the immature DC are cultured for 36 hr in 10% human serum instead, they remained poor stimulators (data not shown). In contrast, the CD11c$^+$ DC were potent APC, whether fresh (Fig. 2a) or cultured (Fig. 2b). Monocytes were weak APC with or without culture in monocyte-conditioned medium.

Morphology of fresh and cultured CD11c$^+$ and CD11c$^-$ subsets compared with monocytes

Cytospins of cells cultured for 1 hr in 10% human serum or 36 hr in monocyte-conditioned medium were prepared. In fresh preparations, the CD11c$^-$ cells were medium-sized and round, with simple oval or slightly indented nuclei (Fig. 3). They expressed moderate but heterogeneous levels of HLA-DR. The CD11c$^+$ cells were less round, had mildly ruffled borders, and

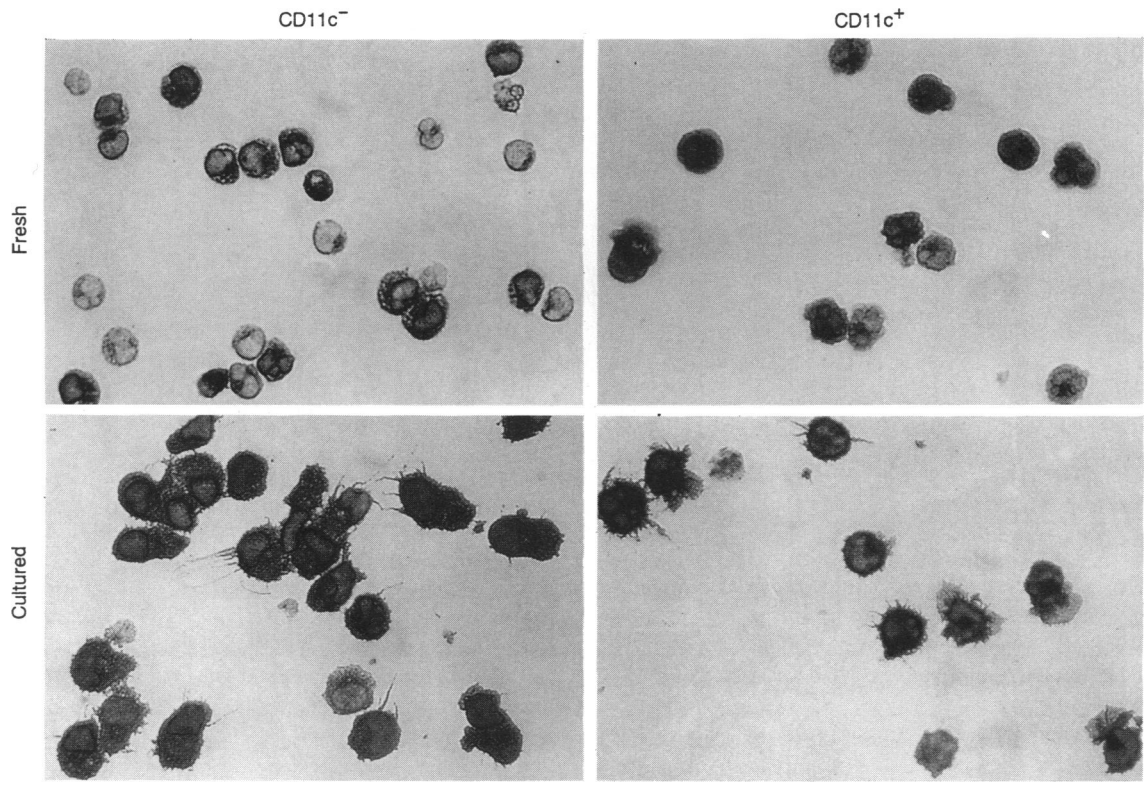

Figure 3. Cytospin morphology of CD11c$^-$ and CD11c$^+$ DCs. Top: cells were prepared without culturing (as in Fig. 1c), then were cultured for 1 hr at 37° in 10% human serum. Bottom: cells were isolated freshly and then cultured for 36 hr in monocyte-conditioned medium. Staining was performed with anti-HLA-DR (ATCC; HB96) of IgG1 isotype. Monocyte-conditioned medium only affected the morphology of the CD11c$^-$ subset.

more complicated or lobulated nuclei. These cells expressed a homogeneous and higher level of HLA-DR. CD14$^+$ cells (data not shown) resembled the CD11c$^+$ cells in morphology, but expressed much lower and variable levels of HLA-DR than either DC subset.

Upon culture in the presence of monocyte-conditioned medium, the CD11c$^-$ cells enlarged, developed processes and up-regulated HLA-DR to a homogeneously high level. If these cells were cultured for 36 hr in 10% human serum instead, some of the cells enlarged to variable extents, and HLA-DR was only partially up-regulated, again to variable levels (data not shown). With culture, the CD11c$^+$ cells also enlarged and developed processes, but to an even greater extent, and also up-regulated HLA-DR to a high level. These changes were not dependent on the monocyte-conditioned medium. On the other hand, the CD14$^+$ cells (data not shown) changed less perceptibly with culture. After culture these cells appear slightly larger, but expressed low and variable levels of HLA-DR.

We conclude that the CD11c$^-$ subset is immunologically immature, but develops typical dendritic morphology and T-cell stimulatory activity in the presence of monocyte-derived cytokines. The CD11c$^+$ cells are mature, but develop more characteristic morphology and phenotype after culture.

Surface phenotype of fresh CD11c$^+$ and CD11c$^-$ DC compared to CD14$^+$ monocytes

In studying the expression of myeloid markers on these three populations, it became apparent that CD11c$^-$ DC expressed only very low levels of CD33, CD32 and CD11b, and lacked CD13 and CD64 (Fig. 4). In contrast, CD11c$^+$ DC shared many antigens with monocytes, including CD13 and CD33. Their levels of expression of the Fc receptors CD32 and CD64 and the β_2-integrin CD11b were lower than those of monocytes.

Among the cocktail$^-$ blood mononuclear cells, there were a few (about 3.5% of total) CD34$^+$ cells present. These were probably not DC, but instead contaminating progenitors. While the total number of cocktail negatives decreased by 50% over 2 days of culture in monocyte-conitioned medium, the CD34$^+$ cells expanded twofold, and did not up-regulate their expression of HLA-DR (data not shown).

All three populations expressed the T-cell antigen CD4. CD11c$^-$ cells expressed more CD4 than CD11c$^+$ cells, which in turn expressed more than monocytes. Low levels of the T-cell antigens CD2 and CD5 were present on the CD11c$^+$ subset.

In general, fresh CD11c$^+$ cells expressed somewhat higher levels of MHC proteins and adhesion molecules than CD11c$^-$ DC or monocytes. Notably, all three cell populations expressed low or undetectable levels of the co-stimulator B7/BB1.

CD11c$^-$ DC appeared to be in a resting state, since they expressed the resting T-cell marker CD45RA, while CD11c$^+$ DC and monocytes both expressed the activation marker CD45RO. None expressed CD25. CD40 was present at low levels on both CD11c$^+$ and CD11c$^-$ DCs, but was barely detectable on monocytes.

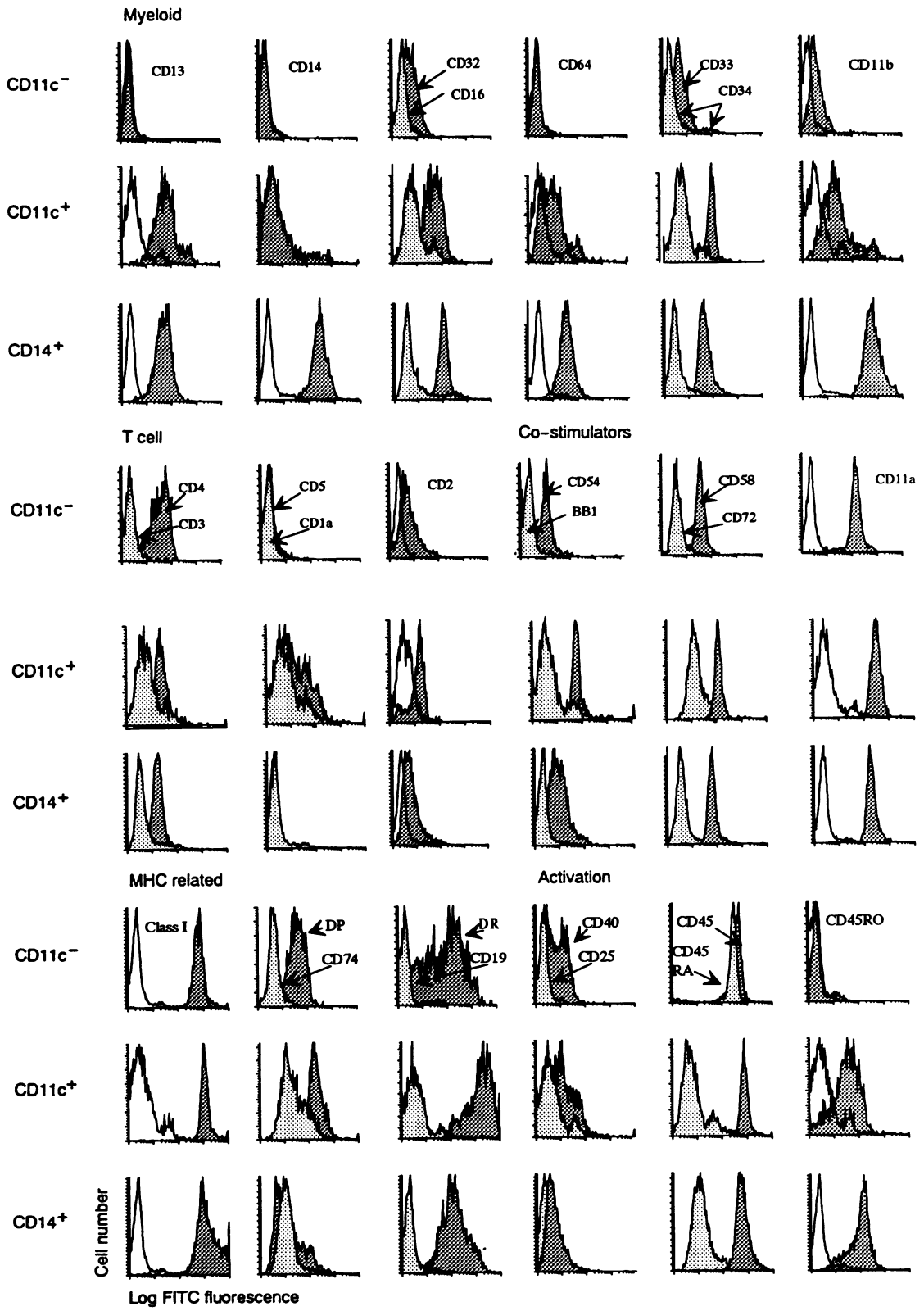

Figure 4. Phenotype of fresh CD11c$^-$ and CD11c$^+$, DC compared with CD14$^+$ monocytes. Cell subsets are labelled on the left. The mAb used for FACS staining against CD antigens are written above each histogram, and were unconjugated. Isotype-matched controls are indicated by the unfilled histograms.

DISCUSSION

Using a modified purification procedure, we have identified two subsets of DC in fresh human blood, the properties of which are summarized in Table 1. We initiated the current study after realizing that significant numbers of candidate DC were lost during isolation of fresh blood DC. This loss occurred during the monocyte-depletion step, wherein cells are applied to plastic dishes coated with γ-globulin at 37°. This method removes some FcR-bearing cells and cells that actively adhere to plastic.

The enrichment of DC from human blood is usually attained by serially depleting other leucocyte lineages. Monocytes are often depleted by attachment of strongly FcR^+ cells to dishes coated with human γ-globulin ('FcR panning'),[8] while other cells which express lower levels of Fc receptors (B cells and CD16-bearing NK cells) do not adhere.[11] By monitoring T-cell stimulatory activity in different cell fractions during the purification, we found that adherence to γ-globulin-coated Petri dishes depleted APC activity (data not shown). This suggested that a population of FcR^+, or transiently plastic adherent, allostimulatory DC was being removed. When we then eliminated γ-globulin adherence from our purification scheme, we were able to isolate a second, mature, FcR^+, $CD11c^+$ population of blood DC. Most prior purifications have utilized a culture period prior to FcR panning. Under these circumstances, FcR panning does enrich for APC activity;[8] thus, we suspect that mature DC in the cultured population have lost their adhesivity. The first description of human blood DC emphasized the transient nature of their adherence to plastic,[7] and the fact that DC become non-adherent after overnight culture. In lieu of FcR panning, we simply added an anti-CD11b mAb to the cocktail of mAb against non-DC used in the antibody panning procedure. CD11b is expressed on both monocytes and NK cells at high levels, but is expressed at low levels on DC. CD11b panning is known to deplete NK cells well.[15] We found anti-CD11b to be much more effective than anti-CD14 for removing monocytes. We do not fully understand this finding, since both antigens are abundant on monocytes, and both mAb share the same isotype, IgG2b. Possibly our anti-CD14 is a low-affinity mAb, or has a fast off-rate.

It is striking that $CD11c^+$ DC are potent stimulators, even though they express relatively low levels of adhesion proteins. Adhesion/co-stimulator proteins are only marginally elevated on fresh $CD11c^+$ DC compared to monocytes and immature DC, yet these cells are far superior APC. Most probably, these proteins, especially B7/BB1, are up-regulated by $CD11c^+$ cells after establishing contact with allogeneic T cells. There is precedent for this, since cultured DC up-regulate many co-stimulators after exposure to allogeneic T cells.[16] Triggering for B7 up-regulation may be induced after class II binds the T-cell receptor[17] or after CD40 binds to gp39 on activated T cells.[18] Furthermore, we found a dramatic up-regulation of MHC proteins and co-stimulators on the $CD11c^+$ population after 36 hr of culture. $CD11c^-$ DC underwent a similar strong up-regulation in the presence of monocyte-conditioned medium, but in culture medium not supplemented with cytokines, expression increased only weakly, and to variable levels.

Most previous studies of DC were performed on cultured cells, and emphasized their morphology, motility,[19] and later their unique ability to initiate primary immune responses.[1] The majority of DC purified by standard methods from cultured human blood mononuclear cells are $CD11c^+$, while a minority (10–30%) are $CD11c^-$.[10] DC that are isolated after 2 days of culture have the characteristic features of this lineage: irregular shape, high class II MHC and accessory proteins, and strong stimulatory activity. The $CD11c^+$ subset in fresh blood expresses all of the features of classical DC during culture, suggesting that it gives rise to the standard, day 2-cultured DC population. The $CD11c^-$ subset only develops into typical DC in the presence of monocyte-derived cytokines. The poor viability of the $CD11c^-$ subset with culture is likely to explain why a variable minority of conventional day 2 DC are $CD11c^-$, while at least half of $HLA\text{-}DR^+$, lineage-negative day 0 blood mononuclear cells are $CD11c^-$. In fact, we have found that some commercial sera (which we used in a previous paper)[10] are particularly poor at maintaining the viability of the $CD11c^-$ subset.

Clearly, the cells described by Thomas *et al.*[9] contained the $CD11c^+$ subset but not the $CD11c^-$ subset, since they selected for $CD33^+$, $CD14^{dim}$ cells. It is possible that their sorted population contained other cells besides the $CD11c^+$ subset. Using their criteria ($CD33^+$ and $CD14^{dim}$), they found 2–3% of PBMC were DCs, while using our criteria (lineage-negative and $HLA\text{-}DR^+$), we found that only 1% of PBMC were DC.

It is evident that many of the DC isolated from certain tissues,[20,21] especially skin,[22–24] are immunologically immature. Skin[25,26] and other tissue[27] DC are bone marrow-derived. *In vivo*, Langerhans' cells express low levels of CD11c.[28] In contrast, a strongly $CD11c^+$ population of DC is found in lymphoid tissues, especially spleen.[29,30] Furthermore, it is known that DC from non-lymphoid tissues can migrate to the spleen, as in the case of cardiac allografts.[31] This suggests to us that the two subsets of DC that we have identified in fresh blood could be migrating in opposite directions: the $CD11c^-$ subset to non-lymphoid tissues such as skin, presumably originating from the bone marrow, and the $CD11c^+$ subset to spleen or lymph nodes, possibly originating from tissues after being stimulated by contact with antigens.

Table 1. Summary of traits that distinguish immunologically immature and mature DCs from monocytes

Characteristics	Subpopulation of fresh cells		
	$CD11c^-$ DC	$CD11c^+$ DC	$CD14^+$ Monocytes
Class II MHC, e.g. HLA-DP	+	+ +	+/−
CD45RA ('resting' marker)	+ +	−	+/−
CD45RO ('activated' marker)	−	+	+ +
Lineage markers for T, NK and B: CD3, CD16, CD19	−	−	−
γ-globulin adherence	−	+	+
Allostimulation when freshly isolated	−	+	−
Allostimulation after culture	+*	+	−
Up-regulation of MHC and co-stimulators after culture	+*	+	−

*Occurs only in the presence of monocyte-conditioned medium.

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