Human granulocyte and monocyte isolation procedures: impact on functional studies

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ABSTRACT

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 a disturbed innate immunity, and relating granulocyte and monocyte function to patient genotype has become an important part of contemporary research. Although 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 upon three enrichment protocols: 1) Ficoll density gradient centrifugation, 2) anti-CD15 antibody conjugated microbeads (positive selection) and 3) Polymorphoprep™. Primary monocytes were isolated in parallel using 1) anti-CD14 magnetic microbeads, 2) non-monocyte depletion by antibody conjugated magnetic microbeads (negative selection), 3) RosetteSep™ antibody cocktail, and 4) the classical adherence protocol. 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 E. coli bacteria was identical in all isolation procedures tested, granulocyte respiratory burst was higher in positively selected cells. In addition, different granulocyte enrichment procedures affect cell surface receptor expression to a different extent. In toto, we propose that positive selection of granulocytes and monocytes be adopted as the procedure of choice for studies on human granulocyte and monocyte function, but caution investigators to be aware of possible alterations in cell phenotypes upon different isolation procedures.
INTRODUCTION

Monocytes represent 3-7 percent of total white blood cells (absolute monocyte count 1.5-7 ×10⁸/ 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: (1) to replenish resident macrophages and dendritic cells in peripheral tissues under normal states, and (2) 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 25-750×10⁸/ 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 inflammation in turn precedes a second wave of migrating monocytes to remove rapidly apoptotic PMNs. Monocytes, macrophages and dendritic cells are also capable of eliciting adaptive immune response via antigen presentation, a role which has also been attributed to granulocytes in inflammatory settings [6;22]. Therefore, PMN and monocytes play a pivotal role in keeping the dynamic balance of 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 of those are rheumatoid
arthritis (RA), systemic lupus erythematosi (SLE), an autoimmunity against DNA, and the pathogenesis of Crohn’s disease (CD), an often severe autoimmunity towards 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 *E. 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 PMN alleviates disease [28]. One of the mechanisms through which granulocytes may exacerbate disease is by the deposition of autoantibodies to 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 forms immune complexes consisting of DNA, antimicrobial peptides and autoantibodies, which subsequently activate plasmacytoid dendritic cells [13;14]. In addition, monocyte function and morphology in SLE has been reported to be altered; however, phagocytosis of bacteria may be either decreased or enhanced, and more studies in order to assess the real contribution of these innate immunity 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 autoimmunities [29], triggering investigations into the properties of innate immune cells in patients, and comparison of their phenotype to the genotype of risk genes relevant for 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 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 called for. 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. PMN were isolated in parallel using (1) Ficoll density gradient centrifugation, (2) polymorphprep™ density gradient centrifugation, and (3) anti-CD15 antibody conjugated magnetic microbeads (positive selection), after which PMN functionality was assessed by \textit{in vitro} phagocytosis and ROS production assays. Monocytes were isolated from peripheral blood in parallel using (1) anti-CD14 antibody conjugated magnetic microbeads (positive selection), (2) non-monocyte depletion by antibody conjugated magnetic microbeads (negative selection), (3) immunorosette based RosetteSep™ antibody cocktail (RosetteSep™), and (4) adherence, aiming to assess their suitability for \textit{in vitro} phagocytosis analysis. We conclude that positive selection of granulocytes and monocytes by anti-CD15 and anti-CD14 antibody conjugated magnetic microbeads, respectively, are 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 regulation 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 manufacturers’ instruction. Additionally, PMN were isolated from EDTA anti-coagulated blood using PolymorphPrep™ (Axis-Shield, Norway). When present, erythrocytes were lysed with ice-cold NH4Cl solution. Before functional testing, PMN were allowed to recover for 30 minutes at 37°C in RPMI 1640 supplemented with 0.5% human serum albumin (HSA; Sanquin, The Netherlands). Cells were resuspended in incubation buffer (20mM HEPES, 132mM NaCl, 6mM KCl, 1mM MgSO4, 1.2mM KH2PO4, 5mM glucose, 1 mM CaCl2 and 0.5% HSA) prior to subjecting them to functional assays. All isolation procedures were done in parallel per healthy donor.

ROS production assay

ROS production was performed as described [8]. Shortly, PMN (2x10⁶ cells/ml) were incubated with Dihydrorhodamine 123 (DHR 123) for 15 minutes and stimulated with
1μM N-formyl-methionine-leucine-phenylalanine (fMLP) for 30 minutes. For priming experiments, cells were pretreated with 5ng/ml GM-CSF for 15 minutes 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 DHR123 to the fluorescent Rhodamine 123 was measured by FACS-analysis within 30 minutes after termination of stimulation.

Monocyte isolation from human peripheral blood

Heparinized blood was obtained from healthy volunteers after informed consent. In order to optimize 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 by 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 adherence method was performed as described previously [7]. Briefly, peripheral blood mononuclear cells were isolated immediately after collection using Lymphoprep™ gradients (Axis-Shield PoC As, Norway). Monocytes were further enriched by virtue of their attachment to a culture plate for 2 hours, washed 3 times with warm phosphate buffered saline (PBS) to remove non-adherent cells, the adherent monocytes were recovered by cell scraper. The purity of monocytes was evaluated by fluorescent staining with CD14-PE monoclonal antibody (UCHM1, murine IgG2a, IQ
products, Netherlands) and FACS analysis. The recovery of monocytes was evaluated by Trypan blue staining and counted using a Zeiss microscope.

Cell culture
Monocytes were cultured in complete medium consisting of RPMI1640 (PAA laboratories, Austria) supplemented with 10% heat-inactivated FCS (PPA) and 10 μg/ml Gentamicine (Centrafarm, Netherlands) at 37°C in 5% CO₂ humidified air.

Phagocytosis assay
GFP expression vector was transformed into E.Coli and grown in LB media until OD of 400 was reached. Bacteria were fixed using 4% formaldehyde. Isolated PMN were resuspended (2 x10⁶/ml) in RPMI containing 10% FCS and 1x10⁶ bacteria were subjected to PMN phagocytosis at 37°C for 15 minutes. The percentage of phagocytosing PMN was determined by flow cytometry by analyzing the percentage of GFP positive PMN. Phagocytosis at 0°C was used as negative control for each experiment. No FITC positive PMN were observed under this condition, confirming active phagocytosis of bacteria rather than attachment of bacteria to PMN membranes. Alternatively, 4% formaldehyde-fixed E. coli were labeled with FITC fluorescence by incubation in 1mg/ml FITC solution containing 0.1M Na₂CO₃ pH 9.5 for 1 hour followed by complete washing. The efficiency of labeling was tested by FACS analysis (see below). Bacterium concentration was quantified using Quantimet HR550 image analysis software (Leica) to analyze microscopic images of FITC-E. coli taken with a Leica (Wetzlar, Germany) DMRXA epifluorescence microscope [30]. For monocytes, the phagocytosis assay was performed
according to Mandell and Hook [19]. In short, monocytes (1 × 10^4/ml) were incubated with FITC-\textit{E. coli} at a 1:5 ratio for 5 or 15 minutes at 37 °C in RPMI1640 medium containing 10% heat-inactivated FCS. Thereafter, phagocytosis was evaluated microscopically by counting both the number of monocytes exhibiting phagocytosis and the number of bacteria phagocytosed per monocyte. At least 300 cells were counted for each slide. The engulfment rather than attachment of \textit{E. coli} to PMN and monocytes was confirmed by confocal microscope (LSM 700, Carl Zeiss, Jena, Germany) and cultures at 4 °C.

\textbf{FACS analysis}

Isolated PMN or whole blood after red cell lysis were stained resuspended in PBS/EDTA/1%FCS. After blocking with FcR blocking reagent from Miltenyi Biotech (Amsterdam, the Netherlands) for 15 minutes, cells were labeled with anti-CD14-PerCP-Cy5.5 (clone M5E2), anti-CD66b-PerCP-Cy5.5 (G10F5), anti-CD15-PE (clone HI98), anti-TLR2-Alexa 647 (clone TL2.1), anti-TLR4-PE (clone HTA125), anti-CD62L-Alexafluor647 (clone DREG56) (all from Biolegend, San Diego, CA). No FcR blocking was performed when staining for FcReceptors using anti-CD16-PE (clone 3G8, Biolegend), anti-CD64-PE (clone 10.1, Biolegend) or anti-CD32-FITC (clone FL18.26 from BD Bioscience, Franklin Lakes, NJ). After washing with PBS, fluorescence was measured by flow cytometry (FACSCanto, BD Bioscience Franklin Lakes, NJ).

\textbf{Analysis of purity}
Analysis of the purities of granulocytes and monocytes by FACS was performed using PerCP-Cy5-labeled CD66b (Clone G10F5, Biolegend) and PE-labeled CD14 monoclonal antibody (clone UCHM1, murine IgG2a, IQ products, Netherlands), respectively. In short, cells were incubated with appropriate antibodies (10 μl per 5 × 10^5 cells) for 30 minutes on ice. Cells were washed twice in PBS/0.1% BSA before FACS analysis. For identifying the platelet contaminants and observing the cytoskeleton of monocytes, enriched monocytes were further stained intracellularly with TRITC conjugated phalloidin (Sigma-aldrich). In short, cells were fixed in PBS/4% formaldehyde for 10 minutes, washed twice in PBS, permeabilized in PBS/0.1% Triton X-100 (Sigma-Aldrich), treated with TRITC conjugated phalloidin (25 μg/ml) for 30 minutes in dark, and washed three times in PBS. Platelets were identified by their morphology under fluorescence microscopy. Contaminating cells in granulocyte isolates were discriminates using anti-CD19-PE, clone IB19 (B-cell), anti-CD3-Amcyan clone SK7 (T-cell) and anti-CD14-PerCP clone M5E2 (Monocyte) antibodies. DAPI was used to exclude dead cells.

Statistics

For comparison of multiple isolation methods, differences between the means of the groups were tested by non-parametric test for related samples (Friedman test) or unpaired samples (Kruskal-Wallis). Where variance between the means of groups was found by Friedman test, post-hoc analysis was performed by Wilcoxon signed Ranks testing, to determine which of the groups differed from each other. Wilcoxon signed Rank non-parametric test for paired samples was also used for comparisons between two groups.
such as the comparison of fMLP-stimulated ROS production with and without priming with GM-CSF.
RESULTS

Comparison of three commonly used isolation protocols for purity of enriched granulocytes.

PMN were isolated from blood from healthy volunteers using, in parallel, three of the most commonly used methods in literature. 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 Polymorphprec™ isolation methods, respectively (Figure 1A, Table 1). This measure, however, excludes non-nucleated 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 Ø >0.5 μM). When results were expressed this way, Ficoll density gradient centrifugation yielded 87±3% granulocyte purity, compared to 88±5% for positive selection (see Figure 1B for representative example). Polymorphprec™ isolation method resulted on average in 76±19% purity, largely due to a higher amount of contaminating debris particles and lymphocytes (Figure 1C). Also, in our hands, the highest variability in purity between isolations was observed when using Polymorphprec™, suggesting that this 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 analyzing the nature of this contaminating mononuclear cell fraction, these were found to be mainly CD3-positive T-cells, with little or no CD19+ B-cell or CD14+ monocytes (not shown).
PMN are amongst the most short-lived cells in the body. They are extremely fragile cells, and prone to apoptosis upon withdrawal from the blood. Importantly, however, no more than 3.7% dying cells were identified by 7AAD (Fig 2D) or DAPI staining (not shown) 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 yield obtained using the three most commonly used PMN isolation protocols

The absolute recovery rates of PMN after the three isolation procedures are shown in Table 1. Typically, isolation of PMN by positive selection, though leading to the highest purity, also yielded the lowest recovery rates; 9.6×10⁵/ml peripheral blood were obtained, which was significantly less then the yields obtained using either Ficoll centrifugation (16×10⁵/ml peripheral blood) or Polymorphprep™ (14.8×10⁵/ml peripheral blood). With a purity of 95.2% and the highest overall yield, granulocyte enrichment by Ficoll centrifugation seems the optimal method when high numbers of cells are required.

Granulocyte functionality in a ROS production assay following different isolation protocols

PMN are easily activated by trace amounts of bacterial lipopolysaccharides or mechanical stressors. To determine whether our different isolation methods give unwanted activation of PMN, we studied their ROS production. Spontaneous PMN ROS production was not
different between isolation procedures (data not shown). In addition, expression of the
neutrophil activation marker L-selectin (CD62L), shedding of which is associated with
PMN activation [26], was similar between groups, confirming that there was no bias in
activation of PMN between isolation methods (Figure 3A, upper left panel).

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

Granulocyte functionality in an *Escherichia coli* phagocytosis assay following different isolation protocols

Ficoll has been shown to change PMN shape and migratory capacity, indicating an effect on cytoskeletal rearrangement [11]. 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 PMN [16]. We therefore studied the *E. coli* phagocytosing capacity of PMN isolated by different methods, using phagocytosis of PMN in whole blood as control. To distinguish PMN in whole blood samples, cells were stained for CD15, and the percentage of PMN containing GFP-positive *E.Coli* was determined by FACS analysis. As expected, phagocytosis of non-isolated PMN 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 percentage of phagocytosing cells were observed in PMN isolated by Ficoll centrifugation, positive selection or Polymorphprep™ (12.6±3%, 10.8±2% and 12.2±3%, respectively, n=6; p = .55, Figure 2C). Engulfment of bacteria rather than adhesion to PMN cell surface was confirmed by confocal microscopy (supplementary movie 1). In addition, the amount of bacteria taken up per cell, as determined by mean fluorescence intensity (MFI) of GFP-positive PMN,
did not differ per isolation method used (MFI of 1172, 1054 and 1231 for Ficoll centrifugation, positive selection or Polymorphprep™, respectively, not shown, p=0.819).

Expression of PMN cell 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 upon different isolation procedures reveals a slight but significant decrease in CD15 and CD66b expression upon Polymorphprep™ isolation as compared to Ficoll isolation and positive selection, respectively. Whereas CD62L expression was comparable between groups, expression of CD66b was highest on PMN isolated by positive selection, followed by Ficoll and Polymorphprep. Although this is not likely to affect phagocytosis (as seen above), it is conceivable that other, untested functions (e.g. migration), could potentially be affected.

We also assessed expression of the CD64 FcγRI, the CD32 FcγRII and the low affinity FcRIII, CD16. Although CD64 expression was higher in positively selected PMN as compared to PMN isolated by Ficoll, significant differences between groups were only observed for CD16. PMN isolated by positive selection showed a small but significant increase in CD16 expression as compared to either Ficoll or Polymorphprep™ isolates.

No differences in CD32 expression were observed between isolation protocols. Next, we analyzed the expression of Toll like receptor (TLR) 2, TLR4 and co-receptor CD14, which bind lipopolisaccharise (LPS), the major component of the outer membrane of
Gram-negative bacteria such as *E. coli* [12]. A dramatic increase in TLR2 and TLR4 expression was observed on PMN isolated by positive selection. This could have substantial consequences for studies into the function of these receptors as well as studies into 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 used 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 were obtained with purities of 98.5%, 97.0%, 67.3% and 64.2% by positive selection, negative selection, adherence and RosetteSep™, respectively (Fig. 4A). Furthermore, visual inspection of monocyte cultures revealed the presence of non-nucleated particles. When results were expressed as percentage of all recorded events (Ø >0.5 μM) the monocyte purity obtained was 95.4%, 48.7%, 58.5% and 35.9% for respectively positive selection, negative selection, adherence and RosetteSep™ (Fig. 4B, representative example). Thus with regard to purity, positive selection yields the bests results for monocyte isolation, both judged by the monocyte fraction of all nucleated cells and as 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 amounts of platelets remained in the enriched monocyte cultures after negative selection. After adherent isolation procedure, the major contaminants are lymphocytes and platelets. After RosetteSep™ procedures, we observed large amounts of lymphocytes, platelets, and non-specific cellular aggregates, which significantly compromise the purity of this monocyte culture.

Comparison of monocyte yield 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 recoveries of monocytes after positive selection, negative selection, and RosetteSep™ procedures were highly reproducible. During the enrichment procedure by adherence, due to the difficulties to standardize 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 compromised monocyte purity, whereas excess washing has substantial consequences with respect to monocyte recovery yield (not shown). Nevertheless, typically, isolation by adherence enriched monocytes to a purity of 68.1% and a recovery of $2.0 \times 10^5$/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 on a per monocyte 
basis, it remains slightly more expensive (Table 2). Viability of monocytes in all isolates 
was >99%, as determined by fluorescence microscopy of DAPI stained nuclei (for 
example, see Fig. 4D).

Monocyte functionality in a *Escherichia coli* phagocytosis assay following different 
monocyte isolation protocols

Along with TLR4, CD14 acts as a co-receptor on monocytes, detecting bacterial LPS. In 
order to evaluate the effect of CD14 antibody binding during positive selection 
procedures on phagocytic capacity of enriched monocytes, we challenged monocytes 
enriched by positive selection or negative selection with FITC-labeled *E. coli*.

Phagocytosis index is significantly influenced by bacteria/monocyte ratio [17]. For the 
studies aiming to compare phagocytic capacity of monocytes, cells were challenged with 
a bacteria/monocytes ratio of 5:1 for 5 minutes to induce uptake of *E.Coli* in 50%-80% of 
all monocytes, an optimal set-up to show the maximal differences between samples.

Phagocytosis index was calculated as the percentage of monocytes exhibiting *E. coli* 
ingestion as determined by fluorescence microscopy. Importantly, *E. coli* phagocytosis 
of monocytes does 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 hrs instead of 2 hrs prior to phagocytosis 
assay had no impact on *E. coli* phagocytic capacity, nor did the time of *E.Coli* challenge 
(a 2:1 bacteria/monocytes ratio was employed to allow for a longer time of *E. coli* 
challenge).
challenging without risking monocyte saturation) (Fig. 5B). The engulfment of \textit{E. coli} by monocytes rather than attachment to the cell surface was confirmed by confocal microscopy (Fig. 5C and Supplementary movie 2). Importantly, we also observed active phagocytosis of platelets and ghosts (Supplementary Fig. 1), potentially suggesting competing effects between \textit{E. coli} and platelets and stressing the importance of an isolation procedure that does not yield such contaminants in the culture. Therefore, \textit{in toto}, the anti-CD14 antibody-conjugated magnetic microbeads (positive selection) protocol appears the best suited monocyte isolation method for this type of phagocytosis analysis.
DISCUSSION

Although some studies into cell-populations may be performed on whole blood, thus preventing 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 are markedly different for the different procedures. We view especially purity as a concern, as this may be important for functional studies. We observed when challenging monocyte cultures with FITC-labeled *E. coli*, that contaminating lymphocytes markedly compromise the accuracy of phagocytosis quantification. This was due to two reasons: first, as a result of the extremely similar morphology 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 surface (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 amount of bacteria is loaded. Furthermore, we observe that co-purified thrombocytes and cell ghosts are also subject to phagocytosis, with unknown effects on monocyte physiology and 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 employing positive selection and anti-CD14 conjugated microbeads appears the
technology of choice, at least for phagocytosis analysis. However, due to the high costs of commercial reagents and required instruments for positive selection, in studies where the purity of monocytes is not strictly required, traditional adherent isolation procedures remain an option. For instance, in studies using monocyte-derived dendritic cells, the lymphocyte contaminants may be less important as the latter will not survive for longer than 1 week in the absence of IL2.

With respect to the absolute yields obtained, the RosetteSep™ comes out superior, but does not yield pure cultures. The other technologies are comparable, including the traditional adherence protocol, the latter is subject to substantial experimentator- and day-to-day-dependent variability, hampering its application and 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 impurities, which can be problematic in certain experiments. Another considerable drawback in the use of Polymorphprep™ is that relatively high purities can only be reached when using EDTA as 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 isolate, as per manufacturer’s datasheet), and a mild yet potentially cell-activating lysis step may therefore nonetheless be required.
Isolates from positive selection methods showed the highest ROS production in response to GM-CSF + fMLP treatment, which may imply that this method of isolation induces the least desensitization of PMN. Hence, positive selection seems 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 [25]. 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 PMN isolated by positive selection. Although this is not likely to affect either the ROS production or *E.Coli* phagocytosis tested here, this increase may nevertheless influence other cellular assays investigators into 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.

In this study, we have 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, this company does not at this time offer granulocyte isolation kits. In addition, using Elutra™ separator, isolates by which monocytes are enriched on the basis of size and to a lesser extent density from an entire apheresis product have been described to result in approximately 75% purity of monocytes. However, this procedure requires an automated system, and may therefore 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 cell function remains to be investigated.

In conclusion, if high purity of cells is required, and limited cells are needed, we would consider that PMN and monocyte isolation using positive selection is the most suitable method. Where high purity of PMN or monocytes is not strictly required, Ficoll density gradient centrifugation and the traditional adherent isolation procedure remain good options for PMN and monocytes purification, respectively. However, investigators into innate cell functions should be well aware of possible alterations in cell phenotypes upon different isolation procedures, and the best isolation procedure may depend on the assays planned to be used.
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Figure 1. The purities of enriched granulocytes. (A) Representative flow cytometry histograms (of n=4) showing the purity of enriched granulocyte isolates, obtained by either 1) Ficoll density centrifugation, 2) anti-CD15 antibody conjugated magnetic microbeads (positive selection), 3) Density centrifugation using Polymorphprep™. Histograms represent CD66b positive cells after gating for nucleated cells using forward and sidescatter profiles. (B) The purities of granulocytes after isolation are expressed as fraction of all cell-like particles (including nucleated cells, non-nucleated cells, and ghosts) after enrichment. (C) Forward and side scatter plots of the enriched samples showing the nature of contaminants as identified using traditional forward and side scatter morphology.
Figure 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- microbeads positive selection. Mean ± SEM of 6 experiments is shown. Asterisks indicate significantly higher ROS production in GM-CSF+fMLP stimulated cells as compared to fMLP alone (P<0.05). (B) Priming capacity was determined by expressing ROS production in GM-CSF cells as a percentage of ROS production in unprimed, fMLP-stimulated granulocytes (Mean ± SEM of 6 experiments). (C) Enriched granulocytes were challenged with FITC-expressing *E.Coli* for 15 min, after which FITC fluorescence of isolates was determined by FACS analysis. Whole blood was used as positive control (Mean ± SEM of 6 experiments). (D) Cell viability of enriched PMN was determined by measuring the percentage of 7AAD positive PMN by flow cytometry. (Mean ± SEM of 6 experiments)
Granulocytes isolated by Ficoll density centrifugation, anti-CD15 antibody conjugated magnetic microbeads (positive selection) or density centrifugation using Polymorphprep™ were subsequently stained for CD62L, CD15, CD66b, CD64, CD16, CD32, CD14, TLR2 and TLR4. Except for 7AAD staining, which was present in less then 5% of PMN, all other markers were present on >98% of all isolated cells, and the median fluorescence intensities of these markers was determined. Mean ± SEM of 6 experiments is shown.
Figure 4. The purities of enriched monocytes. (A) Representative flow cytometry histograms (of n=3) showing the purity of enriched monocyte cultures, obtained by either 1) anti-CD14 antibody conjugated magnetic microbeads (positive selection), (2) non-monocyte depletion by antibody conjugated magnetic microbeads (negative selection), (3) a classical adherence protocol, and (4) immunorosette based RosetteSep™ antibody cocktail, as percentage of monocytes out of nucleated cells. (B) The purities of monocytes after isolation are expressed as fraction of all cell-like particles (including nucleated cells, non-nucleated 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).
**Figure 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 minutes, 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 significant difference, although the level of phagocytosis in negative selection group is slightly lower (n=3, p>0.05, values were presented with Mean±SEM) (B) After positive selection procedure, monocytes after 16 hour culture exhibit the same *E. coli* phagocytotic capacity compared to the 2 hour culture (representative of 3 independent experiments). (C) A representative trans-sectional image of monocytes by confocal microscope confirming that *E. coli* are engulfed rather by attached to monocytes, red: cytoskeleton of monocytes (F-actin) stained by TRITC-phalloidin; green: FITC-labeled *E. coli*. 


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bacteria in human fecal samples by fluorescent in situ hybridization and flow
The images depict bar graphs showing the median fluorescence intensity (MFI) for various cell surface markers on different cell populations:

- **CD62L**: Comparison of Ficoll, Positive selection, and Polymorphprep.
- **CD15**: Similar comparison.
- **CD66b**: Similar comparison.
- **CD16**: Similar comparison.
- **CD64**: Similar comparison.
- **CD32**: Similar comparison.
- **CD14**: Similar comparison.
- **TLR4**: Similar comparison.
- **TLR2**: Similar comparison.

The graphs indicate statistical significance with asterisks (*) on some bars.
Table 1. Comparison of the yield and purity of granulocytes, time consumption and costs of the three different isolation procedures evaluated in the present study.

<table>
<thead>
<tr>
<th>Methods</th>
<th>Yield</th>
<th>Purity</th>
<th>Procedure time</th>
<th>Cost(€/10ml blood)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ficoll centrifugation</td>
<td>16±6.3×10^5</td>
<td>96.2±1.7%</td>
<td>1.5 hours</td>
<td>5.4</td>
</tr>
<tr>
<td>Positive selection</td>
<td>9.6±7.9×10^5</td>
<td>99.5±0.5%</td>
<td>2.5 hours</td>
<td>33*</td>
</tr>
<tr>
<td>Polymorphprep™</td>
<td>14.8±4.3×10^5</td>
<td>91.4±4.9%</td>
<td>1 hour</td>
<td>6.1</td>
</tr>
</tbody>
</table>

Note: The cost for positive selection did not include the magnetic separator. Purity shown is of total nucleated cells. *Cost of positive selection depends on the yield of PMN after Ficoll centrifugation, and shown is the amount calculated based on 10^7 total cells. Mean ± SD is shown of 6 independent experiments. Yield of PMN isolation differed significantly between groups (P = 0.04 by Friedman Test). Specifically, Positive selection yielded lower numbers of PMN compared to either Ficoll or Polymorphprep (P = 0.028 and 0.046, respectively. Purity of PMN differed significantly between groups (P = 0.009 by Friedman Test). Post-hoc analysis revealed that purity of PMN isolated by Polymorphprep was significantly lower than per Ficoll centrifugation or Positive selection (P = 0.03 and 0.03, respectively).
Table 2. Comparison of the yield and purity of monocytes, time consumption and costs of the four different isolation procedures evaluated in the present study.

<table>
<thead>
<tr>
<th>Methods</th>
<th>Yield</th>
<th>Purity</th>
<th>Procedure time</th>
<th>Cost (€/10 ml blood)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive selection</td>
<td>$2.2\pm0.3\times10^5$</td>
<td>$98.5\pm0.4%$</td>
<td>2.5 hours</td>
<td>23</td>
</tr>
<tr>
<td>Negative selection</td>
<td>$2.2\pm0.3\times10^5$</td>
<td>$97\pm0.7%$</td>
<td>3 hours</td>
<td>26</td>
</tr>
<tr>
<td>Adherence</td>
<td>$1.9\pm0.6\times10^5$</td>
<td>$67.3\pm3.6%$</td>
<td>4 hours</td>
<td>8</td>
</tr>
<tr>
<td>RosetteSep®</td>
<td>$4.2\pm0.6\times10^5$</td>
<td>$64.2\pm4.3%$</td>
<td>1.5 hours</td>
<td>46</td>
</tr>
</tbody>
</table>

Note: The cost for positive selection and negative selection did not include the magnetic separator. The yield of monocytes enriched by adherence was shown for the optimal washing condition. Purity shown is of total nucleated cells. Mean of 5 experiments ± SD is shown. The yield and purity of monocytes were significantly different among procedures ($P = 0.009$ and $P = 0.001$, respectively by Kruskal-Wallis test).