Impact of Human Granulocyte and Monocyte Isolation Procedures on Functional Studies

Lu Zhou, a,b Rajesh Somasundaram, a Rosa F. Nederhof, a Gerard Dijkstra, a Klaas Nico Faber, a Maikel P. Peppelenbosch, a and Gwenny M. Fuhler a

Department of Gastroenterology and Hepatology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands a; Department of Gastroenterology and Hepatology, Tianjin Medical University General Hospital, Tianjin Medical University, Tianjin, People's Republic of China b; and Department of Gastroenterology and Hepatology, Erasmus Medical Center, Erasmus University of Rotterdam, Rotterdam, The Netherlands a

One of the first lines of defense against infection is the activation of the innate immune system. It is becoming clear that autoimmune diseases, such as rheumatoid arthritis and Crohn’s disease, may be caused by disturbed innate immunity, and relating granulocyte and monocyte functions to the patient genotype has become an important part of contemporary research. Although it is essential to move this field forward, a systematic study comparing the efficacy and suitability for functional studies of the various available protocols for the isolation of these immune cells has not been performed. Here, we compare human granulocyte functionality under three enrichment protocols: (i) Ficoll density gradient centrifugation, (ii) anti-CD15 antibody-conjugated microbeads (positive selection), and (iii) Polymorphoprep. Primary monocytes were isolated in parallel using (i) anti-CD14 magnetic microbeads, (ii) non-monocyte depletion by antibody-conjugated magnetic microbeads (negative selection), (iii) RosetteSep antibody cocktail, and (iv) the classical adherence protocol. The best results in terms of purity and cell functionality were obtained with positive selection by magnetic microbeads for both human granulocytes and monocytes. Whereas phagocytosis of Escherichia coli bacteria was identical in all isolation procedures tested, the granulocyte respiratory burst was higher in positively selected cells. In addition, different granulocyte enrichment procedures affect cell surface receptor expression to different extents. In toto, we propose that positive selection of granulocytes and monocytes be adopted as the procedure of choice for studies of human granulocyte and monocyte functions but caution investigators to be aware of possible alterations in cell phenotypes with different isolation procedures.

Monocytes represent 3 to 7% of total white blood cells (absolute monocyte count, $1.5 \times 10^8$ to $7 \times 10^8$/liter blood) in healthy human adults. Circulating monocytes, which are derived from myelomonocytic stem cells in bone marrow, have two main functions in the immune system: (i) to replenish resident macrophages and dendritic cells in peripheral tissues under normal states and (ii) to patrol healthy tissues through long-range crawling on the resting endothelium (10). In response to inflammatory signals, monocytes quickly move to sites of infection in the tissues, engage in phagocytosis of foreign substances, and initiate an early immune response through the recruitment of neutrophils and other polymorphonuclear leukocytes (PMNs). PMNs constitute the most abundant of peripheral white blood cells, i.e., 40 to 60% (absolute count, $25 \times 10^8$ to $750 \times 10^8$/liter blood). Their bactericidal activity is essential in the proper clearance of infectious agents and stems from their exocytosis of lysozyme- and protease-containing granules, phagocytosis of bacteria, and the concomitant production of reactive oxygen species (ROS) (respiratory burst). Extravasation of PMNs to the site of infection in turn precedes a second wave of migrating monocytes to remove rapidly apoptotic PMNs. Monocytes, macrophages, and dendritic cells are also capable of eliciting an adaptive immune response via antigen presentation, a role that has also been attributed to granulocytes in inflammatory settings (6, 22). Therefore, PMNs and monocytes play a pivotal role in maintaining the dynamic balance of the human immune system (29).

Interest in measuring innate immune cell functionality has substantially increased, especially because of a growing acceptance of the notion that defects in innate immunity contribute to the pathogenesis of autoimmune diseases. Examples are rheumatoid arthritis (RA); systemic lupus erythematosus (SLE), an autoimmunity against DNA; and the pathogenesis of Crohn’s disease (CD), an often severe autoimmunity toward the resident gut flora. CD patients exhibit a phagocyte immunodeficiency that combines a primary macrophage defect and a secondary granulocytic defect (2–5, 24). Macrophages from these patients demonstrate impaired cytokine secretion in response to Escherichia coli loading (24). Furthermore, after acute trauma in the gut mucosa and skin, PMN recruitment, bacterial clearance, and ROS production are attenuated (20, 23, 25). With respect to rheumatoid arthritis, granulocytes play an important role in the induction of disease and disease progression, as depletion of PMNs alleviates disease (28). One of the mechanisms through which granulocytes may exacerbate disease is by the deposition of autoantibodies in the joints, although relatively little is known about this phenomenon (21). In SLE, granulocytes may enhance immunological reactions by the formation of neutrophil extracellular traps (NETs) which form immune complexes consisting of DNA, antimicrobial peptides, and autoantibodies, which subsequently activate plasmacy-
toid dendritic cells (11, 14). In addition, monocyte function and morphology in SLE have been reported to be altered; however, phagocytosis of bacteria may be either decreased or enhanced, and more studies to assess the real contribution of these innate immune cells have been called for (15, 18).

Taken together, the body of contemporary biomedical literature strongly supports the concept that innate immune cell dysfunction is associated with the pathogenesis of many autoimmune diseases (29), triggering investigations into the properties of innate immune cells in patients and comparison of their phenotype to the genotype of risk genes relevant to autoimmunity. The results of such studies may well depend on the protocols employed for isolation of the immune cells. A comparison of both the yield and the specificity of the available protocols for monocyte and PMN isolation from patient peripheral blood, as well as the relative performance of cells isolated using these protocols in subsequent functional experimentation, is urgently needed.

These considerations prompted us to perform a systemic evaluation of the most frequently used methodologies for the isolation of granulocytes and monocytes from human peripheral blood. PMNs were isolated in parallel using (i) Ficoll density gradient centrifugation, (ii) polymorphprep density gradient centrifugation, and (iii) anti-CD15 antibody-conjugated magnetic microbeads (positive selection), after which PMN functionality was assessed by in vitro phagocytosis and ROS production assays. Monocytes were isolated from peripheral blood in parallel using (i) anti-CD14 antibody-conjugated magnetic microbeads (positive selection), (ii) non-monocyte depletion by antibody-conjugated magnetic microbeads (negative selection), (iii) immunorosette-based RosetteSep antibody cocktail (RosetteSep), and (iv) adherence, aiming to assess their suitability for in vitro phagocytosis analysis. We conclude that positive selection of granulocytes and monocytes by anti-CD15 and anti-CD14 antibody-conjugated magnetic microbeads, respectively, is best suited for studies in which purity is imperative but that in general the isolation method of choice should depend on the type of functional assay to be used.

MATERIALS AND METHODS

Granulocyte isolation from human peripheral blood. Heparin and EDTA anti-coagulated blood was obtained from healthy volunteers after informed consent and in accordance with the ethical guidelines of the institution. Neutrophils were isolated as described previously (9). In short, mononuclear cells were removed by centrifugation of heparinized blood over Ficoll-Paque (Amersham), followed by erythrocyte lysis with ice-cold NH4Cl solution. For positive selection, granulocytes obtained from Ficoll density gradient centrifugation were subsequently subjected to anti-CD15 microbead isolation (Miltenyi Biotec, Amsterdam, The Netherlands), using manual columns, according to the manufacturer’s instructions. Additionally, PMNs were isolated from EDTA anti-coagulated blood using PolymorphPrep (Axis-Shield, Norway). Before functional testing, PMNs were allowed to recover for 30 min at 37°C in RPMI 1640 supplemented with 0.5% human serum albumin (HSA) (Sanquin, The Netherlands). The cells were resuspended in incubation buffer (20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM MgSO4, 1.2 mM KH2PO4, 5 mM glucose, 1 mM CaCl2, and 0.5% HSA) prior to subjecting them to functional assays. All isolation procedures were done in parallel for each healthy donor.

ROS production assay. ROS production was performed as described previously (8). Briefly, PMNs (2 × 10⁶ cells/ml) were incubated with dihydrorhodamine (DHR) 123 for 15 min and stimulated with 1 μM N-formyl-methionine-leucine-phenylalanine (fMLP) for 30 min. For priming experiments, cells were pretreated with 5 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) for 15 min prior to fMLP stimulation. Stimulation of PMN with fMLP was terminated by washing the cells with ice-cold PBS containing 1% HSA and placing them on ice. Oxidation of DHR 123 to the fluorescent rhodamine 123 was measured by fluorescence-activated cell sorter (FACS) analysis within 30 min after termination of stimulation. Monocyte isolation from human peripheral blood. Heparinized blood was obtained from healthy volunteers after informed consent. In order to optimize the monocyte isolation method, monocytes from the same healthy donor were isolated by four different strategies in parallel. In short, monocyte positive selection by monoclonal CD14 antibody (isotype, mouse IgG2a)-conjugated microbeads (Miltenyi Biotec, Germany) and monocyte negative selection with a monocyte isolation kit II (Miltenyi Biotec) were performed with manual columns strictly according to the manufacturer’s protocol, as was monocyte isolation by RosetteSep (Stemcell Technologies, France). Monocyte isolation by the adherence method was performed as described previously (7). Briefly, peripheral blood mononuclear cells (PBMC) were isolated immediately after collection using Lymphoprep gradients (Axis-Shield POcas, Norway). Monocytes were further enriched by virtue of their attachment to a culture plate for 2 h and washed 3 times with warm phosphate-buffered saline (PBS) to remove nonadherent cells, and the adherent monocytes were recovered with a cell scaper. The purity of monocytes was evaluated by fluorescent staining with CD14-phycocerythrin (PE) monoclonal antibody (UCHM1, murine IgG2a; IQ products, The Netherlands) and FACS analysis. The recovery of monocytes was evaluated by trypan blue staining and counting using a Zeiss microscope.

Cell culture. Monocytes were cultured in complete medium consisting of RPMI 1640 (PAA Laboratories, Austria) supplemented with 10% heat-inactivated fetal calf serum (FCS) (PPA) and 10 μg/ml gentamicin (Centrafarm, The Netherlands) at 37°C in 5% CO2 humidified air. Phagocytosis assay. Green fluorescent protein (GFP) expression vector was transformed into E. coli and grown in LB medium until an optical density (OD) of 400 was reached. Bacteria were fixed using 4% formaldehyde. Isolated PMNs were resuspended (2 × 10⁶/ml) in RPMI containing 10% FCS and 1 × 10⁶ bacteria were subjected to PMN phagocytosis at 37°C for 15 min. The percentage of phagocytosing PMNs was determined by flow cytometry by analyzing the percentage of GFP-positive PMNs. Phagocytosis at 0°C was used as a negative control for each experiment. No Fluorescein isothiocyanate (FITC)-positive PMNs were observed under these conditions, confirming active phagocytosis of bacteria rather than attachment of bacteria to PMN membranes. Alternatively, 4% formaldehyde-fixed E. coli cells were labeled with FITC fluorescence by incubation in 1 mg/ml FITC solution containing 0.1 M Na2CO3, pH 9.5, for 1 h, followed by complete washing. The efficiency of labeling was tested by FACS analysis (see below). The bacterial concentration was quantified using Quantimet HR550 image analysis software (Leica) to analyze microscopic images of FITC-labeled E. coli taken with a Leica (Wetzlar, Germany) DMRXA epifluorescence microscope (30). For monocytes, the phagocytosis assay was performed according to the method of Mandell and Hook (19). In short, monocytes (1 × 10⁶/ml) were incubated with FITC-labeled E. coli at a 1:5 ratio for 5 or 15 min at 37°C in RPMI 1640 medium containing 10% heat-inactivated FCS. Thereafter, phagocytosis was evaluated microscopically by counting both the monocytes exhibiting phagocytosis and the bacteria phagocytosed per monocyte. At least 300 cells were counted for each slide. The engulfment rather than attachment of E. coli to PMNs and monocytes was confirmed with a confocal microscope (LSM 700; Carl Zeiss, Jena, Germany) and cultures at 4°C.

FACS analysis. Isolated PMNs or whole blood after red cell lysis were stained and resuspended in PBS-EDTA-1% FCS. After blocking with Fc receptor (FcR) blocking reagent from Miltenyi Biotec (Amsterdam, The Netherlands) for 15 min, cells were labeled with anti-CD14-peridinin chlorophyll protein (PerCP)-Cy5.5 (clone M5E2), anti-CD66b-PerCP-Cy5.5 (G10F5), anti-CD15-PE (clone H198), anti-Toll-like receptor 2 (1066 cvi.asm.org Clinical and Vaccine Immunology
Comparison of Granulocyte and Monocyte Isolation Methods

RESULTS

Comparison of three commonly used isolation protocols for purity of enriched granulocytes. PMNs were isolated from blood from healthy volunteers using, in parallel, three of the most commonly used methods in the literature. The purity of isolates was analyzed by flow cytometry and proved to be 96.2% ± 2%, 99.5% ± 0.5%, and 91.4% ± 4.9% of nucleated cells for Ficoll, anti-CD15 microbead positive selection, and Polymorphprep isolation methods, respectively (Fig. 1A and Table 1). This measure, however, excludes nonnucleated cells (erythrocytes and thrombocytes), as well as ghosts derived from necrotic cells. Hence, we also determined the percentage of CD66b-positive cells as a function of all cell-like particles (i.e., all particles in which the diameter was >0.5 µm). When results were expressed in this way, Ficoll density gradient centrifugation yielded 87% ± 3% granulocyte purity compared to 88% ± 5% for positive selection (see Fig. 1B for a representative example). The Polymorphprep isolation method resulted, on average, in 76% ± 19% purity, largely due to a larger number of contaminating debris particles and lymphocytes (Fig. 1C). Also, in our hands, the highest variability in purity between isolations was observed when using Polymorphprep, suggesting that the method is somewhat more sensitive to small day-to-day variations. We also noticed a donor-to-donor variation in adherence of the lymphocyte ring to the plastic disposables after centrifugation, which may account for some of the contamination variability observed. When the nature of this contaminating mononuclear cell fraction was analyzed, it was found to be mainly CD19-positive T cells, with few or no CD19+ B cells or CD14+ monocytes (not shown).

PMNs are among the shortest-lived cells in the body. They are extremely fragile cells and are prone to apoptosis upon withdrawal from the blood. Importantly, however, no more than 3.7% dying cells were identified by 7-aminoactinomycin D (7ADD) (Fig. 2D) or DAPI (not shown) staining, as determined by FACS analysis of any of the isolation methods used, indicating that no significant cell death was induced during isolation procedures.

In conclusion, the best results in terms of granulocyte purity were obtained using anti-CD15 microbead positive-selection methods.

Comparison of granulocyte yields obtained using the three most commonly used PMN isolation protocols. The absolute recovery rates of PMNs after the three isolation procedures are shown in Table 1. Typically, isolation of PMNs by positive selection, though leading to the highest purity, also yielded the lowest recovery rates: 9.6 ± 10^7/ml peripheral blood were obtained, which was significantly less than the yields obtained using either Ficoll centrifugation (16 ± 10^7/ml peripheral blood) or Polymorphprep (14.8 ± 10^7/ml peripheral blood). With a purity of 95.2% and the highest overall yield, granulocyte enrichment by Ficoll centrifugation seems to be the optimal method when high numbers of cells are required.

Granulocyte functionality in a ROS production assay following different isolation protocols. PMNs are easily activated by trace amounts of bacterial lipopolysaccharides or mechanical stressors. To determine whether our different isolation methods produce unwanted activation of PMNs, we studied their ROS production. Spontaneous PMN ROS production levels did not differ between isolation procedures (data not shown). In addition, expression of the neutrophil activation marker 1-selectin (CD62L), shedding of which is associated with PMN activation (26), was similar between groups, confirming that there was no bias in activation of PMNs between isolation methods (Fig. 3A, upper left).

ROS production can be triggered by the bacterial peptide analogue fMLP, and fMLP-induced ROS production is significantly enhanced in GM-CSF-primed PMNs. Significant differences were observed when comparing fMLP-triggered ROS production between isolation methods (P = 0.042 by the Friedman test). They were caused by the fact that PMNs isolated by Polymorphprep showed a lag in fMLP-induced ROS production compared to either the Ficoll or positive-selection method (109% ± 8% versus 148% ± 18% and 168% ± 28%, respectively; t = 5 min) (Fig. 2A). Pretreatment of PMNs with GM-CSF resulted in significant enhancement of ROS production for all isolation methods tested. However, we observed significant differences between the groups (P = 0.042 by the Friedman test), with PMNs isolated by positive selection showing significantly higher ROS production than those isolated by the other two isolation methods. These results suggest that the ability of PMNs to be primed for ROS production is highest in PMNs isolated by positive selection, which may argue for lower activation status upon isolation in these cells. Indeed, when the priming of ROS production was compared between groups, significant differences were observed between isolation methods (P = 0.009 by the Friedman test), with cells isolated by positive selection showing a significantly higher priming capacity than PMNs isolated by Ficoll. Polymorphprep isolation induced higher variability in priming of ROS production, but due to its lag in
fMLP-induced ROS production, priming in this group was higher than in Ficoll-isolated PMNs (Fig. 2B). Therefore, for ROS production analysis, positive selection of PMNs may be the best option.

Granulocyte functionality in an E. coli phagocytosis assay following different isolation protocols. Ficoll has been shown to change PMN shape and migratory capacity, indicating an effect on cytoskeletal rearrangement (12). Phagocytosis is dependent on the actin cytoskeleton and, as such, may be affected by Ficoll isolation procedures. Positive selection of neutrophils relies on the antibody binding of CD15, a carbohydrate adhesion molecule. Ligation of CD15 antibodies to this integrin-associated molecule may potentially affect phagocytosis and adhesion of PMNs (16). We therefore studied the E. coli-phagocytosing capacity of PMNs iso-
lated by different methods, using phagocytosis of PMNs in whole blood as a control. To distinguish PMNs in whole blood samples, cells were stained for CD15, and the percentage of PMNs containing GFP-positive *E. coli* was determined by FACS analysis. As expected, phagocytosis of nonisolated PMNs is more efficient than that of isolated granulocytes, with more than 53% ± 4% of granulocytes taking up GFP-positive bacteria. No significant differences in the percentages of phagocytosing cells were observed in PMNs isolated by Ficoll centrifugation, positive selection, or Polymorphprep (12.6% ± 3%, 10.8% ± 2%, and 12.2% ± 3%, respectively; *n* = 6; *P* = 0.55) (Fig. 2C). Engulfment of bacteria rather than adhesion to the PMN surface was confirmed by confocal microscopy (see Movie S1 in the supplemental material). In addition, the number of bacteria taken up per cell, as determined by the mean fluorescence intensity (MFI) of GFP-positive PMNs, did not differ by the isolation method used (MFIs were 1,172, 1,054, and 1,231 for Ficoll centrifugation, positive selection, and Polymorphprep, respectively; data not shown; *P* = 0.819).

**Expression of PMN surface markers following different isolation procedures.** Isolation of granulocytes may affect their expression of cell surface receptors and other molecules and thereby alter specific granulocyte functions. CD15, CD66b, and CD62L (L-selectin) are adhesion molecules involved in PMN phagocytosis and chemotaxis. Analysis of CD15 expression under different isolation procedures revealed a slight but significant decrease in CD15 and CD66b expression upon Polymorphprep isolation compared to Ficoll isolation and positive selection, respectively. Whereas CD62L expression levels were comparable between

### TABLE 1

Comparison of yields and purities of granulocytes, time consumption, and costs of the three different isolation procedures evaluated in the study

| Method          | Yield a | Purity (%) b | Procedure time (h) | Cost/10 ml blood c |
|-----------------|---------|--------------|--------------------|--------------------|
| Ficoll centrifugation | 16 × 10^6 ± 6.3 × 10^5 | 96.2 ± 1.7 | 1.5 | $6.84 (€5.4) |
| Positive selection | 9.6 × 10^5 ± 7.9 × 10^5 | 99.5 ± 0.5 | 2.5 | $41.80 (€33) |
| Polymorphprep    | 14.8 × 10^5 ± 4.3 × 10^5 | 91.4 ± 4.9 | 1   | $7.73 (€6.1) |

a Means ± SD of 6 independent experiments. The yields of PMN isolation differed significantly between groups (*P* = 0.04 by the Friedman test). Specifically, positive selection yielded lower numbers of PMNs than either Ficoll or Polymorphprep (*P* = 0.028 and 0.046, respectively).

b Means ± SD of 6 independent experiments. The purity shown is that of total nucleated cells. The purities of PMNs differed significantly between groups (*P* = 0.009 by the Friedman Test). Post hoc analysis revealed that the purity of PMNs isolated by Polymorphprep was significantly lower than that of PMNs isolated by Ficoll centrifugation or positive selection (*P* = 0.03 and 0.03, respectively).

c The cost of positive selection did not include the magnetic separator. The cost of positive selection depends on the yield of PMNs after Ficoll centrifugation, and the amount shown was calculated based on 10^7 total cells.

### FIG 2

Functional testing of enriched granulocyte fractions. (A) Granulocytes isolated by Ficoll density centrifugation, anti-CD15 antibody-conjugated magnetic microbeads (positive selection), or density centrifugation using Polymorphprep were subjected to ROS production analysis. Stimulation with fMLP results in low production of ROS, indicative of resting cells, whereas priming of cells with GM-CSF yields the highest fMLP-induced ROS production in granulocytes isolated by CD15 microbead positive selection. Means ± standard errors of the mean (SEM) of 6 experiments are shown. The asterisks indicate significantly higher ROS production in GM-CSF–plus–fMLP-stimulated cells than in cells stimulated with fMLP alone (*P* < 0.05). Time in minutes is shown. (B) Priming capacity was determined by expressing ROS production in GM-CSF–treated cells as a percentage of ROS production in unprimed, fMLP-stimulated granulocytes (means ± SEM of 6 experiments). *, *P* < 0.05. (C) Enriched granulocytes were challenged with FITC-expressing *E. coli* for 15 min, after which the FITC fluorescence of isolates was determined by FACS analysis. Whole blood was used as a positive control (means and SEM of 6 experiments). (D) Cell viability of enriched PMNs was determined by measuring the percentage of 7AAD-positive PMNs by flow cytometry (means and SEM of 6 experiments).
groups, expression of CD66b was highest on PMNs isolated by positive selection, followed by Ficoll and Polymorphprep. Although this is not likely to affect phagocytosis (as shown above), it is conceivable that other, untested functions (e.g., migration) could potentially be affected.

We also assessed the expression of the CD64 FcγRI, the CD32 FcγRII, and the low-affinity FcRIII, CD16. Although CD64 expression was higher in positively selected PMNs than in PMNs isolated by Ficoll, significant differences between groups were observed only for CD16. PMNs isolated by positive selection showed a small but significant increase in CD16 expression compared to either Ficoll or Polymorphprep isolates. No differences in CD32 expression were observed between isolation protocols. Next, we analyzed the expression of TLR2, TLR4, and coreceptor CD14, which bind lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria, such as E. coli (13). A dramatic increase in TLR2 and TLR4 expression was observed on PMNs isolated by positive selection. This could have substantial consequences for studies of the functions of these receptors, as well as studies of functional LPS responses, and isolation procedures should therefore be carefully considered by investigators undertaking such studies.

**Comparison of the four most commonly used isolation protocols for purity of enriched monocyte cultures.** Monocytes of healthy individuals were isolated from peripheral blood in parallel by the four procedures most commonly reported in the literature. First, we decided to assess the purity of the monocyte fraction following the four different types of enrichment as a percentage of all nucleated cells using FACS. Using this measure, it was observed that monocytes with purities of 98.5%, 97.0%, 67.3%, and 64.2% were obtained by positive selection, negative selection, adherence, and RosetteSep, respectively (Fig. 4A). Furthermore, visual inspection of monocyte cultures revealed the presence of nonnucleated particles. When results were expressed as percentages of all recorded events (diameter > 0.5 μm), the monocyte purities obtained were 95.4%, 48.7%, 58.5%, and 35.9% for positive selec-
tion, negative selection, adherence, and RosetteSep, respectively (Fig. 4B shows a representative example). Thus, with regard to purity, positive selection yields the best results for monocyte isolation, judged both as the monocyte fraction of all nucleated cells and as a fraction of all cell-like bodies. Importantly, after positive-selection procedures during which monocytes were labeled with antibodies for CD14 receptors, abundant CD14 expression was still detected by anti-CD14-PE, indicating a functional condition of these cells with respect to CD14 (Fig. 4D).

Next, we analyzed the nature of the contaminants in the enriched samples obtained using the four different isolation procedures. As shown in Fig. 4C, compared to samples obtained through positive selection, large numbers of platelets remained in the enriched monocyte cultures after negative selection. After adherence isolation procedures, the major contaminants are lymphocytes and platelets. After RosetteSep procedures, we observed large amounts of lymphocytes, platelets, and nonspecific cellular aggregates, which significantly compromise the purity of the monocyte culture.

Comparison of monocyte yields obtained using the four most commonly used monocyte isolation protocols. The absolute recovery rates of monocytes after the four isolation procedures are depicted in Table 2. The purities and recovery rates of monocytes after positive selection, negative selection, and RosetteSep procedures were highly reproducible. During the enrichment procedure by adherence, due to the difficulties in standardizing the washing steps by which monocytes and lymphocytes are separated, outcomes were more variable, at least in our hands. In this respect, we observed that this methodology is sensitive to inadequate washing of cultures, resulting in significantly compro-

**FIG 4** Purities of enriched monocytes. (A) Representative flow cytometry histograms (n = 3) showing the purities of enriched monocyte cultures obtained by either (i) anti-CD14 antibody-conjugated magnetic microbeads (positive selection), (ii) non-monocyte depletion by antibody-conjugated magnetic microbeads (negative selection), (iii) a classical adherence protocol, or (iv) immunorosette based RosetteSep antibody cocktail as percentages of monocytes among nucleated cells. (B) Purities of monocytes after isolation expressed as fractions of all cell-like particles (including nucleated cells, nonnucleated cells, and ghosts) after enrichment. (C) Analysis of the nature of contaminants in the enriched samples as identified using traditional forward and side scatter methodology. (D) Monocytes purified by positive selection were stained for CD14 and nuclei using anti-CD14-PE antibody (red) and DAPI (blue).
mised monocyte purity, whereas excess washing has substantial consequences with respect to the monocyte recovery yield (not shown). Nevertheless, typically, isolation by adherence enriched monocytes to a purity of 68.1% and a recovery of $2.0 \times 10^7$/ml peripheral blood, which is not markedly different from the yields obtained using either positive or negative selection. The RosetteSep procedure leads to substantially better results in this respect and takes less time, but the cultures obtained suffer from impurities (see above), and per monocyte, it remains slightly more expensive (Table 2). The viability of monocytes in all isolates was >99%, as determined by fluorescence microscopy of DAPI-stained nuclei (Fig. 4D).

**Monocyte functionality in an E. coli phagocytosis assay following different monocyte isolation protocols.** Along with TLR4, CD14 acts as a coreceptor on monocytes, detecting bacterial LPS. In order to evaluate the effect of CD14 antibody binding during positive-selection procedures on the phagocytic capacity of enriched monocytes, we challenged monocytes enriched by positive selection or negative selection with FITC-labeled *E. coli*. The phagocytosis index is significantly influenced by the bacterium/monocyte ratio (17). For the studies aiming to compare the phagocytic capacities of monocytes, cells were challenged at a bacterium/monocytes ratio of 5:1 for 5 min to induce uptake of *E. coli* in 50% to 80% of all monocytes, an optimal setup to show the maximal differences between samples. The phagocytosis index was calculated as the percentage of monocytes exhibiting *E. coli* ingestion as determined by fluorescence microscopy. Importantly, *E. coli* phagocytosis of monocytes did not show significant differences, regardless of the isolation procedure used, although positive selection was slightly superior ($n = 3; P > 0.05$) (Fig. 5A). Culturing monocytes isolated by positive selection for 16 h instead of 2 h prior to phagocytosis assay had no impact on *E. coli* phagocytic capacity, nor did the time of *E. coli* challenge (a 2:1 bacterium/monocytes ratio was employed to allow a longer duration of *E. coli* challenge without risking monocyte saturation) (Fig. 5B). The engulfment of *E. coli* by monocytes rather than attachment to the cell surface was confirmed by confocal microscopy (Fig. 5C; see Movie S2 in the supplemental material). Importantly, we also observed active phagocytosis of platelets and ghosts (see Fig. S1 in the supplemental material), potentially suggesting competing effects between *E. coli* and platelets and stressing the importance of an isolation procedure that does not yield such contaminants in the culture. Therefore, *in toto*, the anti-CD14 antibody-conjugated magnetic-microbead (positive selection) protocol appears to be the monocyte isolation method best suited to this type of phagocytosis analysis.

**DISCUSSION**

Although some studies of cell populations may be performed on whole blood, thus avoiding the need for potentially cell-activating isolation procedures, for many studies, including those on the involvement of granulocytes or monocytes in the pathogenesis of autoimmune diseases, isolation of a pure population of cells is essential. Although all four monocyte isolation procedures tested in this study yielded functional phagocytosis-competent monocytes, results with respect to purity and recovery were markedly different for the different procedures. We view purity as a special concern, as it may be important for functional studies. We observed, when challenging monocyte cultures with FITC-labeled *E. coli*, that contaminating lymphocytes markedly compromised the accuracy of phagocytosis quantification. This was due to two factors: first, as a result of the extremely similar morphologies of monocytes and lymphocytes, quantifying the percentage of phagocytosing monocytes by fluorescence microscopy is markedly disturbed by lymphocyte contamination. Second, we observed that lymphocytes are also able to adhere *E. coli* to their cell surfaces (data not shown), which may further hamper quantification. This is especially obvious when monocytes are mixed with a large population of lymphocytes (e.g., PBMC) or a large number of bacteria are loaded. Furthermore, we observe that copurified thrombocytes and cell ghosts are also subject to phagocytosis, with unknown effects on monocyte physiology, thus possibly compromising experimentation. In addition, it has been shown that isolating a pure cell population is important for other functional studies of monocytes, e.g., antigen presentation and cytokine production. For these reasons, isolation of monocytes by employing positive selection and anti-CD14-conjugated microbeads appears to be the technology of choice, at least for phagocytosis analysis. However, due to the high costs of the commercial reagents and instruments required for positive selection, in studies where the purity of monocytes is not strictly required, traditional adherence isolation procedures remain an option. For instance, in studies using monocyte-derived dendritic cells, the lymphocyte contaminants may be less important, as they will not survive longer than 1 week in the absence of interleukin 2 (IL-2).

With respect to the absolute yields obtained, RosetteSep proved superior, but it does not yield pure cultures. The other technologies are comparable, including the traditional adherence protocol, which is subject to substantial experimenter-dependent and day-to-day variability, hampering its application and the comparison of results. Negative selection yields less pure cultures.
and is more expensive per monocyte and thus appears to be a less attractive choice.

Regarding PMN isolation, comparison of three widely used methods showed that isolation by positive selection yielded isolates with the highest purity, followed closely by Ficoll centrifugation. Polymorphprep isolates showed the highest level of impurities, which can be problematic in certain experiments. Another considerable drawback in the use of Polymorphprep is the fact that relatively high purities can only be reached when using EDTA as an anticoagulation agent, which may not always be practical. Theoretically, the main advantage of Polymorphprep is the elimination of erythrocyte lysis steps. However, red blood cell contamination of the PMN ring is often observed (up to 6% of isolates, according to the manufacturer’s data sheet), and a mild yet potentially cell-activating lysis step may therefore be required.

Isolates from positive-selection methods showed the highest ROS production in response to GM-CSF plus fMLP treatment, which may imply that this method of isolation induces the least desensitization of PMNs. Hence, positive selection seems to be the method of choice for studying ROS production, closely followed by Ficoll isolation. ROS production is dependent on the actin cytoskeleton, in that disruption of actin polymerization results in increased ROS levels (1). As CD15 ligation may affect cytoskeletal rearrangement, it is theoretically possible that positive selection using CD15 antibodies can enhance ROS production or affect phagocytosis. However, phagocytosis of E. coli was similar in all three methods tested, and fMLP-induced ROS production in positively selected isolates was equal to that in isolates from Ficoll centrifugation, indicating that CD15 engagement in positive-selection procedures at least does not affect these functions. Although no differences in the PMN activation marker CD62L were observed, our results did clearly show an upregulation of the Toll-like receptors TLR2 and TLR4 on PMNs isolated by positive selection. Although this is not likely to affect either the ROS production or E. coli phagocytosis tested here, the increase may nevertheless influence other cellular assays that investigators of innate immunity may want to study. Careful consideration of different neutrophil isolation techniques is therefore required for every functional study considered by investigators and may depend on the type of assay desired.

**FIG 5** Phagocytic capacity of monocytes. (A) Monocytes obtained through the positive-selection and negative-selection procedures were challenged with FITC-labeled E. coli for 5 min in parallel. Phagocytosis of E. coli is expressed as the percentage of monocytes displaying phagocytosis. E. coli phagocytosis of monocytes after the two isolation procedures did not show a significant difference, although the level of phagocytosis in the negative-selection group was slightly lower ($n = 3$; $P > 0.05$; the values are presented as means and SEM). (B) After the positive-selection procedure, monocytes after 16 h of culture exhibit the same E. coli phagocytic capacity as the 2-h culture (representative of 3 independent experiments). (C) Representative transsectional image of monocytes by confocal microscope, confirming that E. coli cells are engulfed by rather than attached to monocytes. Red, cytoskeleton of monocytes (F-actin) stained by TRITC-phalloidin; green, FITC-labeled E. coli.
In this study, we compared 4 widely used monocyte isolation procedures and 3 commonly used granulocyte isolation methods. However, other isolation procedures have been described that were not covered in this study. For instance, monocytes may be isolated by CD14-positive selection in the magnet-based MagCellect system from R&D Systems. However, at this time, the company does not offer granulocyte isolation kits. In addition, using an Elutra separator, isolates in which monocytes are enriched on the basis of size and, to a lesser extent, density from an entire apheresis product have been reported to result in approximately 75% purity of monocytes. However, the procedure requires an automated system and therefore may not be suitable for every laboratory. For granulocytes, EasySep magnetic isolation kits based on CD66 expression are available from Stemcell Technologies. How these different isolation procedures (CD15 versus CD66b positive selection) affect PMN function remains to be investigated.

In conclusion, if high purity of cells is required and limited numbers of cells are needed, we consider that PMN and monocyte isolation using positive selection is the most suitable method. Where high purity of PMNs or monocytes is not strictly required, Ficoll density gradient centrifugation and the traditional adherence isolation procedure remain good options for PMN and monocyte purification, respectively. However, investigators of innate cell functions should be well aware of possible alterations in cell phenotypes under different isolation procedures, and the best isolation procedure may depend on the assays to be used.

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