CD16\(^+\) and CD16\(^-\) human blood monocyte subsets differentiate \textit{in vitro} to dendritic cells with different abilities to stimulate CD4\(^+\) T cells

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Keywords: cellular differentiation, IL-12 secretion, monocyte subpopulations, \(T_{h1}/T_{h2}\)

Abstract

Experimental protocols for cancer immunotherapy include the utilization of autologous monocyte-derived dendritic cells (moDC) pulsed with tumor antigens. However, disease can alter the characteristics of monocyte precursors and some patients have increased numbers (up to 40\%) of the minor CD16\(^+\) monocyte subpopulation, which in healthy individuals represent 10\% of blood monocytes. At the present, the capacity of CD16\(^+\) monocytes to differentiate into DC has not been evaluated. Here, we investigated the ability of CD16\(^+\) monocytes cultured with granulocyte–macrophage colony-stimulating factor, IL-4 and tumor necrosis factor-\(\alpha\) to generate DC \textit{in vitro}, and we compared them to DC derived from regular CD16\(^-\) monocytes. Both monocyte subsets gave rise to cells with DC characteristics. They internalized soluble and particulate antigens similarly, and both were able to stimulate T cell proliferation in autologous and allogeneic cultures. Nevertheless, CD16\(^+\) moDC expressed higher levels of CD86, CD11a and CD11c, and showed lower expression of CD1a and CD32 compared to CD16\(^-\) moDC. Lipopolysaccharide-stimulated CD16\(^-\) moDC expressed increased levels of IL-12 p40 mRNA and secreted greater amounts of IL-12 p70 than CD16\(^+\) moDC, whereas levels of transforming growth factor-\(\beta1\) mRNA were higher on CD16\(^+\) moDC. Moreover, CD4\(^+\) T cells stimulated with CD16\(^+\) moDC secreted increased amounts of IL-4 compared to those stimulated by CD16\(^-\) moDC. These data demonstrate that both moDC are not equivalent, suggesting either that they reach different stages of maturation during the culture or that the starting monocytes belong to cell lineages with distinct differentiation capabilities.

Introduction

Human blood monocytes are a generalized source for \textit{in vitro} generation of dendritic cells (DC), the most powerful antigen-presenting cells (APC) of the immune system (1). Monocyte-derived DC (moDC) are the chosen cells for designing a vast array of immunotherapy-based protocols, mostly for cancer patients (2). \textit{In vitro}, human DC have been developed from blood monocytes cultured with granulocyte–macrophage colony stimulating-factor (GM-CSF) and IL-4 (3,4). These cells have the characteristics of immature DC and can be further induced to mature by inflammatory stimuli [tumor necrosis factor (TNF)-\(\alpha\), IL-1\(\beta\) and lipopolysaccharide (LPS)] (3,5) or by CD40 ligation (3). Monocytes can also give rise to DC \textit{in vitro} after migration through an endothelial barrier (6) and, \textit{in vivo}, they differentiate into DC after phagocytosis of s.c. injected fluorescent microspheres (7). Heterogeneity within the human monocyte population has been extensively reported (8–10), but few studies have been conducted to address the characterization of DC generated from separate populations of blood monocytes (10,11). Of special interest is the minor CD14\(^-\)CD16\(^+\) circulating pool of monocytes (CD16\(^+\) monocytes), which accounts for 10\% of total monocytes in healthy individual (8), but their numbers are drastically increased in some pathological conditions with respect to regular monocytes (CD14\(^+\)CD16\(^-\)). Most of those diseases

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Experimental protocols for cancer immunotherapy include the utilization of autologous monocyte-derived dendritic cells (moDC) pulsed with tumor antigens. However, disease can alter the characteristics of monocyte precursors and some patients have increased numbers (up to 40\%) of the minor CD16\(^+\) monocyte subpopulation, which in healthy individuals represent 10\% of blood monocytes. At the present, the capacity of CD16\(^+\) monocytes to differentiate into DC has not been evaluated. Here, we investigated the ability of CD16\(^+\) monocytes cultured with granulocyte–macrophage colony-stimulating factor, IL-4 and tumor necrosis factor-\(\alpha\) to generate DC \textit{in vitro}, and we compared them to DC derived from regular CD16\(^-\) monocytes. Both monocyte subsets gave rise to cells with DC characteristics. They internalized soluble and particulate antigens similarly, and both were able to stimulate T cell proliferation in autologous and allogeneic cultures. Nevertheless, CD16\(^+\) moDC expressed higher levels of CD86, CD11a and CD11c, and showed lower expression of CD1a and CD32 compared to CD16\(^-\) moDC. Lipopolysaccharide-stimulated CD16\(^-\) moDC expressed increased levels of IL-12 p40 mRNA and secreted greater amounts of IL-12 p70 than CD16\(^+\) moDC, whereas levels of transforming growth factor-\(\beta1\) mRNA were higher on CD16\(^+\) moDC. Moreover, CD4\(^+\) T cells stimulated with CD16\(^+\) moDC secreted increased amounts of IL-4 compared to those stimulated by CD16\(^-\) moDC. These data demonstrate that both moDC are not equivalent, suggesting either that they reach different stages of maturation during the culture or that the starting monocytes belong to cell lineages with distinct differentiation capabilities.

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involve altered cytokine production, such as sepsis (12), AIDS (13) or solid tumors (14) and, thus, the expansion of CD16+ monocytes has been associated with acute or chronic inflammation. In support of this hypothesis, CD16+ monocytes constitutively produce pro-inflammatory cytokines in HIV-infected patients (13) and, in vitro, they secrete very low or undetectable levels of IL-10 after LPS stimulation (15). Recently, it has been shown that a subset of CD64+CD16+ blood monocytes exhibits phenotypical and functional DC characteristics (16).

The moDC belong to the so-called myeloid subset of DC (17). They express several myeloid-associate markers (CD11b, CD11c and CD33) (3), immature myeloid DC exhibit a high capacity of antigen uptake (3), depend on GM-CSF for survival (3–5) and can be converted into macrophages after cytokine withdrawal or by culture with macrophage colony-stimulating factor (5). In addition to myeloid DC, there is a subset of cells in human lymph nodes, tonsils and blood, the plasmacytoid cells (18), which appears to be of lymphoid origin. Functionally, moDC and plasmacytoid-derived DC can skew an allogeneic primary response to the Th1 or Th2 origin. Functionally, moDC and plasmacytoid-derived DC can skew an allogeneic primary response to the Th1 or Th2 pathway respectively (19). Thus, different populations of DC exist, which could apparently determine the type of immune response by some yet unknown mechanisms.

Human monocytes have been extensively considered as a homogeneous cell population in terms of DC generation. However, the existence of monocyte and DC subsets has prompted us to investigate whether different monocyte subpopulations give rise to DC with unique characteristics. Regarding the expansion of the CD16+ monocyte subset in some diseases in which monocytes are employed to generate DC for immunotherapy, we performed a comparative study of the putative DC generated from these cells and the DC derived from the major subset of human blood monocytes (CD16-). Our data support the concept that these DC are not phenotypical or functionally equivalent.

**Methods**

**Media and reagents**

The culture medium was RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids, (HyClone, Logan, UT), 1% penicillin/streptomycin and 50 μM 2-mercaptoethanol (Gibco/BRL, Grand Island, NY), referred to complete medium (CM). For monocyte culture, 5% heat-inactivated pooled human sera was added to the CM, as we found that this combination of sera gave the best yields of moDC (data not shown). The following recombinant human cytokines were shown). The following recombinant human cytokines were used: GM-CSF (PharMingen, San Diego, CA), IL-4 (Calbiochem, La Jolla, CA) and TNF-α (R & D Systems, Minneapolis, MN).

**Cell separation**

Peripheral blood mononuclear cells (PBMC) were isolated fromuffy coats of healthy volunteers by Ficoll-Hypaque (Gibco/BRL) density-gradient centrifugation. Subsequently, CD56+CD16+ cells and CD56-CD16-CD14+ cells (referred to as CD16+ monocytes and CD16- monocytes) were separated by magnetic cell sorting, using MACS isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, PBMC were first incubated with MACS anti-CD56 antibody conjugated to paramagnetic microbeads in order to eliminate the NK (CD16+) cell fraction. NK-depleted PBMC were further incubated with MACS anti-CD16 antibody to isolate CD16+ monocytes. CD56-CD16+ PBMC were finally incubated with MACS anti-CD14 antibody to obtain the CD16+CD14+ monocyte fraction. Autologous and allogeneic CD4+ T lymphocytes were separated by negative selection using the MACS CD4+ T cell isolation kit (Miltenyi Biotec). Naive CD4+ T cells were obtained from umbilical cord blood.

**In vitro differentiation of PBMC**

Isolated CD16+ and CD16- monocytes were cultured in CM plus 5% human serum at 10⁶ cells/ml in polystyrene six-well plates (Costar, Cambridge, MA), supplemented with GM-CSF (1000 U/ml) and IL-4 (15 ng/ml). The cultures were fed with fresh medium and cytokines every 2 days. After 6 days, non-adherent cells were harvested and replated at 0.5×10⁶ cells/ml in medium containing GM-CSF, IL-4 and TNF-α (40 ng/ml) for an additional 2 days.

**Morphological analysis**

Freshly isolated monocytes and moDC (day 8 of culture) were centrifuged onto slides using a Cytospin 3 (Shandon Lipshaw, Pittsburgh, PA). Then, cells were fixed in methanol for 3 min, stained with May–Grünwald–Giemsa solution and analyzed by light microscopy on an Olympus BX60 microscope.

**Immunofluorescence assays**

The following mAb were used: HLA-DR (TU36), CD1a (HJ149), CD2 (MT910), CD8 (RPA-T8), CD11a (SPV-L7), CD11b (ICRF44(44)), CD11c (B-ly6), CD14 (M5E2), CD16 (3G8), CD19 (B43), CD32 (FLI8. 26(2003)), CD40 (SF10), CD49d (9F10), CD56 (10.1), CD64 (10.1), CD80 (HB15a), CD83 (IT2.2) and CD206 (19.3), all from PharMingen, except CD2 and CD56 (Dako, Glostrup, Denmark), CD11a (Zymed, San Francisco, CA), and CD83 (Immunotech, Marseille, France). Monocytes were analyzed immediately after isolation by direct labeling with anti-CD16, anti-CD14, anti-CD2 and anti-CD56 antibodies conjugated to FITC. After overnight culture (day 0) and at the end of the culture (day 8), monocytes and moDC were respectively stained with the mAb shown above followed by FITC-conjugated goat F(ab’)2 anti-mouse Ig polyclonal antibody (Dako). For two-color assays, freshly isolated CD56-CD16+ monocytes were incubated with FITC-labeled anti-CD16, anti-CD11b or anti-CD64 mAb and phycoerythrin (PE)-conjugated anti-CD14 mAb. Samples were analyzed on a FACSort (Becton Dickinson, Mountain View, CA) using CellQuest software. The cytometer was calibrated with Sphero™ fluorescent particles (Becton Dickinson).

**Endocytosis assays**

FITC-coupled dextran (FITC–DX; M = 40,000) and zymosan (FITC–Zym) (Molecular Probes, Eugene, OR) were diluted in PBS, and used at 0.5 and 0.125 mg/ml respectively. Freshly isolated monocytes and moDC at different days of culture were incubated with FITC–DX or FITC–Zym for 30 min at
37°C; then, uptake was stopped by washing the cells 4 times with ice-cold PBS containing 0.1% BSA and 0.01% sodium azide. Controls of antigen internalization were established by incubating the cells with FITC–DX or FITC–Zym at 0°C. Samples were analyzed on a FACSRef and values obtained at 0°C were subtracted from those obtained at 37°C.

Cytokine detection on LPS-stimulated moDC

moDC (1 × 10^6; day 8 of culture) were stimulated with LPS (Escherichia coli 0111:B4; Sigma, St Louis, MO) at 0.5 µg/10^6 cells/ml for 6 h. Appropriate controls without addition of LPS were set. RNA was isolated from cell lysates using Trizol reagent (Gibco/BRL), and cDNA was synthesized by reverse transcription with oligo(dT)15 and Superscript RNase H-reverse transcriptase (Gibco/BRL). PCR was performed in a 25 µl volume using 5 µl cDNA, 2.5 µl 10×PCR reaction buffer, 0.5 µl MgCl2 (50 mM), 0.5 µl dNTPs (10 mM), 10 pmol of the 5′ and 3′ amplification primers, and 1.5 U Taq polymerase (Gibco/BRL). PCR was conducted for 40 cycles at 94°C for 1 min, 60°C for 50 s and 72°C for 1.5 min. β-Actin mRNA was amplified as a control of RNA integrity. Oligonucleotide primers (15,20) and the expected sizes of PCR products are: β-actin: 5′-gtggtgggcccccagcaca and 3′-cctcttaagtgtcagcagtttc (539 bp); IL-1β: 5′-atgtattgcttattacggtgaat and 3′-ttcactcataattgtgtccttc (777 bp); IL-6: 5′-agtccctttccttggttgaag and 3′-tgggtgaagctgacccagccc (777 bp); TNF-α: 5′-atggtccttgcttcc and 3′-tgagggctcttcggcaaat (621 bp); IL-10: 5′-atgcccaagtgagaacaagccca and 3′-tggtgtaagctgacccagcccct (352 bp); IL-12 (p40 subunit): 5′-ggaggcctcggagaattg and 3′-agttgaggctcgtggaggg (655 bp); TGFB: 5′-aagcagagtagaca-cacagcatatatgctc and 3′-attggagcttgagcagagcata-cagcaag (645 bp); and TNF-α: 5′-gttctccagcttctctcct and 3′-actatctggtggaggtcttct (607 bp). PCR products were separated on 1.5% agarose gels and ratios of mRNAβ-actin band densities were determined by densitometry in an ImageMaster MasterLab software (Amersham, Little Chalfont, UK).

Production of IL-12 p70 in the supernatants of LPS-stimulated moDC was quantified by ELISA. Immature moDC (10^5; day 6 of culture) and moDC at different times of maturation with TNF-α (3, 12, 24 and 48 h) were incubated for 24 h with LPS (50 ng/10^6 cells). ELISA kit was obtained from PharMingen (lower limit of detection: 4 pg/ml).

T cell activation

For proliferation assays, purified CD4^+ T cells (1.5 × 10^5) either from allogeneic or autologous PBMC were seeded into 96-well round-bottom microplates (Costar) and cultured with graded numbers of stimulator cells (monocytes or mature moDC; day 8 of culture) in CM. Proliferation of T cells was measured on day 5 after an 18-h pulse with [3H]thymidine (0.5 µCi/well; Amersham). Tests were conducted in triplicate and results are shown as the mean c.p.m. of triplicates. For cytokine production assays, 1.5 × 10^6 purified CD4^+ cord blood T cells were co-cultured in 24-well plates (Costar) with allogeneic mature moDC at 10:1 or at 100:1 ratios in CM. Controls were set without addition of DC. After 6 days of priming, T cells were re-stimulated for 6 h (for intracellular staining of IL-4 and IFN-γ) or 24 h (for quantitation of cytokine secretion) with immobilized anti-CD3 antibody (5 µg/ml, clone UCHT1; PharMingen).

Fig. 1. Comparative cytofluorographic and morphological analysis of magnetically isolated monocytes. (A) Forward and side light scatter profile of total PBMC and isolated CD16^+ and CD16^- monocytes. (B) Staining of monocytes with FITC-conjugated anti-CD14 and anti-CD16 mAb (shaded histograms). Empty grey profiles represent the staining with FITC-labeled anti-CD2 (on CD16 histograms) and with anti-CD56 (on CD14 histograms). Discontinuous lines are isotype-matched irrelevant antibodies used as negative controls. Percentage of CD14^- or CD16^- cells and mean fluorescence intensity (MFI) are indicated on the top of the histograms. (C) Light microscopic analysis of freshly isolated CD16^- and CD16^- human blood monocytes stained with May–Grunwald–Giemsa solution. (D) Two-color immunofluorescence analysis of CD16^- monocytes. Cells were labeled with FITC-conjugated anti-CD16, anti-CD11b or anti-CD64 mAb and PE-conjugated anti-CD14. Quadrants were set to exclude cells that stained with appropriate isotype-matched control antibodies. Percentages of positive cells in each quadrant are indicated. Results are representative of one out of 10 experiments in (A), (B) and (C), and of one out of four experiments in (D).

Analysis of IL-4 and IFN-γ production

For intracellular staining of IL-4 and IFN-γ, GolgiPlug™ (PharMingen) was added to the cultures 2 h before cells were collected, to prevent cytokine secretion. Then, cells were fixed and permeabilized with a Cytofix/Cytoperm kit (PharMingen) and incubated with FITC-labeled anti-IL-4 (clone 4S.B3) and PE-labeled anti-IL-4 (clone BD4-8) antibodies (PharMingen). Samples were analyzed on a FACSRef. Quantitation of IFN-γ and IL-4 in the supernatants was performed by a sandwich ELISA. ELISA kits were purchased
from PharMingen. The lower limits of detection were 4 pg/ml for both IL-4 and IFN-γ.

Statistical analysis
The statistical significance of the data was determined by the Student’s paired t-test. Each experiment was performed a minimum of 3 times.

Results

Purification and phenotypical analysis of CD16+ and CD16- monocytes

Blood monocyte subsets were isolated as described in Methods. The yields obtained were 11.4 ± 1 and 1.73 ± 0.2% of CD16- and CD16+ monocytes from total PBMC respectively. Figure 1(A and B) shows the purity of both populations, which was determined on freshly isolated cells by using anti-human CD16 and anti-CD14 mAb. Purity of isolated cells was usually >95%. CD16+ monocytes showed a more heterogeneous forward and side light scatter profile than regular CD16- monocytes, but they were localized basically into the monocyte region (Fig. 1A) and they displayed a typical monocyte morphology (Fig. 1C).

Comparative phenotypical analysis of CD16+ and CD16- monocytes was conducted, and results are summarized in Table 1. Both cell populations stained negatively for CD2, CD56 (Fig. 1B), CD8 and CD19 (not show), CD16+ monocytes expressed lower levels of the myeloid markers CD14 and CD33, and of Fcγ receptor I (FcγRI, CD64) than regular CD16- monocytes, and some CD16- monocytes were negative for these markers. The levels of molecules related to antigen presentation and co-stimulation (HLA-DR, CD40, CD80 and CD86) were elevated on CD16+ monocytes compared to CD16- monocytes. CD16+ monocytes also showed significantly increased levels of CD11a and CD64, equivalent levels of CD11c, and lower expression of CD11b than CD16- monocytes. In relation to DC markers, CD16+ and CD16- monocyte subsets were CD1a- and a few cells showed low levels of CD83 (mean of 7% on CD16- monocytes and 18% on CD16+ monocytes), although differences were not significant.

As mentioned above, the expression of CD14, CD11b and CD64 was heterogeneous on CD16+ monocytes. Thus, we performed double-labeling assays on these cells in order to characterize their different phenotypic profiles (Fig. 1D). As expected, there is an inverse correlation among the levels of CD14 and CD16 on that monocyte subset (8). On the other hand, a high percentage of CD11b+CD16+ monocytes and CD64+CD16+ monocytes co-expressed CD14 (59 and 47% respectively). However, additional CD16+ monocyte subpopulations can be identified: CD11b+CD14- (20%), CD11b-CD14- (20%), CD64+CD14+ (13%) and CD64+CD14- (39%).

Stimulation of allogeneic CD4+ T cells with CD16+ or CD16- monocytes

In order to determine whether the initial phenotypic differences between CD16+ and CD16- monocytes had an effect on inducing primary T cell proliferation, we carried out allogeneic mixed lymphocyte reactions (alloMLR) by using overnight cultured monocytes as stimulator cells and allogeneic CD4+ T cells (Fig. 2). In a series of experiments, we found that CD16+ and CD16- monocytes induced T cell proliferation similarly, and only slight variations can be seen in different experiments. Therefore, no correlation was found between

Table 1. Expression of surface markers on freshly isolated monocytes (day 0) and after eight days of culture with GM-CSF, IL-4 and TNF-α.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Day 0</th>
<th>Day 8</th>
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<tr>
<td></td>
<td>CD16- monocytes (%)</td>
<td>CD16+ monocytes (%)</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>91 ± 12 (57 ± 6)</td>
<td>95 ± 4 (291 ± 78)</td>
</tr>
<tr>
<td>CD40</td>
<td>60 ± 27 (16 ± 5)</td>
<td>82 ± 13 (51 ± 30)</td>
</tr>
<tr>
<td>CD80</td>
<td>16 ± 18 (12 ± 3)</td>
<td>32 ± 15 (14 ± 3)</td>
</tr>
<tr>
<td>CD86</td>
<td>63 ± 18 (17 ± 7)</td>
<td>85.5 ± 9 (48 ± 16)</td>
</tr>
<tr>
<td>CD11a</td>
<td>100 (96.5 ± 18)</td>
<td>100 (186 ± 23)</td>
</tr>
<tr>
<td>CD11b</td>
<td>98 ± 1.5 (87 ± 38)</td>
<td>52 ± 10 (35 ± 6)</td>
</tr>
<tr>
<td>CD11c</td>
<td>100 (47 ± 5)</td>
<td>100 (38 ± 5)</td>
</tr>
<tr>
<td>CD14d</td>
<td>45 ± 26 (12 ± 2)</td>
<td>60 ± 20 (17 ± 2)</td>
</tr>
<tr>
<td>CD33</td>
<td>88.5 ± 9</td>
<td>71 ± 6</td>
</tr>
<tr>
<td>MR</td>
<td>95 ± 2</td>
<td>25 ± 9</td>
</tr>
<tr>
<td>CD16</td>
<td>2 ± 1</td>
<td>100 (46.5 ± 25)</td>
</tr>
<tr>
<td>CD32</td>
<td>100 (73.5 ± 26)</td>
<td>100 (75.5 ± 16)</td>
</tr>
<tr>
<td>CD64</td>
<td>91 ± 9 (17 ± 7)</td>
<td>32 ± 15 (16 ± 5)</td>
</tr>
<tr>
<td>CD1a</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CD83</td>
<td>7 ± 2 (13 ± 3.5)</td>
<td>18 ± 17 (18 ± 12)</td>
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</table>
Characterization of DC generated in vitro from CD16⁺ blood monocytes

The high accessory phenotype of CD16⁺ monocytes and their ability to stimulate an increased rate of T cell proliferation.

CD16⁺ and CD16⁻ monocytes can be driven into cells with dendritic morphology

CD16⁺ and CD16⁻ monocytes cultured with GM-CSF, IL-4 and TNF-α developed a DC morphology (Fig. 3). Cells rapidly (1–2 days of culture) became non-adherent to plastic. They were bigger in size, with eccentric round nuclei and a higher cytoplasm/nucleus ratio than monocytes precursors. Most of the CD16⁻ and CD16⁺ moDC developed long and sharp dendritic processes. However, some of the CD16⁺ moDC showed pseudopodia-like projections instead of developed dendrites (Fig. 3).

Phenotype of CD16⁺ and CD16⁻ moDC

CD16⁺ and CD16⁻ moDC showed a typical phenotypic profile of moDC (Table 1). They down-regulated several myeloid surface markers (CD14, CD33) and FcγR (CD16, CD64). CD32 was the only remaining FcγR expressed on moDC (74% of CD16⁻ moDC and 55% of CD16⁺ moDC, P < 0.05). During differentiation, both cell types progressively increased their levels of co-stimulatory molecules CD40, CD80 and CD86, and CD16⁻ moDC augmented its expression of HLA-DR as well. Thus, levels of CD40, CD80 and HLA class II tended to become equal on both DC populations at the end of the culture, despite the initial differences. Only CD86 remained significantly higher on CD16⁺ moDC (P < 0.05).

CD16⁺ moDC greatly increased their levels of CD11b compared to those monocyte precursors. Levels of CD11c were strongly up-regulated on both moDC during differentiation, but its expression became significantly higher on CD16⁺ moDC (P < 0.005). On the other hand, the expression of CD11a diminished after differentiation, although it was still greater on CD16⁺ moDC (P < 0.05).

One of the most striking differences between CD16⁻ and CD16⁺ moDC was the expression of CD1a. Most of CD16⁻ moDC were CD1a⁻ and they expressed this receptor at high density on their cell surface, whereas CD16⁺ moDC expressed CD1a at very low levels (82 versus 38%, P < 0.005 and 165 versus 70 mean fluorescence intensity, P < 0.05). At this time, an average of 60–77% of DC was CD83⁺. No significant differences were found between CD16⁻ and CD16⁺ moDC regarding CD83 surface expression.

Antigen internalization by CD16⁺ and CD16⁻ monocytes and moDC

Freshly isolated monocytes and moDC collected at different days of culture (days 3, 6 and 8) were analyzed for their
ability to capture a fluoresceinated soluble antigen, DX, and for their capacity to phagocytose particulate antigens, such as Zym. The ability of CD16+ monocytes to accumulate both DX and Zym was severely diminished in relation to freshly isolated CD16+ monocytes (Fig. 4A and B). However, as the differentiation from monocytes to moDC took place, both cell types similarly increased their ability for antigen uptake. Cells reached the maximum of antigen accumulation as immature moDC (day 3–6) and uptake was reduced after TNF-α-induced maturation (day 8) (Fig. 4A and B). Since DX and Zym uptake can be mediated by the mannose receptor (CD206, MR) (21,22), we examined the expression of this molecule on fresh monocytes (day 0), and on immature (day 6) and mature (day 8) moDC (Fig. 4C). CD16+ monocytes barely expressed MR, while CD16+ monocytes expressed it at high levels. Immature moDC showed the highest expression of MR, whereas it was greatly diminished on mature moDC.

Cytokine production by LPS-stimulated moDC
Mature CD16- and CD16+ moDC were stimulated with LPS, and production of IL-1β, IL-6, IL-10, IL-12 (p40), TNF-α and TGF-β1 mRNA was evaluated (Fig. 5A and B). All cytokines were detected in both moDC populations. Importantly, LPS-stimulated CD16- moDC produced higher levels of IL-12 mRNA than CD16+ moDC (64 ± 8 versus 34 ± 4 arbitrary units, P < 0.01). In contrast, the levels of TGF-β1 mRNA detected in CD16+ moDC were always increased compared to CD16- moDC (65 ± 18 versus 30.5 ± 12 arbitrary units, P < 0.01).

Further, we wanted to corroborate the data obtained for IL-12 mRNA at the protein level. Thus, we examined the secretion of IL-12 p70 heterodimer in cultures of 24-h LPS-stimulated moDC. Moreover, since a differential production of IL-12 by DC has been reported according to their stage of maturation (23), we evaluated the secretion of IL-12 by immature moDC (day 6 of culture) and by moDC induced to mature for 3, 12, 24 or 48 h with TNF-α (Fig. 5C). After LPS stimulation, immature CD16+ and CD16- moDC secreted the maximum levels of IL-12, and this production declined with time of maturation. However, secretion of IL-12 by CD16+ moDC was greatly augmented compared to CD16+ moDC at each time point evaluated. Secretion of IL-12 by CD16+ moDC was below the limit of detection of our assay (4 pg/ml) between 12 and 48 h of maturation, whereas production of IL-12 by CD16- moDC only became undetectable after 48 h of maturation. Unstimulated controls did not show detectable production of IL-12.

Stimulation of primary allo- and autoMLR by CD16- and CD16+ moDC
A unique feature of DC is to initiate antigen-specific T cell activation. Specifically, induction of autoMLR is an intrinsic property of DC. Therefore, we wanted to evaluate the ability of CD16- and CD16+ moDC to prime autologous and allogeneic CD4+ T cells in terms of proliferation rate and cytokine production.

Proliferation assays were established as shown in Fig. 6. CD16+ moDC induced a slight increase in the proliferation rate on autoMLR assays in relation to CD16- moDC, although differences were usually minimal (Fig. 6A). No differences were found in the allostimulatory ability of both moDC (Fig. 6B).

Further, we analyzed whether CD16- and CD16+ moDC induced a polarized Th1 response, as reported for myeloid DC in alloMLR (19). We established co-cultures of naive CD4+ T cells from cord blood with allogeneic mature moDC. The T cell cytokine profile evaluated at the single-cell level indicates that CD16- and CD16+ moDC induced a mixed Th1/Th2 response, and a few lymphocytes produced both IL-4 and IFN-γ (Th0 response) (Fig. 7A and Table 2).

IFN-γ and IL-4 were simultaneously detected in the culture supernatants regardless of the nature of DC (Fig. 7B), although the amount of IL-4 increased greatly at lower DC/T cell ratio (1/100). Remarkably, CD16+ moDC triggered increased
secretion of IL-4 by CD4+ T lymphocytes, ranging from 52 to 700% of the IL-4 values obtained after stimulation with CD16− moDC (Fig. 7C). The enhanced production of IL-4 was more evident at 1:100 DC/T cell ratio and, at that point, differences on the percentage of IL-4+ lymphocytes reached statistical significance (Table 2). That increase was also observed when we used adult CD4+ T lymphocytes as responding cells in allo- and autoMLR (data not shown). CD16+ moDC also augmented the amount of IFN-γ in the supernatants, but this increase was much lower than the observed for IL-4 (range 34–147%, Fig. 7C).

**Discussion**

The minor subset of circulating human CD16+ monocytes has been associated with mature tissue macrophages (8) and, recently, it has been shown that they share some features with DC (16). In this work, we isolated the CD56−CD16+ cellular fraction from total PBMC and we compared their ability to generate DC in vitro in relation to CD56−CD16−CD14+ regular monocytes (4). The data presented here indicated that CD56−CD16+ cells are constituted essentially by monocytes based on morphology and cell surface markers. Phenotypical analysis of CD16+ and CD16− monocytes confirmed most of the data already reported, i.e. CD16+ monocytes expressed higher levels of HLA class II, CD11a and CD49d, and lower levels of CD14, CD33, CD64 and CD11b, compared to CD16− monocytes (8, 24). However, heterogeneity in cell size as well as in the expression of classical myeloid markers (CD14, CD11b, CD33 and CD64) account for a more heterogeneous population than originally described (25). Nevertheless, the expression of other markers (HLA-DR,
Characterization of DC generated in vitro from CD16+ blood monocytes

CD86, CD11a, CD11c and CD32) was fairly homogeneous, which may indicate that this monocyte subset belongs to a single cell lineage. It was already reported that CD16+ monocytes could be further subdivided into CD64+ and CD64− (9,16). In addition, a new subset of putative human blood (M-DC8+) DC, which expressed the CD16 marker, has been identified (26). However, M-DC8+ cells were subsequently included as a subpopulation of CD14+CD16+ monocytes, accounting for 30–50% of cells within this monocytes subset (11). Hence, our CD16+ cells would contain the M-DC8+ leukocyte fraction.

Although there are conflicting reports in relation to the T cell stimulatory ability of CD16+ monocytes (9,11,26,27), we could not detect any significant difference between the accessory function of CD16+ and CD16− monocytes in MLR. Those discrepancies might rely on cell isolation procedures, i.e. the use of fluorescence versus magnetic cell sorting, and the application of different mAb for cell separation. Thus, different works might be analyzing unique monocyte subsets. Furthermore, the steady-state of CD16+ and CD16− monocytes may change depending upon the isolation technique, and, although CD16+ monocytes expressed higher levels of HLA class II, B7 and CD40, they could induce hyporesponsivity on T cells through altered secretion of stimulatory/inhibitory cytokines and/or factors involved in T cell survival.

When we cultured CD16+ and CD16− monocytes with GM-CSF, IL-4 and TNF-α, they gave rise to cells with the phenotypical characteristics of myeloid DC, which strongly expressed two critical myeloid DC markers: CD11b and CD11c. Despite the great phenotypical differences found between CD16+ and CD16− monocytes, moDC were similar in most of the markers analyzed. There were, however, some remarkable differences. Thus, CD16+ moDC bore higher surface levels of CD86, CD11a and CD11c, and showed a reduced expression of CD1a and CD32 compared to CD16− moDC. CD32 is the main Fcγ receptor found in vivo on DC (28) and the loss of CD32 during monocytes to DC differentiation has been correlated with a higher degree of maturation, since mature DC decrease their antigen-capturing ability (3). In addition, immature moDC expressed the highest level of CD1a in vitro and its expression decreases after maturation (29). Consequently, based on CD32 and CD1a expression, we could conjecture that CD16+ moDC reached a more advanced level of maturation compared to CD16− moDC. The augmented expression of CD86 would also support this hypothesis, as it has been extensively proved that DC maturation is accompanied by an increase in their accessory function (3,5,29). On the other hand, the differential expression of those markers was already evident at the immature stage of moDC (day 6, data not shown). Therefore, we can consider that the characteristics of CD16+ monocytes, which resembles mature tissue macrophages (8), may influence their subsequent development through the DC pathway. In fact, it has been speculate that CD16+ monocytes could be generated by maturation of CD16+ monocytes (8). In that sense, Piemonti et al. (30) reported a marked decrease in the expression of CD1a on human moDC differentiated in vitro in the presence of vitamin D₃, which promotes the differentiation of monocytes.

Fig. 6. Lymphoproliferative capacity of moDC in auto- and alloMLR. Graded doses of mature CD16+ and CD16− moDC were incubated with 1.5×10⁵ autologous or allogeneic CD4+ T cells for 5 days, followed by an 18-h pulse of [³H]thymidine. Data are presented from three out of five independent experiments. Results are shown as [³H] incorporation (c.p.m.) and are averages of triplicates. Values of c.p.m. incorporation by lymphocytes in the absence of DC were subtracted.
into macrophages. Based on that fact, we could speculate that, among the subpopulations of CD16+ monocytes described here, those with the closest phenotype to regular CD16+ monocytes (i.e. CD14highCD11b+ or CD14highCD64+) would be able to express CD1a and those with more mature phenotype (CD14low/CD11blow-, CD14low/CD64low-) would not.

At this point, it is still unclear whether all CD16+ monocytes subsets became DC or only few phenotypes are selected during the culture. CD16+ moDC are morphologically more heterogeneous than CD16- moDC and some cells developed thick projections similar to pseudopodia instead of sharp dendrites, which might be related to the cellular heterogeneity within the starting population of monocytes discussed above. Therefore, we hypothesized that the heterogeneity of CD16+ moDC described here could lie in the existence of, at least, two types of CD16+ monocyte precursors with different levels of maturation.

The cytokine profile expressed by DC depends upon the stimuli and the DC subpopulation evaluated (20). Here, we analyzed the expression of cytokine mRNA on immature and mature moDC stimulated with bacterial LPS. Our assays revealed that LPS-stimulated CD16+ moDC consistently produced lower levels of IL-12 p40 transcripts and secreted lower amounts of IL-12 p70 than CD16- moDC. It has been demonstrated that maturation of DC is followed by impaired responsiveness to bacterial LPS at the level of IL-12 production (23), presumably because immature DC down-regulate their Toll-like receptors after treatment of with TNF-α (31). This data would strengthen the hypothesis that CD16+ moDC have reached a more mature level of differentiation than CD16- moDC in our cultures. Additionally, autocrine stimulation by other cytokines secreted during the culture could influence the production of IL-12 by CD16+ moDC. In this respect, CD16+ moDC have the potential to secrete increased amounts of TGF-β1 compared to CD16- moDC, as their levels of mRNA transcripts are significantly elevated in the former cell population and TGF-β1 is an effective suppressor of IL-12 production (32).

CD16+ and CD16- moDC stimulated T cell proliferation similarly in allo- and autoMLR. However, when we analyzed the ability of that moDC to polarize the response of naive lymphocytes in allogeneic assays, we found that CD16+ moDC consistently induced a higher secretion of IL-4 by T cells compared to that induced by CD16- moDC (100%) in three different experiments. (A) and (C) Results from co-cultures at 1:100 DC/T cell ratio.

**Table 2.** Percentage of IL-4/IFN-γ-expressing lymphocytes stimulated by CD16+ and CD16- moDC in allogeneic MLR (culture conditions are described in Fig. 7)

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<th>CD16+ moDC (%)</th>
<th>CD16- moDC (%)</th>
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<tr>
<td>1/10</td>
<td>6.3 ± 1.5a</td>
<td>8.5 ± 3.5b</td>
</tr>
<tr>
<td>1/100</td>
<td>15.5 ± 8.5</td>
<td>13 ± 1.4</td>
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<tr>
<td>1/10</td>
<td>2 ± 0.3</td>
<td>3.2 ± 1.7</td>
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<td>1/100</td>
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<td>6.5 ± 3.6</td>
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*P < 0.05.

Fig. 7. Cytokine production by naive cord blood CD4+ T lymphocytes primed with allogeneic mature moDC. (A) Two-color immunofluorescence analysis of IL-4 and IFN-γ expression on lymphocytes stimulated by CD16+ (left) or CD16- (right) moDC. CD4+ T cells were cultured with allogeneic moDC at 1:10 or 1:100 DC/T cell ratio for 6 days followed by a 6-h re-stimulation with immobilized anti-CD3 mAb. Results are from one out of three experiments. (B) Analysis of IL-4 and IFN-γ secretion on supernatants of cultures re-stimulated for 24 h with anti-CD3. Allogeneic cultures were established as in (A). Detection of cytokines was performed by ELISA and results represent one of the three independent experiments performed. Production of cytokines by lymphocytes cultured in the absence of moDC was 0–12% of the shown values and it was subtracted. (C) Percentage of the cytokine production (IL-4 and IFN-γ) by CD16+ moDC-stimulated T cells compared to that induced by CD16- moDC (100%) in three different experiments. (A) and (C) Results from co-cultures at 1:100 DC/T cell ratio.
showed that DC1 could actually induce the development of T_{h}2 effectors in alloMLR at a low-density DC/T cell ratio. Furthermore, Cella et al. (35) demonstrated that blood plasmacytoid cells induced IL-4, as well as IFN-γ-producing T cells, also in alloMLR. Our results are in agreement with those reported by Tanaka et al. (34), as both CD16+ and CD16- moDC stimulated the production of IL-4 and IFN-γ in MLR. Furthermore, we could evidence the increase of IL-4 production at lower DC/T cell ratio (1:100 versus 1:10, see Fig. 7B).

The greater induction of IL-4 that we found on CD16+, moDC-stimulated lymphocytes might be linked to the reduced ability of CD16+ moDC to produce IL-12. Nevertheless, the APC employed for MLR assays were moDC matured for 48 h with TNF-α, that showed no secretion of IL-12 after LPS stimulation. Although we could not discard an induction of IL-12 by other stimuli when moDC are co-cultured with T cells (CD40 ligation or IFN-γ itself) (23), perhaps other factors rather than IL-12 are involved in the differential induction of IL-4. In that sense, CD16+ moDC expressed higher levels of the co-stimulatory molecule CD86 compared to CD16- moDC and CD86 co-stimulation has been extensively involved in the generation of IL-4-producing T cells (36-38). On the other hand, it has been established that limiting doses of antigen preferentially supported IL-4 over IFN-γ production (34,39). In our system, the antigen density does not seem to be an important difference between CD16+ and CD16- moDC, due to their similar expression of HLA-DR.

As this paper was under review, the characterization of DC derived from M-DC8- cells (a putative subpopulation of CD16+ monocytes) was described (11). Our data derived from CD16+ monocytes are inconsistent with those reported for M-DC8- cells. When they differentiate into DC, LPS-stimulated M-DC8+ cells secreted higher levels of IL-12 than M-DC8- moDC, they induced a greater proliferative response in alloMLR and they generated T_{h}1 responses. We believe that such differences could arise from the fact that the CD16+ monocyte subset comprises M-DC8+ as well as M-DC8- cells, hence the data are not fully comparable.

Taken together, the data presented above indicated that the type of moDC could influence by itself the nature of T_{h}1 cell responses to some extent, in an independent way from the cytokine environment in which they have been generated. It remains to be elucidated whether the divergent features of moDC subsets originate from a differential expression of cytokine receptors on monocyte precursors and/or from the involvement of different molecules in the signaling pathways triggered after cytokine–receptor engagement. CD16+ monocytes are phenotypically related to pulmonary alveolar macrophages and some authors have speculated a commitment of these blood monocytes to go into the lung (40). Although this point is still unclear, there is an intriguing possibility that CD16+ monocytes colonize tissues where, in the absence of strong T_{h}1-polarizing signals, T_{h}2 responses are predominant (41).

In summary, we have shown that, in vitro, different populations of human blood monocytes can generate DC with unique characteristics. It remains to be determined whether this phenomenon has a physiological relevance. However, it should be considered when monocytes are used to obtain DC for immunotherapy, since the type of immune response elicited by those DC could vary depending on the predominant subpopulation in the cultures.

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Abbreviations

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<tr>
<th>Abbreviation</th>
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<tr>
<td>alloMLR</td>
<td>allogeneic mixed lymphocyte reaction</td>
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<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
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<tr>
<td>autoMLR</td>
<td>autologous mixed lymphocyte reaction</td>
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<td>CM</td>
<td>complete medium</td>
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<td>DC</td>
<td>dendritic cell</td>
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<td>DX</td>
<td>dextran</td>
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<td>GM-CSF</td>
<td>granulocyte–macrophage colony-stimulating factor</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>monocyte-derived DC</td>
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<td>MR</td>
<td>mannose receptor</td>
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References

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