

# Detection and properties of the human proliferative monocyte subpopulation

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**Abstract:** Peripheral blood monocyte subpopulations have been reported and can give rise to diverse, differentiated phenotypes. A subpopulation(s) of human monocytes can proliferate in vitro in response to macrophage-colony stimulating factor (M-CSF; or CSF-1). This population, termed the proliferative monocyte (PM), is presumably less mature than other monocytes; however, it has not been defined further. Previous studies monitoring the frequency of the slowly cycling PM from different donors indicated that the assay for their reproducible measurement required improvement. We demonstrate that for optimal PM detection, high 5-bromo-2'-deoxyuridine concentrations are required over a delayed and wide time-frame. Surface marker phenotyping by flow cytometry showed that freshly isolated PM are CD14<sup>+</sup> and could be distinguished from two other human monocyte subpopulations, namely, the CD14<sup>lo</sup>CD16<sup>+</sup> and CD14<sup>lo</sup>CD64<sup>-</sup> subsets. PM express relatively high levels of CD64 and CD33 but have relatively low CD13 expression; they are also c-Fms<sup>+</sup> and human leukocyte antigen-DR<sup>+</sup>. Labeling with carboxyfluorescein diacetate succinimidyl ester (CFSE) enabled the estimation of the number of PM divisions over time. Following CFSE labeling and culture, PM were sorted from the nonproliferating population and shown to have a distinctive, spindle-shaped morphology and higher capacity to form multinucleated, tartrate-resistant acid phosphatase<sup>+</sup> cells in the presence of M-CSF and receptor activator of nuclear factor- $\kappa$ B ligand. The phenotype and properties of the PM subpopulation were examined as a prelude to determining its role in disease using methods that can be applied to clarify human monocyte heterogeneity. *J. Leukoc. Biol.* 79: 757–766; 2006.

**Key Words:** macrophages · cell proliferation · cell differentiation

## INTRODUCTION

Peripheral blood monocytes derive from bone marrow precursors and in turn, migrate into tissues and differentiate locally

into functionally distinct macrophages [1]. A key regulator in this development is macrophage-colony stimulating factor (M-CSF; or CSF-1) [2–4]. Monocytes mature to some extent in the blood, migrate into tissues, and undergo further maturation and activation, for example, at sites of inflammation [5]. The developmental program, including the definition of subpopulations, is less well-defined for this lineage as for lymphoid lineages.

It is now being appreciated that monocytes are heterogeneous, and subpopulations have been identified based on physical, functional, and surface marker criteria. For example, a monocyte subpopulation comprising ~10% of peripheral blood monocytes is defined by CD14<sup>lo</sup>CD16<sup>+</sup> expression [6]. Another subpopulation has been phenotyped as CD64<sup>-</sup>, which comprises <10% of the total monocytes [7]. In cultured monocytes, a highly autofluorescent macrophage arises with increased interleukin (IL)-1 $\alpha$  production [8]. The heterogeneity of monocytes is also reflected in the range of differentiated macrophage-lineage cells, such as osteoclasts and dendritic cells (DC), which can derive from monocyte subpopulations [9, 10], and in the recent human and murine data, indicating subpopulations with a so-called inflammatory phenotype [11, 12]. These various subpopulations described in the literature presumably represent different maturational and/or activation states, which require better characterization [13]. In disease states, there is evidence that there is a shift in subpopulations, including the CD14<sup>lo</sup>CD16<sup>+</sup> monocytes, reflecting a perturbed host response to the disease, for example, in sepsis and cancer [14, 15]. Perturbation in subpopulations has been noted in rheumatoid arthritis and other inflammatory diseases [16, 17]. Exercise has been shown to alter the steady state of subpopulations by mobilizing the marginal pool [18].

Human monocytes are usually perceived as being a nonproliferating cell type [19]. However, we and others have shown that a small percentage of human monocytes can enter the cell cycle in vitro in response to agents such as M-CSF and granulocyte M-CSF (GM-CSF) [20–22]. We have termed this pre-

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sumably immature subpopulation proliferative monocytes (PM) [21–24] and have proposed they could enter sites of inflammation and contribute to the local macrophage proliferation, which has been observed clinically and in animal models of inflammation [25–28]. Surface marker analysis has been carried out following in vitro culture of PM [23], but their characteristics upon isolation and relationship to other subpopulations have not been examined.

In the present study, culture conditions were optimized [5-bromo-2'-deoxyuridine (BrdU) concentration, labeling time, and cell concentration] prior to phenotyping. Upon isolation, the PM were shown to be far less abundant in the CD14<sup>lo</sup>CD16<sup>+</sup> monocyte subpopulation than in the corresponding CD14<sup>+</sup> monocytes. Differences in the relative proliferative potential of eight “subsets” of CD14<sup>+</sup> monocytes were also demonstrated. Carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling allowed an estimate of the number of cell divisions that the PM population could undergo as well as morphologic characterization upon subsequent sorting.

## MATERIALS AND METHODS

### Peripheral blood mononuclear cell (PBMC) isolation

Buffy packs were obtained from the Australian Red Cross Blood Service. The contents of the buffy pack (~70 mL) were transferred to a sterile container and diluted with phosphate-buffered saline (PBS) to ~120 mL. The sample was then layered over Ficoll Paque Plus (Amersham Biosciences, Uppsala, Sweden), which was then spun at ~1000 g for 20 min without braking. The PBMC layer was removed. These cells were resuspended to 50 mL in PBS, washed once at 200 g, and washed twice at 400 g. The PBMC were resuspended in fluorescein-activated cell sorter (FACS) wash [PBS containing 1% fetal bovine serum (Invitrogen Corp., Grand Island, NY) and 1 mM EDTA (Ajax Chemicals, Auburn, NSW, Australia)] and counted.

### Cell sorting

PBMC were stained with 10 µL of the desired antibody (Table 1) for 25 min on ice, after which, they were washed three times with FACS wash. PBMC stained with isotype controls were used to set gates for cell sorting using a FACS Advantage SE (BD Biosciences).

### Cell culture

Human blood from healthy volunteers was collected and allowed to clot at room temperature for 1 h. After centrifugation (1400 g for 15 min), the serum layer

TABLE 1. Antibodies Used to Sort Monocyte Subpopulations Prior to Culture

Antibody	Clone	IgG subclass	Supplier & number
CD11b-PE	D12	IgG <sub>2a</sub>	BD347557
CD13-PE	L138	IgG <sub>1</sub>	BD347837
CD14-FITC	MφP9	IgG <sub>2b</sub>	BD347493
CD14-PE	MφP9	IgG <sub>2b</sub>	BD347947
CD14-APC	MφP9	IgG <sub>2b</sub>	BD340436
CD16-FITC	NKP15	IgG <sub>1</sub>	BD347523
CD33-PE	P67.6	IgG <sub>1</sub>	BD347787
CD64-FITC	10.1	IgG <sub>1</sub>	BD555527

IgG, Immunoglobulin G; PE, phycoerythrin; BD, BD Biosciences, San Jose, CA; FITC, fluorescein isothiocyanate; Mφ, macrophage; APC, allophycocyanin.

was removed, pooled, filtered, and stored at -20°C. Except where specified, the culture medium was α-minimum essential medium (MEM; JRH Biosciences Inc., Lenexa, KS) containing 3% human serum (HS), penicillin/streptomycin [100 U/mL and 100 mg/mL final concentration, respectively (Invitrogen Corp.)], and 2 mM Gluta-MAX-1 (Invitrogen Corp.). When used, M-CSF (Chiron, Emeryville, CA) supplemented this medium at a concentration of 5000 U/mL. Sorted monocytes were cultured in polypropylene 96-well round-bottom plates (Corning Inc., Corning, NY). Except where specified, the cell concentration was 2.5 × 10<sup>5</sup> cells/mL in 200 µL medium.

### BrdU pulsing

The BrdU pulsing solution had equal concentrations of BrdU (Sigma Chemical Co., St. Louis, MO) and cytidine (Sigma Chemical Co.). At the days specified, 10 µL BrdU pulsing solution was added to each well. The concentrations of BrdU and cytidine were dependent on the assay undertaken. In each assay, nonpulsed monocytes were used as negative controls.

### Fixing and permeabilization

At the end of the BrdU pulse, the tissue-culture plates were spun at 400 g for 5 min. The medium was then carefully removed, and cells were washed twice and then resuspended in 200 µL 0.5% paraformaldehyde (Sigma Chemical Co.). After 4 h at room temperature in the dark, 44 µL 5% Tween 20 (APS, NSW, Australia) was added, and the plate was placed in the dark at 4°C overnight.

### Flow cytometry

Cells were stained in the wells in which they had been cultured, fixed, and permeabilized. The cells were washed twice with PBS and then resuspended in 30 µL staining solution, the composition of which was PBS containing anti-BrdU antibody (8.3 µg/mL, BD Biosciences) and DNase I (3.3 µg/mL, Sigma Chemical Co.). They were incubated at room temperature in the dark for at least 1 h and then at 4°C for 3 h. Where necessary, 3 µL propidium iodide (PI) solution (1 mg/mL, Sigma Chemical Co.) was added immediately prior to the 4°C incubation. Cells were resuspended in 200 µL FACS buffer, transferred to 1.5 mL polypropylene tubes, and analyzed on a FACSCalibur flow cytometer (BD Biosciences). Acquisition and analysis of triplicates of each condition, usually in excess of 10,000 events, excluded cellular debris based on forward-scatter (FSC) versus side-scatter (SSC) profile.

### CFSE labeling

CFSE labeling was done prior to or after cell sorting for kinetics experiments, whereas unsorted PBMC were used in other experiments. The cells were resuspended at a concentration of 10<sup>7</sup> cells/mL in PBS. Carboxyfluorescein diacetate-succinimidyl ester (Molecular Probes, Eugene, OR) was added to a final concentration of 2.5 µM. The solution was mixed well and incubated at room temperature for 10 min. An equal volume of α-MEM/5% serum was used to quench the reaction, after which, the cells were washed with PBS/1% serum.

For kinetic studies, CFSE-labeled CD14<sup>+</sup>-sorted monocytes were cultured and harvested at Days 4, 6, and 9. Using CellQuest software (BD Biosciences), the number of dividing cells in each peak was determined. The distance between peak apexes was used to set the gate size with the gates beginning and ending halfway between these apexes. PM in the starting population were estimated using the method proposed by Lyons [29]

In other experiments, PBMC were CFSE-labeled, seeded onto nontreated, 100 mm dishes (Iwaki, Japan) at a concentration of 5 × 10<sup>7</sup> cells per dish, and allowed to adhere for 48 h. Nonadherent cells were washed away, and new medium was added. The medium was replaced every 2–3 days, and after 9–10 days, the cells were detached and sorted into cells that had divided and those that had not. Cells were replated in α-MEM containing 3% HS, 1% penicillin/streptomycin, and 2 mM Gluta-MAX-1.

Photographs of cells were taken with a digital camera mounted on an inverted microscope (Axiovert 25, Carl Zeiss, Germany) using phase-contrast microscopy. Image acquisition was with Axiovision software (Version 4.0, Carl Zeiss).

### Tartrate-resistant acid phosphatase (TRAP) staining

CFSE-labeled cells that had been sorted into proliferative or non-PM at Day 9 were added to a 96-well Multiwell plate in the presence of 50 ng/ml soluble

receptor activator of nuclear factor- $\kappa$ B ligand (RANKL; PeproTech, Rocky Hill, NJ) and 5000 U/ml recombinant human M-CSF (Chiron) or M-CSF alone. Cells were cultured in duplicate or triplicate for each condition.

These cultures were incubated for up to 21 days, and the culture medium was changed every 3–4 days, at which time, fresh factors were added. TRAP staining was performed as described previously [30]. Briefly, the cells were fixed with 4% paraformaldehyde for 3 min. After removing the paraformaldehyde and allowing to air-dry, a 1:1 solution of ethanol and acetone was placed on the cells for 30 s and then removed, and the cells were again allowed to air-dry. TRAP stain (20  $\mu$ g/mL naphthol AS-MX phosphate, Sigma Chemical Co.), dissolved in N,N-dimethylformamide (Sigma Chemical Co.), 600  $\mu$ g/mL Fast Red Violet LB salt (Sigma Chemical Co.) in buffer (50 mM sodium acetate, Merck, Kilsyth, Victoria, Australia), and 40 mM potassium sodium tartrate (BDH Laboratory Supplies, Poole, UK) in H<sub>2</sub>O, was applied for 30 min and then washed away. Osteoclast numbers were evaluated by counting the number of TRAP-positive, multinucleate ( $\geq 3$ ) cells.

## Statistics

A *t*-test was used to determine statistical differences, and  $P < 0.05$  was considered significant. When comparing proliferation between donors, the average percentage of BrdU<sup>+</sup> cells in a subpopulation was normalized relative to the average percentage of BrdU<sup>+</sup> cells from the CD14<sup>+</sup> population cultured in the same conditions.

## RESULTS

### PM proliferation assay

We modified the BrdU incorporation assay to overcome previously reported difficulties when studying human monocyte proliferation [21–23]. Briefly, these challenges include the small percentage of PM in PBMC and even elutriation-purified monocytes, the lag period prior to the commencement of S-phase, and the variation between donors in monocytes, which can be assayed as PM, as well as the variation in response to M-CSF [21–23]. As the aim was to use flow cytometry to measure proliferation of monocyte subpopulations sorted prior to culture, this assay protocol was optimized before commencing the phenotypic analysis.

We have previously shown that at least following culture, the PM were CD14<sup>+</sup> [23]; preliminary sorting indicated that they also resided in the CD14<sup>+</sup> population of PBMC prior to culture (see also below).

#### *BrdU labeling time*

When sorted CD14<sup>+</sup> monocytes were cultured for 4 days and then pulsed with BrdU for 18 h, as done previously [23], the response to M-CSF was variable and nonexistent for a number of donors (**Fig. 1a**). Increasing the pulse window from 18 h to 2–4 days increased the number of cells captured in cycle; data for three donors are given in Figure 1b. However, as also shown in Figure 1b, the response to M-CSF was still inconsistent, and it is interesting that the sum of the percent BrdU incorporation in the 2-day periods (Days 4–6 and Days 6–8) was more than the percent BrdU incorporation from Days 4 to 8 in the absence or presence of M-CSF. Although this last observation suggested that the BrdU was limiting at the concentration used (50  $\mu$ M) when added over the longer period of 4 days from Days 4 to 8, a similar effect was also observed at a higher BrdU concentration (300  $\mu$ M), as shown in Figure 1b. As discussed below, the higher BrdU concentration gave superior delineation of flow data.

#### *BrdU concentration and culture conditions*

We therefore increased the BrdU concentration from 50  $\mu$ M, a concentration often used in proliferation studies [23, 31], to 300  $\mu$ M and higher. We found, under this modified labeling protocol, that incorporation was highest between Days 4 and 8 (**Fig. 1c**), with a consistent M-CSF-induced increase in monocytes entering S-phase compared with serum alone over this period.

The cell concentrations were also varied over the range of  $1.25 \times 10^5$  cells/mL– $7.5 \times 10^5$  cells/mL. As the cell concentration rose, the absolute number of detectable CD14<sup>+</sup> PM increased, especially in the absence of M-CSF; also, at the higher cell densities, the relative stimulatory effect of M-CSF tended to be less (data not shown). Likewise, at  $2.5 \times 10^5$  cells/mL, if the serum concentration were increased to 6%, the proliferation of CD14<sup>+</sup> cells increased, and again, the impact of M-CSF tended to be reduced (data not shown); the proliferation in 1% HS was suboptimal.

When examining the effect of varying the cell number from  $2.5 \times 10^5$  cells/mL to  $7.5 \times 10^5$  cells/mL, we observed that a BrdU concentration  $\geq 300$   $\mu$ M gave optimal labeling at both cell concentrations over the period of Days 4–8, as judged by the clear delineation of the BrdU<sup>+</sup> and BrdU<sup>-</sup> populations following flow cytometric analysis (**Fig. 2a**). The suboptimal detection of PM using 50  $\mu$ M BrdU can be observed, especially in the higher density cultures, where more cells were entering S-phase. The higher PM:BrdU ratio in the higher density cultures is more akin to the condition likely to be faced when enriching the PM when phenotyping. A toxic effect of BrdU concentration, such as reduced cell numbers, was not observed at the higher concentrations during the course of the assay.

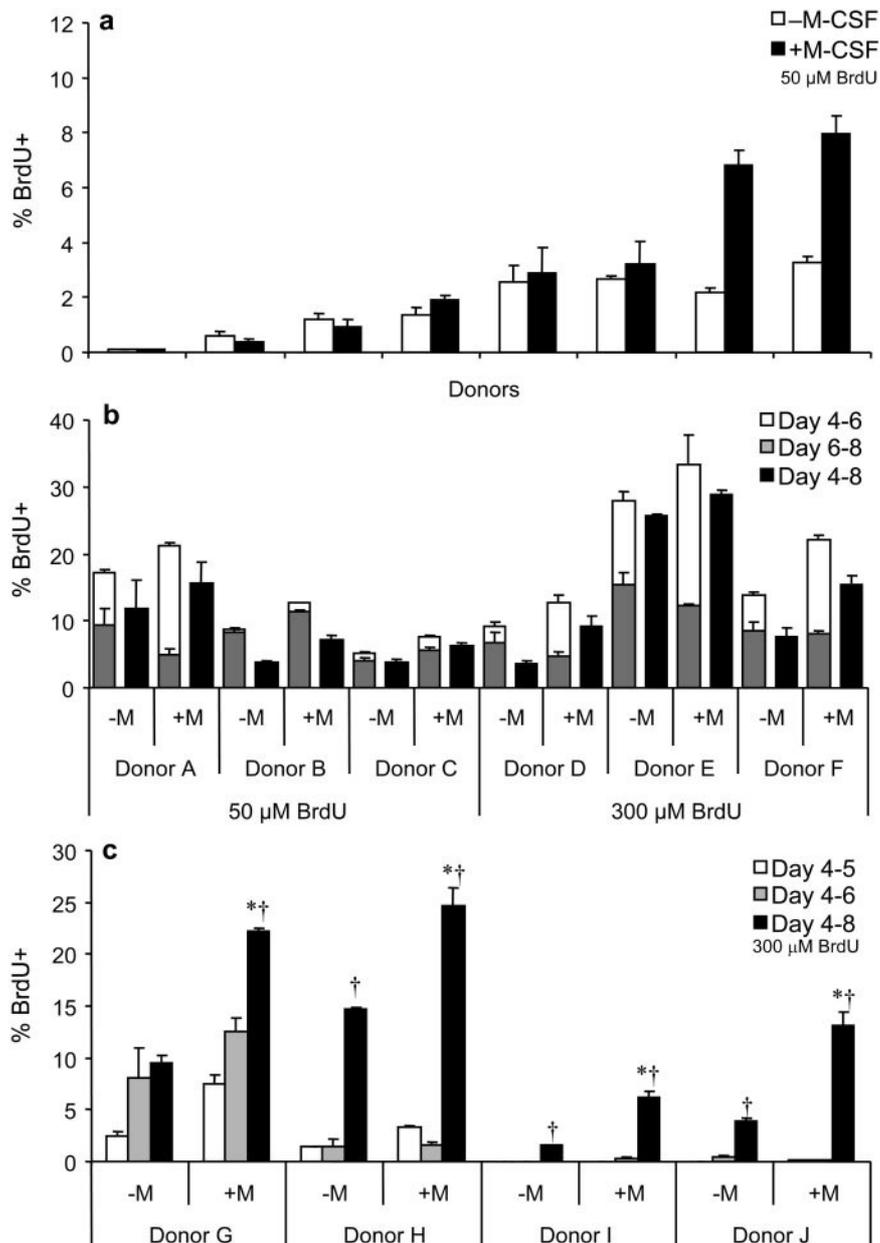
As a result of all of the above findings, for subsequent BrdU incorporation studies, a BrdU concentration of 300  $\mu$ M was used for Days 4–8 labeling of  $2.5 \times 10^5$  cells/mL in 3% HS  $\pm$  M-CSF and adopted as a standard protocol. With this protocol, the mean M-CSF-induced increase in BrdU incorporation compared with HS alone was approximately twofold ( $n=40$ ).

#### *PM proliferation*

Closer examination of the BrdU<sup>+</sup> cells in cultures pulsed with the high ( $\geq 300$   $\mu$ M) BrdU concentration revealed in fact two bands, which were identified by DNA content staining with PI as S-phase (2N) and G2 (4N; **Fig. 2b**), suggesting that some, if not all, of the PM was actually dividing and not merely undergoing DNA synthesis.

CFSE labeling has some advantages over BrdU labeling in that it identifies cells that have undergone cell division, and it does not require cell fixation, thereby enabling cell sorting by fluorescence and subsequent analysis of live cells (reviewed in ref. [29]). The cell division implied by the prior PI staining (**Fig. 2b**) was confirmed using CFSE-labeled CD14<sup>+</sup> monocytes cultured in M-CSF; CFSE fluorescence levels were determined after 4, 6, and 9 days. The gradual appearance of a peak with half the fluorescence intensity, indicating cells that have undergone one division [32], can be observed (**Fig. 2c**). This observation was repeated with four donors, and cell division was also observed in the absence of M-CSF. Using this loss of fluorescence to monitor cell division, it was calculated with six donors at Day 9 that  $\sim 30\%$  of cells had divided once, and 7%

**Fig. 1.** Optimization of PM bioassay. (a) CD14<sup>+</sup> monocytes ( $2.5 \times 10^5$  cells/mL) from eight donors were sorted and cultured in the absence or presence of M-CSF for 5 days with an 18-h BrdU pulse (50  $\mu$ M) prior to harvest (Materials and Methods). (b) CD14<sup>+</sup> monocytes from six donors were sorted and cultured ( $2.5 \times 10^5$  cells/mL) in the presence (+M) or absence (-M) of M-CSF and were pulsed with a final BrdU concentration of 50 or 300  $\mu$ M for 2 days (Days 4–6, Days 6–8, stacked bars) or 4 days (Days 4–8) prior to harvest (Materials and Methods). (c) CD14<sup>+</sup> monocytes from four donors were cultured ( $2.5 \times 10^5$  cells/mL) in the absence (-M) or presence (+M) of M-CSF and pulsed with BrdU (300  $\mu$ M) from Days 4 to 5, 4 to 6, or 4 to 8. There is a significant difference in BrdU incorporation between cultures with and without M-CSF when pulsed from Days 4 to 8 (\*,  $P < 0.05$ ). Furthermore, for three of the four donors, there is a significant increase in BrdU incorporation from Days 4 to 8 when compared with Days 4–6 in both culture conditions ( $\dagger$ ,  $P < 0.05$ ). Data are mean values  $\pm$  SEM of percent BrdU<sup>+</sup> cells from triplicate cultures.



had divided twice. The mean original precursor population was calculated to be 13.7% for these donors [29].

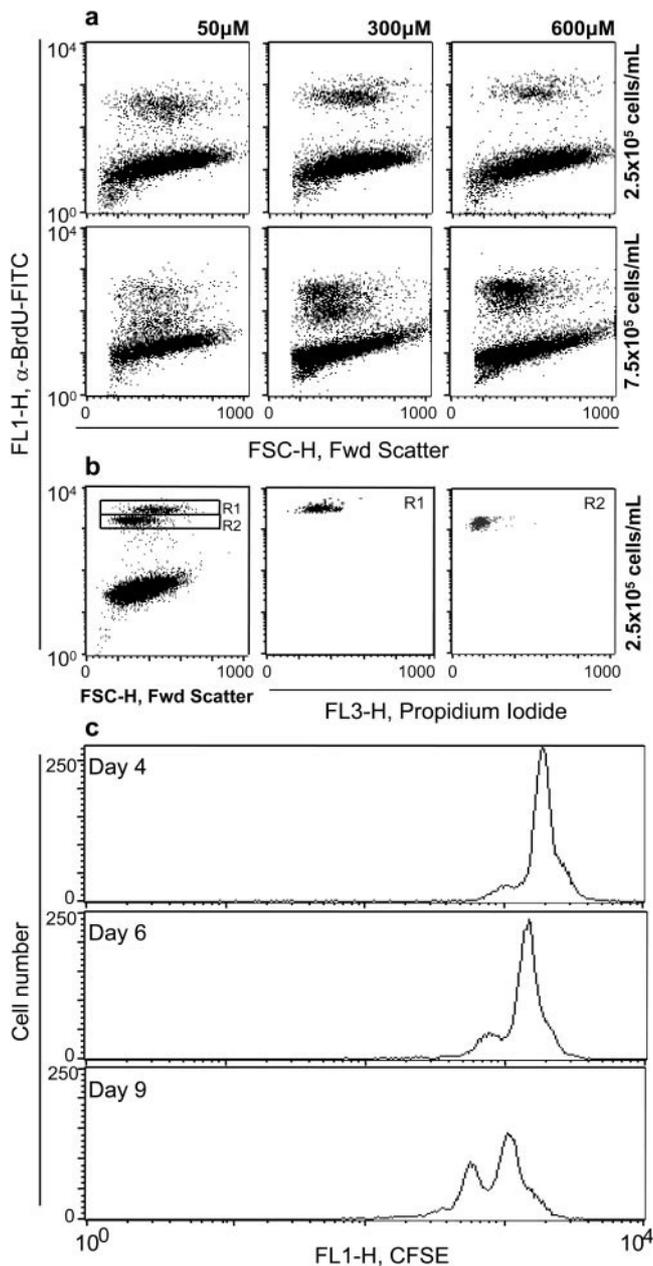
### PM surface marker expression upon isolation

Previous reported phenotyping of PM was carried out on cells at the end of the BrdU labeling period and after 5 days culture in M-CSF [23]. As human monocyte marker expression alters following culture [33, 34], monocyte populations were therefore sorted based on surface marker expression prior to culture, and DNA synthesis was measured using the modified protocol.

#### CD14<sup>med/lo</sup> subpopulations

Monocytes can be divided into CD14<sup>+</sup> and CD14<sup>med/lo</sup> subpopulations, in a ratio of  $\sim 9:1$ , with most of the latter expressing CD16 and with reduced expression of CD64 [7, 35]. As the frequency of the CD14<sup>lo</sup>CD16<sup>+</sup> cells and PM is similar [7, 35], we assessed whether they are the same by sorting them (Fig.

3a) and measuring entry into the cell cycle. As shown in Figure 3b for the three donors presented, the proliferation of the CD14<sup>lo</sup>CD16<sup>+</sup> subpopulation is approximately half that of the CD14<sup>+</sup> population. In Figure 3c, these data are expressed relative to the BrdU incorporation of the CD14<sup>+</sup> monocytes for each donor, which was arbitrarily set at a value of one. If the CD14<sup>med/lo</sup> subpopulation were composed principally of PM, it would be expected that the relative proliferation of these cells would be several times greater than that of the general monocyte population. The data for Donor A, for example, where the prevalence of CD14<sup>+</sup> cells within monocytic cells (based on FSC vs. SSC) is 83% (Fig. 3a), and the percentage of CD14<sup>+</sup> cells, which incorporated BrdU in culture, is 26% (Fig. 3b), indicate that 21% of monocytic cells, which incorporated BrdU in culture, are CD14<sup>+</sup> at Day 0; this is compared with 0.8% of monocytic cells, which incorporate BrdU, and are CD14<sup>lo</sup>CD16<sup>+</sup> at Day 0. It should be noted that the variation



**Fig. 2.** PM proliferation. (a)  $CD14^+$  monocytes ( $2.5 \times 10^5$  cells/mL and  $7.5 \times 10^5$  cells/mL) were cultured for 8 days in the presence of M-CSF, and over the last 4 days, BrdU was added at three concentrations (50, 300, and 600  $\mu$ M). Data are representative of four donors. (b)  $CD14^+$  monocytes ( $2.5 \times 10^5$  cells/mL) were cultured as in a and labeled with 300  $\mu$ M BrdU over the same time period. Two distinct regions (R1 and R2) were distinguishable within the  $BrdU^+$  population, which by PI staining for DNA content, were demonstrated to represent 4N and 2N content, respectively (Materials and Methods). (c)  $CD14^+$  monocytes were labeled with CFSE and cultured in M-CSF ( $2.5 \times 10^5$  cells/mL), and CFSE intensity was measured at Days 4, 6, and 9 (Materials and Methods). Data are representative of four donors. For six donors at Day 9, the proportion of cells that had divided once was  $30.97 \pm 3.6\%$  and for cells that had divided twice,  $6.88 \pm 1.8\%$ , indicating that  $13.66 \pm 1.03\%$  of cells at Day 0 was PM (mean values  $\pm$  SEM).

between donors was reduced when the proliferation of the  $CD14^lo CD16^+$  subpopulation was expressed relative to that of the general  $CD14^+$  population rather than absolute values. Other  $CD14^{med/lo}$  subsets ( $CD64^lo$  or  $Flt-1^+$ ) also had reduced relative proliferation (data not shown).

### $CD14^+$ subsets

Potential subsets of  $CD14^+$  monocytes were then investigated using the myeloid markers, CD13, CD64, CD11b, and CD33. With the antibodies used, there were no discrete subpopulations within the  $CD14^+$  monocytes that were distinctly positive or negative for these surface antigens; therefore, for convenience, high and low expression was defined arbitrarily as subsets, as the highest and lowest 15%, respectively (Fig. 4, a–d). For each donor, the proliferation of each subset, in the absence and presence of M-CSF, was again expressed relative to the total monocyte population ( $CD14^+$  cells) and the value averaged for all donors. In other words, the response of each donor population was controlled internally, leading again to a reduction in donor-to-donor variation compared with absolute values. The data for the M-CSF-treated populations are presented in Figure 4e. In the absence of M-CSF, the proliferation of each subset, relative to the proliferation of the total  $CD14^+$  monocyte population, was generally similar to that in the presence of M-CSF (data not shown).

**$CD14^+ CD13^{lo/hi}$**  The hematopoietic expression of CD13 is mainly confined to the committed myeloid progenitors and their progeny (reviewed in ref. [36]). It can be seen in Figure 4e that the proliferative capability of the  $CD14^+ CD13^{lo}$  cells is better than that of the entire  $CD14^+$  population, which was given an arbitrary value of one.

**$CD14^+ CD33^{lo/hi}$**  CD33 is a member of the sialoadhesion family of integrins, which is expressed on myeloid cells, with highest expression on monocytic cells and a putative role in regulation of proliferation or survival of normal and leukemic myeloid cells [37]. It can be seen in Figure 4e that in the presence of M-CSF, the  $CD14^+ CD33^{hi}$  subset has a higher proliferative capability compared with the corresponding cultures of  $CD14^+ CD33^{lo}$  monocytes.

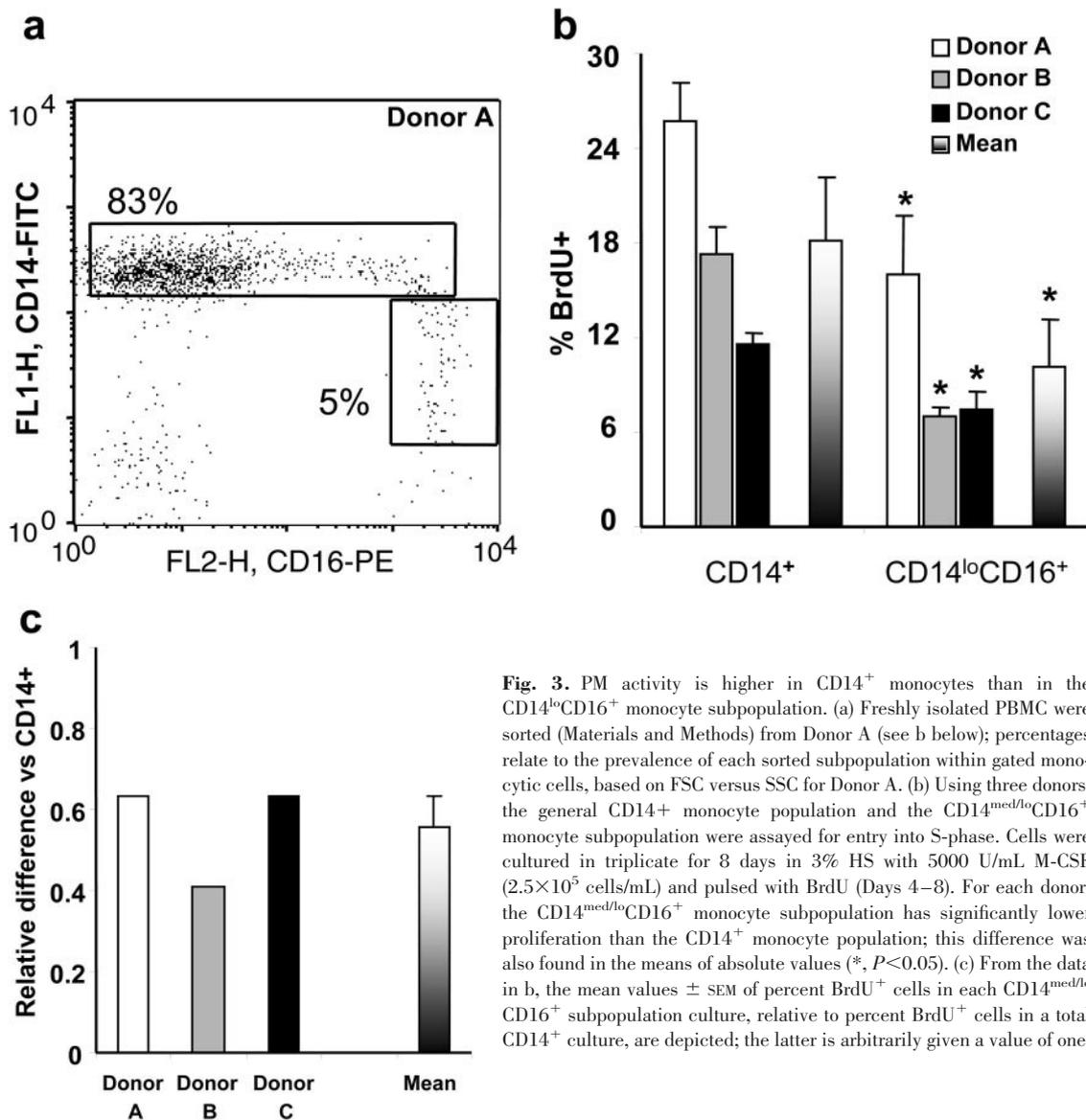
**$CD14^+ CD64^{lo/hi}$**  CD64 is the high-affinity Fc receptor for IgG and is expressed in early myelopoiesis but also on mature cells [38, 39]. As shown in Figure 4e, there is a difference in the proliferative capability between  $CD14^+ CD64^{hi}$  and  $CD14^+ CD64^{lo}$  populations; the former shows higher and the latter, lower BrdU incorporation than the  $CD14^+$  population overall.

**$CD14^+ CD11b^{lo/hi}$**  CD11b is a member of the  $\alpha$ -chain family of integrins, which is highly expressed on monocytes as part of the membrane-activated complex-1 with CD18 (reviewed in ref. [40]). In Figure 4e, it can be observed that there is a small difference in the proliferative capacity between  $CD14^+ CD11b^{lo}$  and  $CD14^+ CD11b^{hi}$  subsets in the presence of M-CSF; the similar difference observed in the absence of M-CSF did not reach statistical significance (data not shown).

**Other monocyte markers** The sorted  $CD14^+$  monocytes were also positive for the M-CSF receptor (c-Fms) and human leukocyte antigen-DR. However, unlike the other surface antigens studied above, we were not able to demonstrate any differential expression on the PM (data not shown).

### PM morphology upon culture

As mentioned above, one advantage of CFSE labeling is that proliferating and nonproliferating populations can be sorted after culture, and the former has a lower fluorescence intensity than the latter [29]. For this particular approach, we cultured CFSE-labeled PBMC long-term in M-CSF on nontreated dishes, separated the PM subpopulation (CFSE<sup>lo</sup>; Fig. 5a), and determined



**Fig. 3.** PM activity is higher in CD14<sup>+</sup> monocytes than in the CD14<sup>lo</sup>CD16<sup>+</sup> monocyte subpopulation. (a) Freshly isolated PBMC were sorted (Materials and Methods) from Donor A (see b below); percentages relate to the prevalence of each sorted subpopulation within gated monocytic cells, based on FSC versus SSC for Donor A. (b) Using three donors, the general CD14<sup>+</sup> monocyte population and the CD14<sup>med/lo</sup>CD16<sup>+</sup> monocyte subpopulation were assayed for entry into S-phase. Cells were cultured in triplicate for 8 days in 3% HS with 5000 U/mL M-CSF ( $2.5 \times 10^5$  cells/mL) and pulsed with BrdU (Days 4–8). For each donor, the CD14<sup>med/lo</sup>CD16<sup>+</sup> monocyte subpopulation has significantly lower proliferation than the CD14<sup>+</sup> monocyte population; this difference was also found in the means of absolute values (\*,  $P < 0.05$ ). (c) From the data in b, the mean values  $\pm$  SEM of percent BrdU<sup>+</sup> cells in each CD14<sup>med/lo</sup>CD16<sup>+</sup> subpopulation culture, relative to percent BrdU<sup>+</sup> cells in a total CD14<sup>+</sup> culture, are depicted; the latter is arbitrarily given a value of one.

whether, following this treatment at least, they might differ in morphology from the bulk of the nonproliferating, adherent monocytes. Differences in morphology were observed between the two populations following short-term reculture. After these treatments, the majority of the PM assumed a fusiform, “spindle” shape, whereas the nonproliferative population was predominantly a rounded, “fried egg” shape (Fig. 5, b and c, respectively).

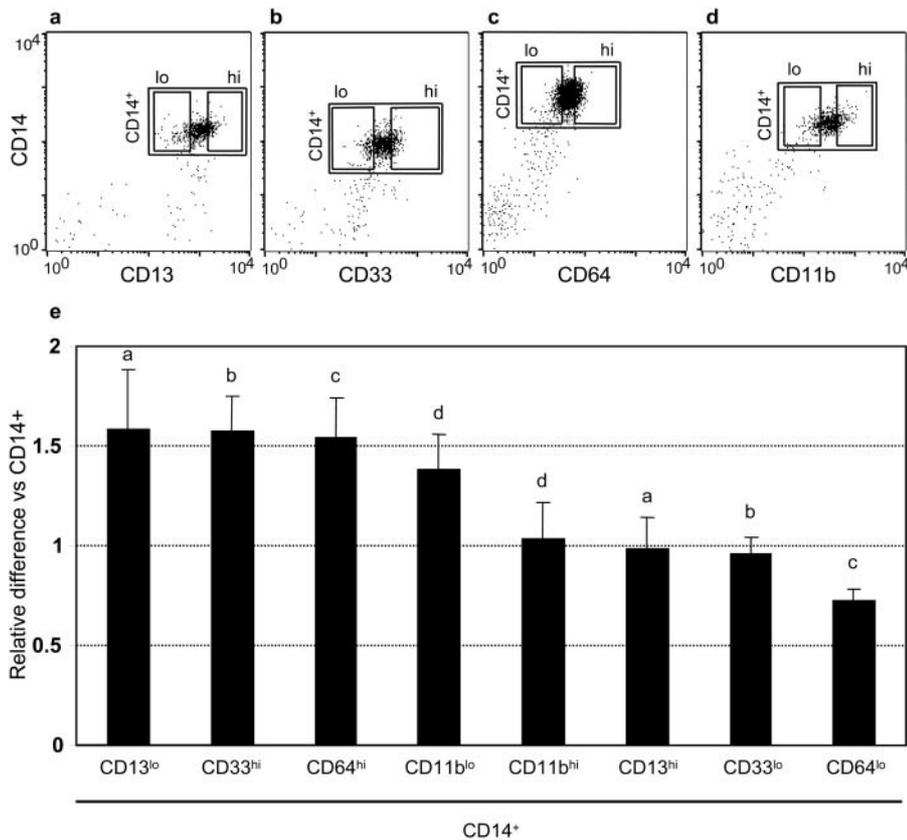
#### PM enriched for osteoclast-like precursors

A poorly characterized subpopulation of CD14<sup>+</sup> human monocytes can be converted into TRAP<sup>+</sup> multinucleated osteoclasts following long-term culture in M-CSF and RANKL [41]. We assessed whether we could obtain any evidence for PM being this subpopulation. CFSE-labeled PBMC were cultured in M-CSF in nontreated dishes, and the PM and nonproliferating

populations were separated and cultured in M-CSF and RANKL for 21 days on a tissue-culture surface. The resultant cells were then TRAP-stained (Fig. 6, a–d). We found that cells derived from the PM population had significantly more multinucleated ( $n \geq 3$ ) TRAP<sup>+</sup> cells when cultured in M-CSF and RANKL compared with the nonproliferating cells (Fig. 6e). The number of osteoclast-like cells from either source of cells cultured in M-CSF alone was lower than from nonproliferating cells cultured in RANKL and M-CSF (data not shown).

## DISCUSSION

An advantage of a functional assay, namely proliferation, over surface marker phenotyping is that it avoids the difficulty of



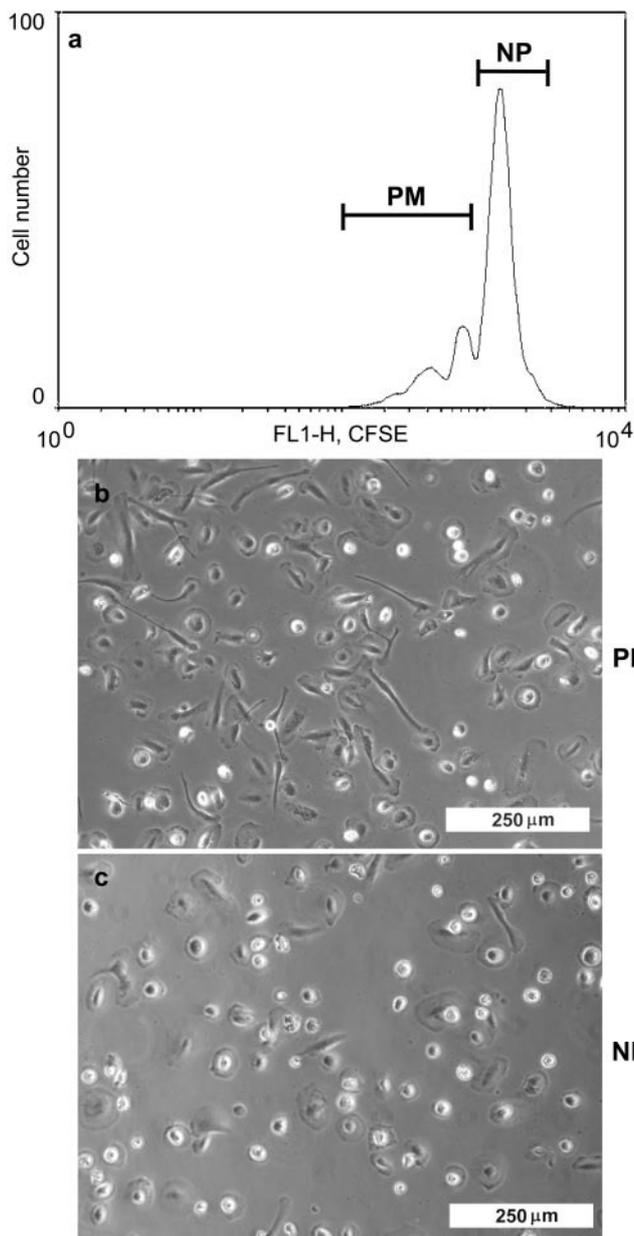
**Fig. 4.** PM activity of CD14<sup>+</sup> subsets. (a–d) Freshly isolated PBMC were sorted, gating on monocytic cells, cultured ( $2.5 \times 10^5$  cells/mL) for 8 days in the presence of M-CSF, and pulsed with BrdU for Days 4–8. (e) Triplicate cultures of monocyte subsets were assayed for entry into S-phase, and mean values of percent BrdU<sup>+</sup> cells were expressed relative to mean values of the triplicate cultures of total CD14<sup>+</sup> monocytes for each donor, the latter, arbitrarily given a value of one. Data are means  $\pm$  SEM of relative values from n number of donors. Data with the same letter indicate a significant difference in proliferation between two subsets with highest and lowest expression of a particular marker. CD13<sup>hi</sup> and CD13<sup>lo</sup> subsets (n=7 donors; bars a,  $P=0.02$ , CD14<sup>+</sup>CD13<sup>lo</sup> vs. CD14<sup>+</sup>CD13<sup>hi</sup>). CD33<sup>hi</sup> and CD33<sup>lo</sup> subsets (n=7 donors; bars b,  $P=0.02$ , CD14<sup>+</sup>CD33<sup>lo</sup> vs. CD14<sup>+</sup>CD33<sup>hi</sup>). CD64<sup>hi</sup> and CD64<sup>lo</sup> subsets (n=8 donors; bars c,  $P=0.005$ , CD14<sup>+</sup>CD64<sup>hi</sup> vs. CD14<sup>+</sup>CD64<sup>lo</sup>). CD11b<sup>hi</sup> and CD11b<sup>lo</sup> subsets (n=7 donors; bars d,  $P=0.029$ , CD14<sup>+</sup>CD11b<sup>lo</sup> vs. CD14<sup>+</sup>CD11b<sup>hi</sup>). Figure labels for subsets shown in FACS dot-plots (a–d, 4) correlate with subset data labels in e (4).

distinguishing between whether a particular marker reflects differentiation or activation. We found that a high concentration of BrdU ( $\geq 300 \mu\text{M}$ ) gave higher values for the degree of DNA synthesis for CD14<sup>+</sup> cells than typically used lower concentrations. Furthermore, a relatively wide BrdU labeling window between Days 4 and 8 gave the most consistent results for a range of different donor populations. These conditions would appear to be essential if optimal PM detection by BrdU labeling is to occur. In cultures with a higher cell concentration, BrdU concentrations of  $300 \mu\text{M}$  and greater were required to clearly delineate BrdU<sup>+</sup> cells. We suggest that this is an important and relevant finding, as higher PM concentrations in culture result when the PM are enriched following successful phenotyping. The requirement for high BrdU concentrations during a relatively long labeling period is consistent with previously reported observations that human monocyte <sup>3</sup>H-thymidine incorporation was less if the label were added at the initiation of the cultures, which was considered likely to be a result of its breakdown [42, 43]. At the 3% concentration of pooled HS, normally used in our studies, M-CSF generally enhanced the proliferative response. The basal HS response tracked the M-CSF response during the various cell fractionations described above, suggesting that the same population is proliferating under the two conditions. This basal response may not be a result of endogenous M-CSF, as preliminary experiments with neutralizing anti-M-CSF antibody failed to block it (data not shown). Whether the signal transduction pathways governing the M-CSF-dependant PM proliferation are similar to those for the well-studied mouse macrophages (reviewed in ref. [44]) is under current investigation.

As shown with a combination of BrdU labeling and PI staining, the cells that appear to have gone through the process of cell division, i.e., with less intense BrdU and lower total DNA staining, constitute a significant proportion of BrdU<sup>+</sup> cells and would account for the increased numbers of BrdU<sup>+</sup> cells at 8 days. CFSE labeling provided a method to measure the number of cycles that the PM population could undergo within a certain time-frame. Evidence was obtained for at least three cell divisions in some experiments over the time examined (9 days), even given the lag period for cell-cycle entry.

Surface marker phenotyping demonstrated for the first time that prior to culture, the PM are predominantly CD14<sup>hi</sup> cells, thereby distinguishing them from the CD14<sup>lo</sup>CD16<sup>+</sup> and other overlapping CD14<sup>med/lo</sup> subpopulations, which are present at similar proportions in human monocytes as the PM [7, 35]. The CD14<sup>lo</sup>CD16<sup>+</sup> subpopulation is considered to be a relatively mature monocyte [35], and the assumption that the PM are relatively immature because of their proliferative capability [23] is consistent with the reduced relative, proliferative capacity of the CD14<sup>lo</sup>CD16<sup>+</sup> cells. The PM may mature to become the CD14<sup>lo</sup>CD16<sup>+</sup> population upon differentiation in vivo, although we reported previously that at the end of 5-day cultures in M-CSF, they did not express CD16 [23].

By normalizing the data relative to the proliferative response of the total CD14<sup>+</sup> cells from each donor, rather than focusing on the absolute proportion of cycling cells, we were able to demonstrate higher proliferative capability amongst CD14<sup>+</sup> cells expressing lower CD13 levels and higher CD33 and CD64 levels. We suggest that our comparison of the CD14<sup>+</sup> subsets relative to the total CD14<sup>+</sup> monocyte population gives a clearer

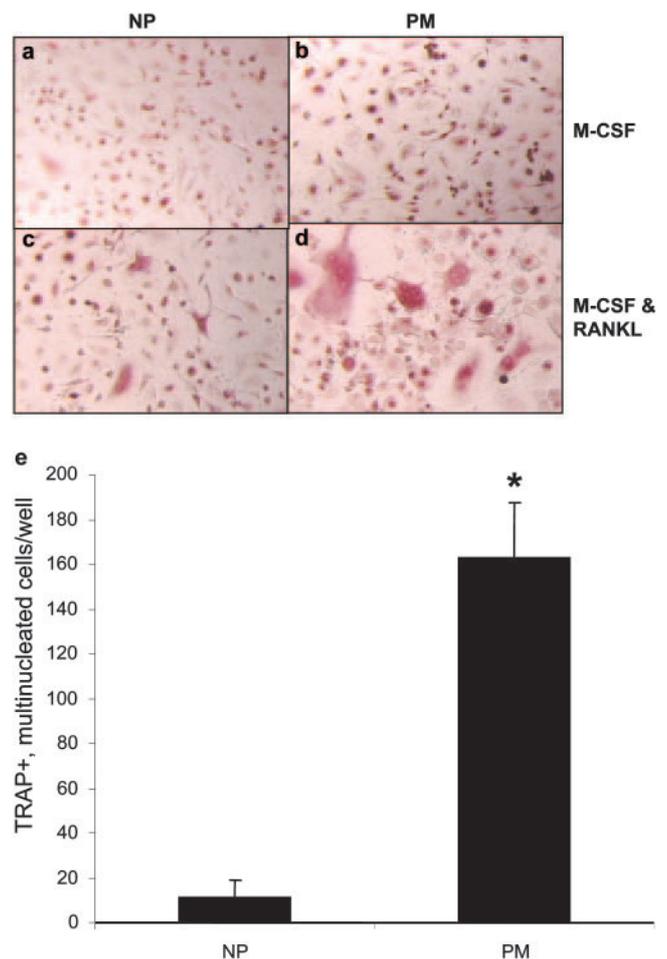


**Fig. 5.** PM morphology following culture. CFSE-labeled PBMC were cultured in 3% HS and M-CSF ( $5 \times 10^6$  cells/mL) in nontreated dishes for 9–10 days. (a) The adherent cells were then sorted based on their CFSE fluorescence intensity as PM (CFSE<sup>lo</sup>) and nonproliferating (NP; CFSE<sup>hi</sup>) monocytes (Materials and Methods). (b) PM (CFSE<sup>lo</sup>) cells were then cultured again in 3% HS overnight (original magnification,  $\times 180$ ). (c) Nonproliferating (CFSE<sup>hi</sup>) cells were cultured as in b at the same original magnification. Data are from a representative experiment, which was repeated with three donors.

depiction of the proliferative capacity and therefore maturity within the CD14<sup>+</sup> cells. From our findings, CD13 expression is predicted to increase, and that for CD33 and CD64 would decrease as monocyte differentiation proceeds. Support for this concept comes from our finding that the more mature CD14<sup>lo</sup>CD16<sup>+</sup> subpopulation [35] exhibited relatively reduced proliferative capacity. The delineation of the subsets lends itself to further work in elucidating the degree to which the subsets differ in other monocyte attributes such as response to lipopolysaccharide, motility, phagocytic ability, and differentiation potential.

Assuming the PM are less mature than the bulk of the monocyte population, they are likely to have more potential to differentiate. In this context, there has been a deal of interest in the ability of CD14<sup>+</sup> human peripheral blood populations to be precursors of bone-resorbing osteoclasts [45], DC (reviewed in refs. [46, 47]), and even of nonhaemopoietic lineages, for example, smooth muscle cells, osteoblasts, and adipocytes [48]. CFSE labeling enabled the sorting, culture, and morphologic characterization of the PM upon subsequent culture as distinctly spindle-shaped, delineating them by this criterion from the bulk of the CD14<sup>+</sup> monocytes.

The morphologic differences between the spindle and fried-egg cells are similar to those caused by growth factors such as M-CSF, GM-CSF, and IL-3 [49]. As the nonproliferating and PM were cultured in the same medium, a more likely proposition is that the morphology is intrinsic to the PM. Monocyte-derived fusiform cells have been hypothesized to be an intermediate stage in osteoclast differentiation (a “pro-osteoclast”; ref. [50]), as our data also suggests, and even have pluripotent



**Fig. 6.** Osteoclast-like potential of the PM. CFSE-labeled PBMC were sorted after 9 days in culture as PM (CFSE<sup>lo</sup>; b, d) and nonproliferating monocytes (CFSE<sup>hi</sup>; a, c) and were further cultured for 21 days in M-CSF (a, b) or M-CSF and RANKL (c, d). TRAP staining was performed. (e) TRAP<sup>+</sup> multinucleated (“osteoclast-like”) cells were counted for three donors. The number of osteoclast-like cells was significantly higher in the PM-derived cells cultured in RANKL and M-CSF compared with the nonproliferating-derived cells cultured in the same way (\*,  $P=0.042$ ).

potential [48]. Whether culturing monocytes in other growth factors, such as GM-CSF, alters the PM morphology or the potential to form osteoclast-like cells is currently under investigation.

Additional culture in the presence of RANKL and M-CSF further characterized the PM as having higher capacity to differentiate to TRAP+, multinucleated, osteoclast-like cells. We propose that this finding is consistent with a recent one describing the generation of osteoclastic cells from proliferating DC precursors in human peripheral blood [51]. Whether the PM can become bone-resorbing osteoclasts or possess other osteoclastic properties such as RANK expression awaits further analysis.

As previously put forward, the PM, upon migration into tissues, could represent precursors of macrophage lineage populations, which can proliferate locally, thereby contributing to the cell accumulation at a site of inflammation [24]. Peripheral blood "inflammatory" monocyte subpopulations have recently been proposed for humans [11] and mice [12]. It is therefore possible that the PM bears some relationship to the human inflammatory subpopulation, as there is recent evidence for a less mature subset(s), which can migrate and mature at a site of inflammation in the mouse [52]. As it has also been reported in murine models that an inflammatory reaction can skew the blood monocytes toward a higher frequency of immature cells [12], it would be of interest to know if PM levels are altered in inflammatory diseases.

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