

Identification and Characterization of a Novel Monocyte Subpopulation in Human Peripheral Blood

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With the aid of two-color immunofluorescence and flow cytometry, a new subset of cells coexpressing CD14 and CD16 antigens can be identified in human peripheral blood. Using the monoclonal antibody My4, these CD14⁺/CD16⁺ cells account for 2.2% of the mononuclear cells and form about 13% of all cells identified by the monocyte-specific CD14 monoclonal antibody. The CD14⁺/CD16⁺ cells can be assigned to the monocyte lineage based on typical morphology, on expression of additional monocyte-associated molecules, on the ability to form reactive oxygen intermediates and on the expression of monocyte-specific NaF-sensitive esterase. Light scatter analysis revealed lower forward angle and right angle light scatter for the CD14⁺/CD16⁺ cells compared with the regular monocytes, and the

average cell size was determined to be 13.8 and 18.4 μm , respectively. Expression of class II antigens on these "small monocytes" was twofold higher compared with the regular monocytes. By contrast, the capacity to perform adherence to plastic surfaces, as well as the ability to phagocytize antibody-coated erythrocytes was clearly reduced in the CD14⁺/CD16⁺ monocyte subset as compared with the regular monocytes. Hence the CD14⁺/CD16⁺ cells appear to represent a new monocyte subset with a distinct functional repertoire. A survey of various tissues revealed that a large proportion of the alveolar macrophages, but not of the peritoneal macrophages, express the CD14⁺/CD16⁺ phenotype.

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CELLS OF THE monocyte lineage produce a wide spectrum of regulatory molecules like tumor necrosis factor (TNF), interleukin-1 (IL-1), prostaglandin E₂ (PGE₂) and complement¹; in the various tissues they mature into distinct functional subsets like inflammatory macrophages, Langerhans cells, and osteoclasts.² The obvious heterogeneity at the level of the mature macrophage leads to the question of whether there are also distinct subsets in the blood monocyte population, either as cells of different maturation level or as cells committed to a specific function or to a specific target tissue.

In this report we characterize a novel subset of blood monocytes that can be clearly identified by two-color fluorescence.³ These cells are characterized by low density expression of the monocyte-specific CD14 antigen and by the coexpression of the CD16 antigen, which represents a specific type of Fc-receptor initially described for Nk-cells and granulocytes. Although these CD14⁺/CD16⁺ cells bear many characteristics of the regular blood monocytes in terms of antigen expression, the decreased ability of this subset to perform Fc-receptor-mediated phagocytosis of erythrocytes and the increased class II antigen expression points toward a distinct functional role of these cells.

MATERIALS AND METHODS

Preparation and storage of cells. Mononuclear cells from peripheral blood of healthy adults, from cord blood of healthy newborns, and from bone marrow samples of healthy bone marrow donors were obtained by Ficoll-Hypaque (Pharmacia, Freiburg, FRG) density-gradient sedimentation.⁴ Tonsils were obtained from patients undergoing tonsilectomy after chronic tonsillitis, thymus specimens from children who had open heart surgery, and spleens from patients undergoing splenectomy after trauma. The samples were dissected by forceps and the resulting cell suspensions purified by the density-gradient sedimentation.

Alveolar macrophages (AM) were obtained from patients undergoing diagnostic bronchial lavage. The diagnoses were epidermoid cancer (n = 2), oesophageal tumor (n = 1), chronic bronchitis (n = 1), and no detectable abnormality (n = 1). Peritoneal macrophages (PM) were obtained from patients undergoing diagnostic peritoneal lavage after trauma. Only samples without visible blood contamination were considered. The cells were collected by low speed centrifugation and subsequently purified by density-gradient

sedimentation. All samples were frozen under controlled conditions in the presence of 10% dimethylsulfoxide (DMSO) and 20% fetal calf serum (FCS) using a freezing machine (Messer-Griesheim, Krefeld, FRG). Immediately before use cells were thawed rapidly in a 37°C water bath and washed twice with RPMI 1640 with 10% FCS.

Monoclonal antibodies. The CD16 antibody VEP 13 (immunoglobulin [Ig] M reacts with a low-affinity Fc-receptor expressed on natural killer (NK) cells and neutrophils (Dr Kraft, University of Vienna, Austria).^{5,6} The CD14 antibodies LeuM3 (immunoglobulin G₂ [IgG₂]; Becton Dickinson, Heidelberg, FRG) and My4 (IgG₁; Coulter Electronics, Krefeld, FRG) react with the CD14 cell surface glycoprotein expressed exclusively on mature human monocytes^{7,9}; Leu7 (IgM; Becton Dickinson) reacts with a subpopulation of granular lymphocytes¹⁰ and Leu2a (IgG₁; Becton Dickinson) with suppressor and cytotoxic T cells and with 50% of the large granular lymphocytes (LGL).^{6,11} The CD11b antibody M522 (IgG1), reacts with the C_{3b}-receptor, and T151 is specific for CD4 antigen (E.P. Rieber, Institute for Immunology, University of Munich, FRG).^{12,13} The anti-HLA-DR antibody L243 was purified from supernatants of the respective hybridoma cell line (ATCC, Rockville, MA).¹⁴ The CD2 antibody T11 (IgG1) detects the sheep red blood cell (SRBC) receptor present on T and NK cells¹⁵ (Coulter Electronics).

Immunofluorescence. For two-color immunofluorescence, 1 × 10⁶ cells were incubated for 30 minutes at 4°C simultaneously with a fluorescein isothiocyanate (FITC)-conjugated CD14 MoAb (My4 or LeuM3) and a biotinylated antibody (VEP13, L243, M522, T151, T11, Leu7) or a phycoerythrin-conjugated antibody (Leu2a) under

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saturation conditions. After two washes with phosphate buffered saline (PBS) containing 2% FCS and 0.02% azide the cells were fixed at once with 1% paraformaldehyde dissolved in PBS if a directly conjugated antibody was used. Otherwise the cells were incubated for 30 minutes with 50 μ L avidin-phycoerythrin 1:2.5 (Becton Dickinson), washed twice and then fixed. For the assessment of the Fc-receptor cells were incubated with heat-aggregated (30 minutes, 62°C), biotinylated human IgG (Miles Laboratories, Uppsala, Sweden) according to the same procedure. As isotype controls we used the irrelevant mouse myeloma immunoglobulins TEPC183 (IgM), UPC10 (IgG2), and MOPC21 (IgG1) (Sigma Chemical Co, St Louis, MO; all biotinylated) or IgG1-FITC (Coulter Electronics), IgG2-FITC, and IgG1-phycoerythrin (Becton Dickinson) as controls for the respective directly conjugated monoclonal antibodies (MoAbs).

Flow cytometry and cell sorting. Flow cytometry analysis was performed by using an EPIPS V flow cytometer (Coulter Electronics) equipped with an argon laser with a 488 nm excitation wavelength and photomultiplier tubes at 900 to 1,200 V. At least 20,000 cells per sample were analyzed and histograms acquired were analyzed with the MDADS software of the EPICS V system. Specific fluorescence intensity represents the difference between the mean channel of the specific MoAb and the mean channel of the control antibody used in a logarithmic scale.

The percentage of positive cells was calculated from specific and control staining. If there was an overlap between the specific MoAb and the irrelevant MoAb of the same isotype at the same concentration we applied a subtraction mode program (Coulter Electronics). When comparing CD14 and CD16 expression on different monocyte populations (peripheral blood mononuclear cell [PBMC], AM, PM) photomultipliers were adjusted such that the negative control stainings were set at comparable levels.

For cell sorting the cells were stained with the CD14 MoAb My4 and the CD16 MoAb VEP 13 as described above, except that azide-free reagents were used. Cells were kept on ice at all times during the sorting procedure. They were collected into tubes containing 4 mL FCS and were spun for 5 minutes at 400 g after each sorting step.

To obtain pure populations of CD16⁺, CD14⁺/CD16⁺, and CD14⁺⁺ cells we first separated CD16-positive and CD16-negative cells from each other. Then the CD16-positive fraction was sorted into two populations, one which contains only the CD16-antigen expressing NK cells (CD16⁺ cells) and one which also stains for the CD14-antigen in a low intensity (CD14⁺/CD16⁺ cells). The CD16 negative fraction was finally used to isolate the regular CD14⁺⁺ monocytes (CD14⁺⁺ cells). Purity of the respective cell populations was ascertained by reanalysis (range, 92% to 98%).

Morphology. For the morphologic analysis 3 \times 10⁴ cells of each sorted population were centrifuged onto microscope slides, using a cytospin centrifuge. The air-dried slides were stained according to Pappenheim and morphologic characteristics were determined by inspection of the slides using conventional microscopy. At least 100 cells were analyzed per population and donor.

Cytochemistry. Air-dried cytospin preparations of the sorted cells were fixed in the vapor phase of formaldehyde for 5 minutes, washed and air dried again. The slides were incubated for 70 minutes at room temperature in PBS (pH 6.8 to 7.0) containing 0.1 mg/mL naphthol-AS acetate (Sigma), 2% propylene glycol (Merck, Darmstadt, FRG), and 2mg/mL fast blue BB salt (Serva, Heidelberg, FRG). For sodium fluoride inhibition 1.5 mg/mL NaF (Merck) was added. After a short wash (1 minute) the slides were air dried and stained with nuclear fast red (Merck) for another 5 minutes and washed and air dried again. The percentage of positive cells was determined by oil immersion microscopy. Cells that contain more than five dark brown granules were considered positive.

Phagocytosis. The ability for phagocytosis was determined by using sorted cells. SRBCs (15 \times 10⁶; Behring, Marburg, FRG) were washed three times with PBS and resuspended in 10 mL PBS containing 60 μ g/mL carbazol (Sigma). Then 2 μ L 30% H₂O₂ (Merck) were added and the SRBCs were incubated for 15 minutes at room temperature. The erythrocytes were washed with PBS and coated with rabbit-anti-sheep-IgG (Amboceptor 6000, Behring) for 15 minutes at 37°C. Cells of each population (3 \times 10⁶) were incubated for 3 hours with 1 \times 10⁶-coated SRBC at 37°C in 0.3 mL RPMI 1640 with 10% FCS. For lysis of the non-phagocytized SRBCs, 1 mL of warm PBS containing 170 mmol/L NH₄Cl, 10 mmol/L KHCO₃, and 1 mmol/L of ethylenediamine tetraacetic acid (EDTA) were added. The cells were incubated for 5 minutes at 37°C and then washed twice with RPMI 1640 with 10% FCS. This step was repeated until all SRBCs were lysed. The percentage of phagocytic cells was determined by phase contrast microscopy.

Adherence. Tissue culture flasks (25 cm²; Becton Dickinson) were precoated with heat-inactivated FCS for 1 hour at 37°C. PBMCs (5 \times 10⁶), prestained by double-marker immunofluorescence as described above, were incubated in 5 mL RPMI 1640 10% FCS for 1 hour at 37°C in a 5% CO₂ incubator.

Nonadherent cells were removed by two washes with RPMI 1640 10% FCS. The remaining cells were incubated for 10 minutes in 5 mL containing 1 mmol/L EDTA and recovered by gentle treatment with a rubber policeman. Then the cells were washed twice with PBS 2% FCS and analyzed with the EPICS V flow cytometer. The number of cells recovered was used to calculate the percentage of adherent cells.

Reactive oxygen. For measuring the formation of reactive oxygen, we modified a method described by Bass et al.¹⁶ PBMCs (5 \times 10⁶) were incubated for 30 minutes at 4°C in PBS 2% FCS with LeuM3-Pe (Becton Dickinson) under saturating conditions and then washed twice with PBS 2% FCS. 2.4 mg 2',7'-dichlorofluorescein diacetate (DCF-DA; Molecular Probes, Eugene, OR) were dissolved in 1 mL ethanol and diluted with PBS 1% gelatin (PBSg; 0.1%) to a concentration of 5 μ mol/L DCF-DA. The cells were resuspended in 4.5 mL RPMI 1640 10% FCS and incubated at 37°C in a water bath. Then 0.5 mL DCF-DA PBSg was added to reach a final concentration of 0.5 μ mol/L. To control background fluorescence, we took a sample of 5 \times 10⁵ cells after 1 minute and added at the same time phorbol myristate acetate (PMA; Sigma) to reach a final concentration of 100 ng/mL. After 30 minutes of incubation, we took another sample and analyzed it at once with the EPICS V flow cytometer in two-color fluorescence.

Because the quantity of reactive oxygen produced corresponds to the intensity of the green fluorescence we gated on the CD14-, the CD14⁺, and the CD14⁺⁺ cells, respectively, and determined the mean channel of their green fluorescence in a logarithmic scale. Specific fluorescence intensity represents the difference between the mean fluorescence intensity of cells incubated for 1 minute and of cells incubated for 30 minutes.

Statistics. Results are indicated as mean \pm SD. Statistical significance was evaluated by the use of a Student's *t* test.

RESULTS

When mononuclear cells are stained with the CD14 MoAb My4 and the CD16 MoAb VEP 13 in two-color immunofluorescence the flow cytometry analysis reveals a population of strongly positive CD14 cells (CD14⁺⁺) and a population of CD16⁺ cells. In addition to the singly stained cells, a population coexpressing CD14 and CD16 can be identified (Fig 1). These cells express the CD14 antigen in low intensity and are termed CD14⁺/CD16⁺. The percentage of CD14⁺/

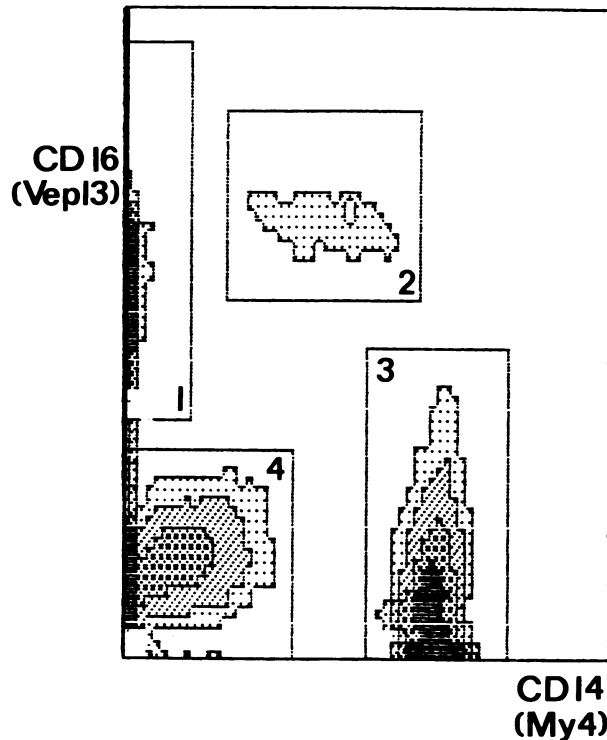


Fig 1. Two-color immunofluorescence of human PBMCs with CD14 and CD16 MoAbs. Cells were stained with biotin-conjugated VEP13 and with My4-FITC followed by avidin-phycoerythrin and were then analyzed by flow cytometry gated on all mononuclear cells. In this example, the percentage of CD16⁺ (area 1) is 17%, of CD14⁺⁺ (area 3) is 27%, and of CD14⁺/CD16⁺ (area 2) is 3%.

CD16⁺ cells among the PBMC is $2.2 \pm 0.8\%$ ($n = 20$); the average absolute number is 45 ± 34.8 cells/ μL blood (Table 1).

Relative to the regular CD14⁺⁺ monocytes ($14.1 \pm 4.7\%$ of the PBMCs), the CD14⁺/CD16⁺ cells account for 13% and also form approximately 13% of the CD16⁺ cells ($14.4 \pm 5.2\%$ of the PBMCs), which previously were thought to exclusively represent NK cells. The CD14⁺/CD16⁺ cells appear to represent the entire low-intensity CD14⁺ subset. Clear enumeration of the CD14⁺ cells in a single-color immunofluorescence for CD14, however, is difficult because there is a spillover of the CD14⁻ and the CD14⁺⁺ cells into the borderline areas of the CD14⁺ cells. The CD14 MoAb My4 appears to detect higher numbers of CD14⁺/CD16⁺ cells compared with the CD14 MoAb LeuM3, since the average percentage of LeuM3⁺/CD16⁺ cells is 1.1% of all

Table 1. Enumeration of CD14⁺/CD16⁺ Cells in Peripheral Blood Using MoAb My4 and LeuM3

	Positive Cells (mean \pm SD, $n = 20$)	
	% of all PBMCs	Cells (μL)
CD16 ⁺ *	14.4 ± 5.2	288.3 ± 208.4
My4 ⁺ /CD16 ⁺	2.2 ± 0.8	45.4 ± 34.8
My4 ⁺⁺	14.1 ± 4.7	265.6 ± 126.7
LeuM3 ⁺ /CD16 ⁺	1.1 ± 0.3	19.4 ± 9.3
LeuM3 ⁺⁺	13.6 ± 4.9	250.3 ± 113.0

*The CD16 MoAb used was VEP13.

Table 2. Expression of Monocyte-Associated Molecules on CD14⁺ Cells

	Specific Fluorescence Intensity (mean \pm SD; $n = 4$)			
	Class II	Fc-Receptor	CD4	CD11
CD14 ⁺	$28.3 \pm 6.9^*$	$20.7 \pm 5.5^\dagger$	$9.5 \pm 2.5^\dagger$	$9.8 \pm 3.3^*$
CD14 ⁺⁺	22.4 ± 6.0	17.8 ± 5.1	7.1 ± 1.3	17.4 ± 2.6

Given is the difference between the specific MoAb staining and isotype control in channels on a log scale. The antibody used was LeuM3.

*Difference between CD14⁺ and CD14⁺⁺ is significant with $P < .05$.

A six-channel difference on the log scale denotes a twofold increase in antigen density.

†Difference is not significant.

PBMCs (Table 1). In contrast, additional CD16 MoAbs (Leu11a, Leu11c) gave percentages of CD14⁺/CD16⁺ cells similar to VEP13.

Since the CD14⁺/CD16⁺ cell population is characterized by markers used to define monocytes and NK cells, respectively, we studied by two-color immunofluorescence additional cell surface molecules on the low intensity CD14⁺ cells. As demonstrated in Table 2 molecules associated with monocytes like class II- and Fc-receptors are clearly expressed on the CD14⁺ cells. Class II antigen density was two times higher than the regular CD14⁺⁺ monocytes. The CD14⁺ cells also express low level CD4 and the CD11b

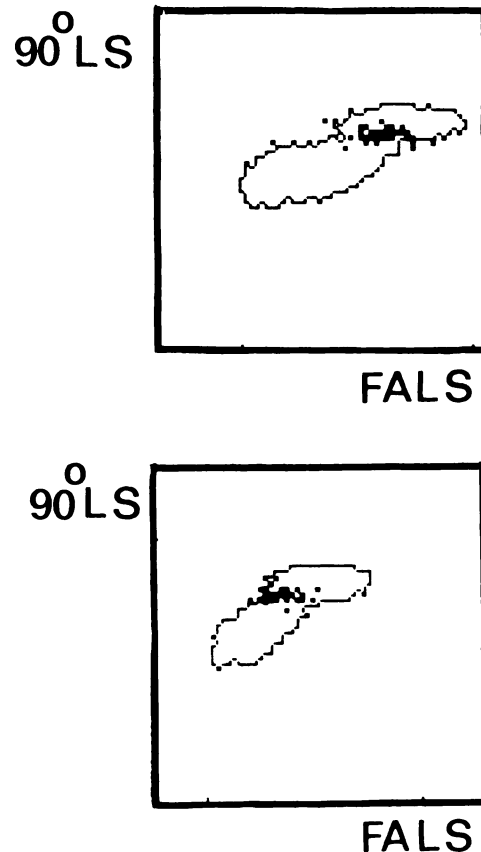


Fig 2. Light scatter distribution for CD14⁺/CD16⁺ cells. The light scatter profiles of the CD14⁺/CD16⁺ cells (dark areas) are superimposed with distribution of all mononuclear cells (marked by the dark lines) in two representative examples.

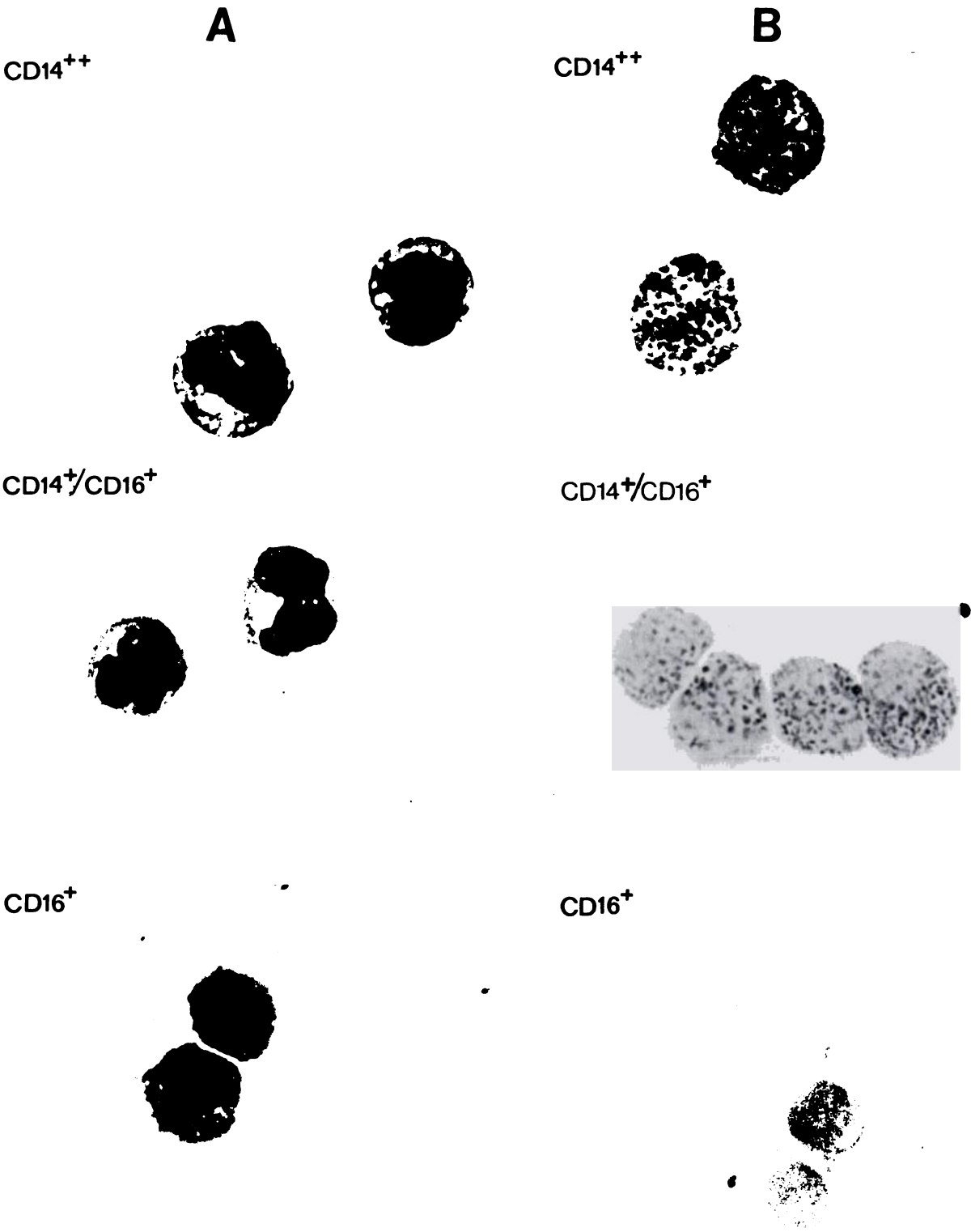


Fig 3. Morphology and cytochemistry of CD14⁺/CD16⁺ cells. The respective populations were isolated by cell sorting and then either stained with Pappenheim stain (A) or for naphthol-AS-acetate-esterase (B).

expression is present to a lesser extent than on the CD14⁺⁺ cells. In contrast, antigens found on NK cells but not on monocytes could not be detected on the CD14⁺ cells. The CD2 antigen, which defines the SRBC-receptor expressed on all NK cells, was absent from the CD14⁺ cells as were the Leu7 antigen and the Leu2a antigen (data not shown). More direct analysis of antigen expression on CD14⁺/CD16⁺ cells was performed by three-color immunofluorescence using Texas Red staining and dye laser excitation with rhodamine-6G. In these studies we could confirm the higher class II expression (n = 5), the lower CD11b expression (n = 3), and, in addition, we could demonstrate that the NK cell associated NKH-1 antigen is absent from the CD14⁺/CD16⁺ cells (n = 6).

Monocytes exhibit increased size and granularity compared with the lymphocytes, and these properties upon flow cytometry analysis are reflected in higher forward-angle light scatter (FALS) and 90° light scatter (90°LS), respectively. Figure 2 shows two examples of light scatter profiles for PBMC with monocytes representing the upper right and lymphocytes the lower left cell population. Superimposed on these histograms is the distribution of the CD14⁺/CD16⁺ cells (dark area), demonstrating that these cells localize primarily into the monocyte area.

The morphology of the CD14⁺/CD16⁺ cells was analyzed on populations purified by cell sorting. Microscopic determination of cell size confirmed the evidence from light scatter analysis in that the diameter for CD14⁺⁺ regular monocytes was $18.4 \pm 1.7 \mu\text{m}$, and for CD14⁺/CD16⁺ cells it was $13.8 \pm 1.4 \mu\text{m}$. Furthermore, both cell types exhibited light blue cytoplasm and an irregular and indented nucleus (Fig 3, panel A). By contrast, the CD16⁺ cells were characterized by considerably lower size, abundant cytoplasm, and multiple cytoplasmic granules.

The pattern of expression of cell surface molecules, the light scatter properties, and the morphology favor a monocyte nature of the new cell population of CD14⁺/CD16⁺ cells. Hence, we set out to study functional properties associated with monocytes. Nonspecific esterase staining was evident on sorted, highly purified CD14⁺/CD16⁺ cells and on CD14⁺⁺ cells, but it was absent from CD16⁺ cells (Fig 3, panel B). This staining was sensitive to NaF treatment (Table 3).

The ability to generate reactive oxygen was studied in flow cytometry using DCF-DA. Green fluorescence induced after 30 minutes incubation with PMA gave little activity for the CD14 cells compared with the control incubation. By con-

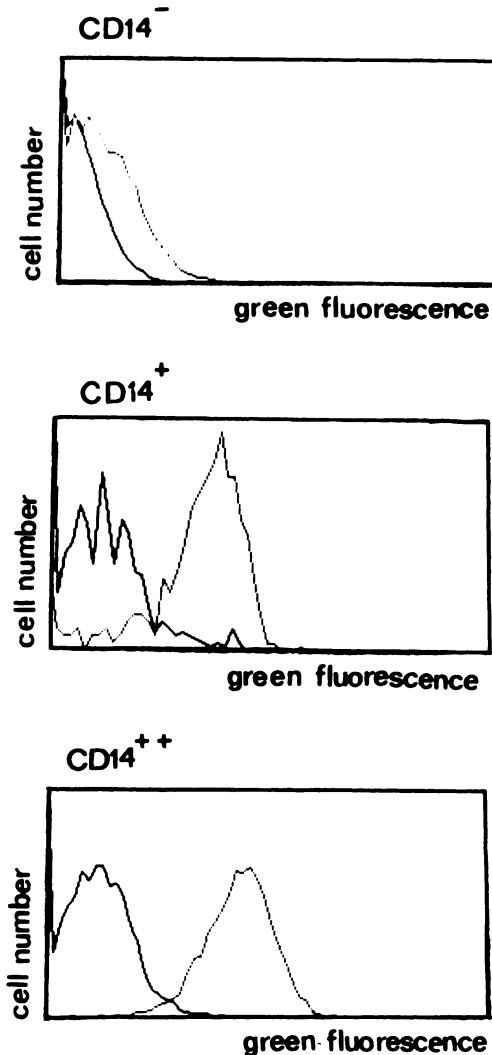


Fig 4. Generation of reactive oxygen species by CD14⁺ cells. Cells were stained with LeuM3-phycoerythrin, loaded with DCF-DA, and incubated for 1 minute (control, left curve) or for 30 minutes in the presence of PMA. Given is the green fluorescence gated on yellow fluorescence. Gates for CD14⁺ cells were limited to the central portion of the CD14⁺ population to avoid spillover from CD14⁻ and CD14⁺⁺ cells. Average specific staining for five donors was 2.8 ± 2.0 channels for CD14⁻, 11.4 ± 2.8 channels for CD14⁺, and 17.4 ± 3.9 channels for CD14⁺⁺.

trast, the CD14⁺ cells and the CD14⁺⁺ cells both gave a strong signal (Fig 4), although, in terms of mean specific fluorescence intensity, staining was somewhat less intense for the CD14⁺ cells (see legend to Fig 4).

Adherence to plastic surfaces, a typical feature of blood monocytes, was found in approximately 80% of the CD14⁺⁺ cells; whereas for the CD14⁺/CD16⁺ small monocytes, only about 50% of the cells were recovered in the adherent fraction (Fig 5A). Next, we studied phagocytosis of antibody-coated erythrocytes using sorted cells, with a purity of greater than 92%. These cells were allowed to phagocytize

Table 3. Nonspecific Esterase Reactivity of Sorted CD14⁺/CD16⁺ Cells

NaF-Inhibition	Positive Cells (%)		
	CD16 ⁺	CD14 ⁺ /CD16 ⁺	CD14 ⁺⁺
-	8.3 ± 3.0	98.3 ± 1.3	96.6 ± 2.3
+	7.6 ± 0.7	3.1 ± 3.6	4.2 ± 8.5

Results are mean \pm SD of four donors. Cells with five or more granules were considered positive.

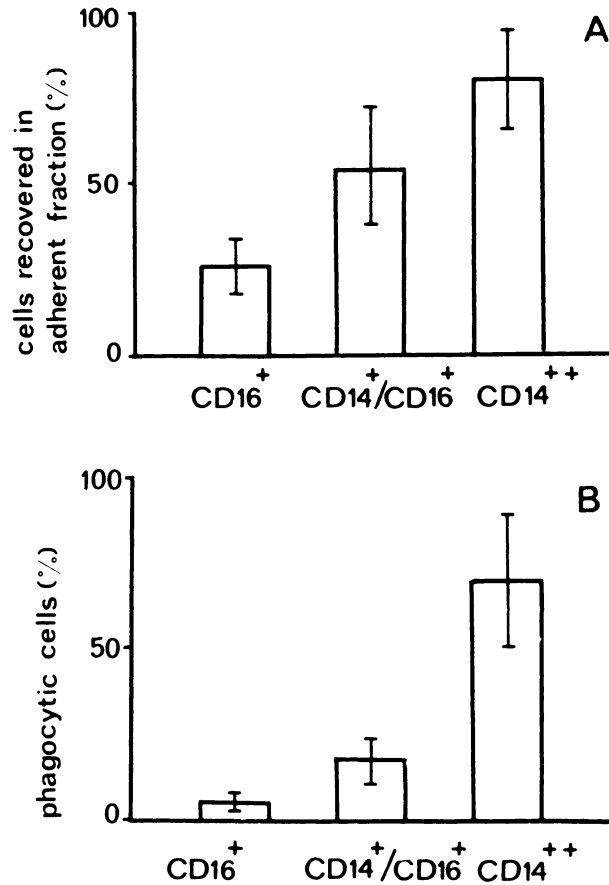


Fig 5. Adherence and phagocytosis by CD14⁺/CD16⁺ cells. (A) Given is the recovery of CD14⁺/CD16⁺ cells after adherence to a plastic surface. Results are from four donors. (B) Phagocytosis was tested on populations isolated by two-step cell sorting. The purified subsets were incubated for 3 hours with antibody-coated erythrocytes, and after lysis of noningested erythrocytes, results were read with light microscopy. Results are from four donors.

and, after lysis of noningested erythrocytes, the results were analyzed by light microscopy. Whereas 70% of the CD14⁺⁺ cells contained one or more erythrocytes, only 15% of the CD14⁺/CD16⁺ cells showed phagocytosis (Fig 5B).

Thus, the CD14⁺/CD16⁺ cells express NaF-sensitive nonspecific esterase and were able to produce reactive oxygen comparable to the regular CD14⁺⁺ monocytes. The ability of the CD14⁺/CD16⁺ subpopulation to adhere to plastic surfaces, however, is reduced and there is no or only minimal phagocytosis.

Next we screened different tissues of the lymphohematopoietic system for representation of CD14⁺/CD16⁺ cells using MoAbs My4 and VEP13. In cord blood, bone marrow, tonsils, and spleen percentages of CD14⁺/CD16⁺ cells were in a range comparable to that of adult peripheral blood (Table 4). In the thymus less than 0.1% CD14-positive cells were detected, precluding a more detailed analysis.

Finally, we analyzed the CD14 and CD16 antigen expression of tissue macrophages recovered from alveolar space and from peritoneal cavity in comparison to peripheral blood monocytes. Figure 6 demonstrates that AM exhibit a low

Table 4. Tissue Distribution of CD14⁺/CD16⁺ Cells

Tissue	Positive Cells (%)		
	CD16 ⁺	CD14 ⁺ /CD16 ⁺	CD14 ⁺⁺
Cord blood (n = 5)	15.8 ± 5.6	2.6 ± 1.3	25.8 ± 7.3
Bone marrow (n = 4)	6.7 ± 5.5	0.8 ± 0.5	5.8 ± 2.4
Tonsils* (n = 5)	1.4 ± 0.6	0.4 ± 0.3	0.6 ± 0.2
Spleen (n = 4)	9.8 ± 4.5	2.6 ± 0.8	15.5 ± 15.1
Thymus (n = 4)	<0.1	<0.1	<0.1

NOTE. All values are mean ± SD.

*Single cell suspensions from the lymphoid organs were obtained after dissection with forceps and density-gradient centrifugation.

expression of CD14 antigen comparable to the CD14⁺/CD16⁺ cells (Figs 6a and b). The average CD14 antigen density expressed in mean specific fluorescence intensity was determined to be 8.2 ± 0.8 and 19.3 ± 6.0 for AM and CD14⁺/CD16⁺ monocytes, respectively (n = 3). By contrast, PMs show a high CD14 antigen expression comparable to the CD14⁺⁺ monocytes in peripheral blood (Figs 6a and c). Mean specific fluorescence intensity for CD14 was 29.5 ± 0.2 and 36.5 ± 4.5 for PM and CD14⁺⁺ monocytes, respectively (n = 3).

Expression of the CD16 antigen was evident on all CD14⁺ AMs (mean, 98%; n = 3), although antigen density was somewhat lower compared with the staining of NK cells and CD14⁺/CD16⁺ cells in PBMC. By contrast, the CD16 antigen expression on PMs was virtually absent (Fig 6F). Thus, with respect to these two cell surface antigens of the CD14 and CD16 cluster, the CD14⁺/CD16⁺ monocyte subset in peripheral blood resembles the tissue macrophage of the alveolar space.

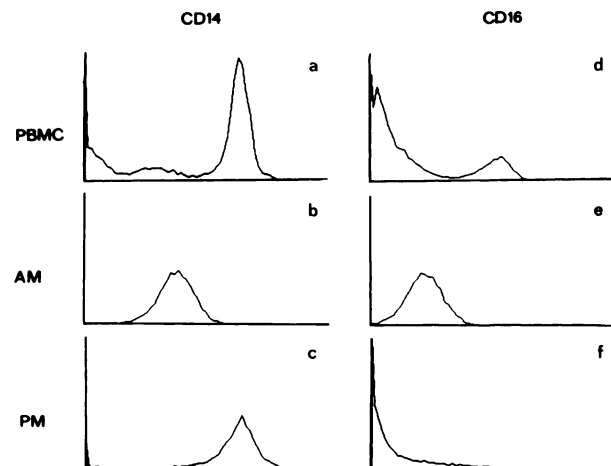


Fig 6. Expression of CD14 and CD16 antigens on AM and PM. Expression of the CD14 antigen as detected by the My4 MoAb (a-c) and the CD16 antigen as detected by the VEP13 MoAb (d-f) is given with fluorescence intensity on the x-axis and cell number on the y-axis.

DISCUSSION

In the present study we report on a new monocyte subpopulation in human peripheral blood recognized by two-color fluorescence and flow cytometry. The population is characterized by low-density expression of the 55 Kd cell surface molecule defined by the CD14 MoAb My4 or LeuM3. The broad distribution in CD14 antigen density on the CD14⁺ cells, however, results in overlap with the negative and the strongly positive CD14⁺⁺ cells such that a clear discrimination is not possible. The additional staining of the cells with CD16 MoAb VEP13 allows for a clear definition and enumeration of the subpopulation (Fig 1).

Analysis of additional cell surface molecules like class II and C_{3b}i receptor support the monocyte nature of the CD14⁺/CD16⁺ cells but on its own such results form only indirect evidence for the lineage assignment. Interestingly, the CD14⁺/CD16⁺ cells express twofold higher amounts of class II antigen, suggesting that they might be potent in antigen presentation. Still a relationship to the peripheral blood dendritic cells is unlikely, because the latter are reported to lack CD14 antigen and Fc-receptors and they have dendritic morphology.^{17,18} Since dendritic cells are usually isolated by an overnight adherence procedure a direct comparison, however, is difficult. Analysis of light scatter properties indicates that the CD14⁺/CD16⁺ cells are similar to the CD14⁺⁺ monocytes. Both granularity and cell size were somewhat lower but clearly distinct from lymphocytes.

There have been attempts to isolate monocyte subsets from peripheral blood using countercurrent elutriation. Detailed analysis of such fractions of "intermediate" monocytes in the study by Norris et al¹⁹ revealed a mixture of lymphocyte-size and monocyte-size cells without a clearly discernible, additional population. Using cell sorting, we are able to consistently isolate the CD14⁺/CD16⁺ monocyte subset with greater than 92% purity. In cytospin preparations of these cells the somewhat smaller size was confirmed. In addition, the cells exhibited all morphologic features of monocytes. The ability of the cells to form reactive oxygen and the strong expression of NaF inhibitable nonspecific esterase clearly demonstrates the monocyte nature of the CD14⁺/CD16⁺ cells. Still, these cells appear to be functionally distinct from the regular monocytes. Most prominent is the greatly reduced ability to perform phagocytosis of antibody-coated erythrocytes in spite of the presence of Fc-receptors on the cell surface. One might argue that the sorting procedure differentially impairs the CD14⁺/CD16⁺ cells resulting in reduced phagocytosis. In experiments not shown we analyzed phagocytosis of erythrocytes without cell sorting by measuring the increased FALS signal following ingestion of these large particles. Using this approach we also failed to detect significant phagocytosis. While the question of whether phagocytosis of other particles can be achieved

remains to be analyzed, the current finding already suggests a different functional repertoire of the CD14⁺/CD16⁺ small monocytes compared with the regular CD14⁺⁺ monocytes.

There are two major possibilities for the position of the CD14⁺/CD16⁺ cells within the monocyte system: (1) The CD14⁺/CD16⁺ cells might be closely related to the regular CD14⁺⁺ monocytes only differing in maturation level, or (2) the CD14⁺/CD16⁺ cells might constitute an independent separate subpopulation with a distinct functional repertoire. The lower functional capacity of the CD14⁺/CD16⁺ cells with respect to adherence and phagocytosis supports the idea that these cells might be immature precursor cells. Human promonocytes, however, were reported to be somewhat larger than blood monocytes²⁰ and, on the other hand, the strongly indented nucleus and the higher capacity to produce TNF would argue in favor of a more mature cell type. Studies with *in vitro* maturation, which are currently underway in this laboratory, may help to answer this question. When comparing the CD14⁺/CD16⁺ cells with the monocytes observed by Norris et al in their intermediate fractions of elutriator-isolated cells,¹⁹ it is clear that these cells are distinct. The type of cell reported by Norris et al is phagocytic and low in Fc-receptors, whereas the CD14⁺/CD16⁺ cells are low in phagocytosis, but strongly express Fc-receptors. Thus, it appears that the CD14⁺/CD16⁺ small monocytes have not been recognized previously. The identification of a monocyte subpopulation that strongly expresses the CD16 antigen has implications for the use of CD16 MoAb for identification of NK cells because many studies in the past used this marker in the analysis of the biology of NK cells.^{21,22} The CD14⁺/CD16⁺ cells comprise more than 10% of all CD16-positive cells. Hence, studies on NK cells that exclusively rely on this marker will have to be interpreted with caution.

The analysis of tissue distribution revealed that only in the alveolar space CD14⁺/CD16⁺ cells can be found in large numbers. The CD16 antigen expression on AMs was noted earlier,²³ but the expression of CD16 on blood monocytes has not been described as yet. The comparable expression of CD14 and CD16 antigens on both AMs and CD14⁺/CD16⁺ monocytes might hint toward a specific relationship between these two types of cells. The phenotypic and functional analysis of the CD14⁺/CD16⁺ cells presented here clearly demonstrates that these cells comprise a unique monocyte subpopulation that may be involved in various clinical conditions.

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