The impact of pre-analytical treatment and sorting on human neutrophil function

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ABSTRACT: This study was designed to test the hypothesis that pre-analytical treatment of relatively fragile cells is more important for their viability and function than shear forces and decompression shocks on a flow cytometric sorter. Human neutrophils were enriched using different techniques (sedimentation, hypotonic or ammonium chloride-mediated erythrocyte lysis) and their function was evaluated by measuring their capability of mounting oxidative burst. In contrast to other isolation techniques, neutrophils enriched by spontaneous sedimentation were found to be intact both in terms of their function and relative numbers within the leukocyte population; such preparations were thus run on a FACSAria cell sorter using 70, 85 and 100 μm nozzles and the corresponding internal pressure values. The viability and function of sorted neutrophils with or without subsequent cultivation were re-evaluated using phagocytosis assays. Independent of the nozzle size and internal sorter pressure, the capability of neutrophils of responding to *E. coli* stimulation was impaired after sorting while phorbol myristate acetate stimulation remained intact. In tested samples, only 20 h incubation of sorted cells confirmed the expected influence of the nozzle size on the sorted cell function. We conclude that pre-analytical treatment is more important for cell function than conditions during sorting.

Keywords: flow cytometry; cell sorting; granulocytes; phagocytosis; oxidative burst

Flow cytometry (FCM) is one of the most powerful analytical techniques in biological research and flow sorting is one of the few widely used preparation methods that allows the isolation of well-defined cell subpopulations, their subsets and even individual cells from heterogeneous suspensions like peripheral blood or bone marrow. In parallel, modern medicine uses multicolour FCM as a standard diagnostic tool (Kalina et al. 2012) and high speed sorting of different cell types including stem cells holds potential for therapy (Voltarelli 2000). One of the frequently discussed drawbacks of high-speed flow sorters is cellular sensitivity to decompression which occurs during droplet formation behind the nozzle where a relatively high internal instrument pressure decreases to its atmospheric value. In general, smaller, compact cells like lymphocytes are believed to be less susceptible to decompression-mediated damage than cells with a larger content of cytoplasm (Stovel 1977).

Granulocytes play an essential role in the protection of mammals against pathogens that have penetrated the physical barriers of the body. As an important part of innate immunity they participate in eliminating invading microorganisms and thus reduce the risk of serious complications before other defence mechanisms, for example cellular and humoral components of adaptive immunity, become efficient. Mammalian granulocytes are traditionally divided into three groups according to their function and the content of cytoplasmic granules: neutrophils, eosinophils, and basophils. Neutrophils, constituting the vast majority of granulocytes in circulation, use two major defence

mechanisms against various external agents: the oxygen-dependent pathway involving the NADPH oxidase system, superoxide dismutase, myeloperoxidase and other oxygen-consuming enzymes that play a role in oxidative burst (Hampton et al. 1998), and oxygen-independent machinery consisting mainly of proteolytic enzymes and antibacterial proteins discharged from cytoplasmic granules into the phagocytic vacuole and phagolysosomes (Lehrer et al. 1988). Defects in neutrophil development and function are the cause of several primary and secondary immunodeficiencies (McCusker and Warrington 2011; de Oliveira-Junior et al. 2011). Therefore, determination of granulocyte counts, their functionality, and regulatory mechanisms are important issues in clinical research.

FCM has been used to test the functional properties of granulocytes and in the diagnostics of immunodeficiencies associated with cellular innate immunity (Emmendorffer et al. 1994). Similarly, granulocyte sorting has been used to determine cytokine production (Dorward et al. 2013). In the vast majority of FCM studies focused on the characterisation and functional properties of blood leukocytes including granulocytes, the red blood cell population must be eliminated before or after cell staining and/or activation; red blood cells are approximately 1000x more numerous than the overall leukocyte population and only a few applications can be run in full blood preparations. Similarly, bulk leukocyte sorting is only possible on isolated leukocytes or leukocyte-enriched preparations as sorting from full blood is limited by the high frequency of erythrocytes and unacceptable abort/ sort conflict rates. It has become more and more obvious that sample treatment during leukocyte isolation plays a crucial role in the quality of cell characterisation and the reliability and reproducibility of functional assays (Roos and de Boer 1986; Beliakova-Bethell et al. 2014). Erythrocyte susceptibility to hypotonic environments, the lack of ammonium transporters on the surface of mature red cells as well as the different apparent density of peripheral blood cells have been used in developing standard operating procedures that allow for peripheral blood leukocyte isolation while eliminating undesirable effects and sample-to-sample variation. Fast and reproducible preservation of antigen expression and density has become a key point in case of immunophenotyping while high cell viability and excellent health status is a main goal for functional assays. In the case of neutro-

phils, activation by surfaces, varying conditions during isolation procedures and isolation buffer components may also have an adverse impact on experimental results (Berkow and Dodson 1986).

Two slightly different approaches for cell detection are used in the current flow cytometric sorters that work on the principle of droplet formation. In jet-in-air (or stream-in-air) devices, the cells are measured after they leave the nozzle while the BD FACSAria cell sorters (closed system sorter) resemble FCM analysers in that cells are measured within the flow chamber before they experience decompression when injected into the air. Such a design makes FACSAria easier to operate in terms of excitation/emission optic alignment and allows for lower power lasers at the same excitation efficiency. On the other hand, FACSAria flow cell/nozzle assembly requires higher internal pressure (BD FACS Aria III User's Guide 2012) when compared to jet-in-air FCM sorters and it is conceivable that this high pressure may have detrimental effects on the sorting of fragile cells.

Here, we set out to test how both pre-analytical cell treatment and decompression on the closed system FCM sorter influence the integrity of fragile blood cells. To do so, we first isolated granulocytes on the BD FACSAria II sorter, using nozzles of different orifice sizes, and from leukocytes or leukocyte-enriched preparations obtained from human blood using various isolation techniques. We then tested the ability of the isolated granulocytes to react to *E. coli* and soluble phorbol myristate acetate (PMA).

MATERIAL AND METHODS

Peripheral blood. In three experiments, 5 ml of heparin-treated (Zentiva, Czech Republic) blood was obtained from three volunteers; two of them without any medical treatment that could influence the count of white blood cells (WBCs) or their functional properties during the experiment. The third volunteer suffered from Systemic lupus erythematosus (SLE) and fulfilled five of the 11 diagnostic criteria established by the American College of Rheumatology: non-erosive arthritis, antinuclear antibody test positivity, anti-dsDNA positivity, haemolytic anaemia, and positivity for anti-Ro, anti-La, and anti-U1 RNP. He was treated with Medrol (methylprednisolone, 8 mg/day), Imuran (azathioprine 50 mg/day), and Plaquenil

(hydroxychloroquine sulphate 200 mg/day). The volunteer's disease state was regarded as inactive (Mex-SLEDAI ≤ 2). Written consent was obtained from all volunteers.

Pre-analytical treatment. One of the most important steps in peripheral blood leukocyte and leukocyte subset isolation is the removal of abundant red blood cells. Different approaches have been tested and used in many laboratories including hypotonic erythrocyte lysis, density gradient sedimentation as well as spontaneous sedimentation and buffy coat recovery. We chose four different isolation techniques for neutrophil enrichment before sorting on an FACSAria II (Becton Dickinson, USA).

Ammonium chloride-based lysis. Anticoagulant-treated blood samples were lysed with ammonium chloride in a HEPES-buffered solution containing isotonic EasyLyse™ solution (DakoCytomation, Denmark). Briefly, 1.5 ml of anti-coagulated blood were incubated with 10x excess of EasyLyse™ at room temperature (RT) for 15 min according to the manufacturer's instructions. Samples were then centrifuged (300 × g, 10 min, RT) and the supernatant was discarded. Then, the pellet was resuspended in 4 ml of phosphate buffered saline (PBS), centrifuged again and resuspended in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% foetal calf serum, 0.05% l-glutamine, 150 UI/ml penicillin, 50 μg/ml streptomycin (all chemicals from Sigma, St. Louis, MO) to a final density of 10^6 cells per ml.

Hypotonic lysis. The method of hypotonic lysis by deionised water (first described by Carlson and Kaneko 1973) was used in a modified form: 1.5 ml of anticoagulated blood were added to 45 ml of ice-cold high-quality deionised water and gently mixed by inversion. After 10 s, 5 ml of 10 × concentrated phosphate buffered saline (PBS, Sigma, St. Louis, USA) were added, the suspension was gently mixed by inversion again and centrifuged. The pellet was then resuspended in 4 ml of PBS and sedimented by centrifugation again. Supernatant was discarded, the cells were resuspended in 1.5 ml of IMDM, counted and their density was set to 10^6 cells per ml.

Modified buffy coat method. The buffy coat method (described by Roos and de Boer 1986) was used in a modified form to avoid the exposure of isolated cells to either ammonium chloride ion or hypotonic shock. Ten ml disposable syringes were pre-filled with 2 ml of 6% dextran (SERVA Electrophoresis, Heidelberg, Germany), and 5 ml of anticoagulant treated blood were subsequently aspirated into the syringe. The sample was then sedimented at a 45° angle for 40 min in a humidified (5% CO_2, 37 °C) incubator. After sedimentation, the leukocyte-rich suspension above the dextran layer containing the vast majority of red blood cells was collected by carefully moving the syringe piston and collecting the material released from the needle tip. The presence of granulocytes in the collected material was tested by FCM as described below. The collected suspension (about 2 ml) was diluted in 4 ml of IMDM, centrifuged and washed as described in the previous paragraphs. The final density was set to 10^6 cells per ml.

1 × g sedimentation in insulin syringes. Anticoagulated blood was drawn into a 1 ml insulin syringe which was placed at a 60° angle for 20 min in an upside-down position at room temperature. Importantly, any mixing was prevented as mixing the syringe content after blood collection results in worse red blood cell separation. After sedimentation, the bottom layer containing mainly erythrocytes was removed by pushing the syringe piston and the leukocyte-rich buffy coat layer with residual red blood cells was collected and washed in IMDM as described above. White blood cells were counted using a haemocytometer and their density was set to 10^6 per ml.

Measurement of phagocytic activity. The FagoFlowEx® kit (Exbio, Prague, Czech Republic) was used to analyse the ability of neutrophils to mount oxidative burst just after isolation or 20 h after incubation in IMDM (37 °C, 5% CO_2).

Before the assay, cell suspensions were kept at room temperature in IMDM. For the assay, $5 × 10^5$ cells in 1 ml were incubated with dihydrorhodamine-123 (DHR123) and one of the two activators contained in the kit – either a fixed *E. coli* suspension (phagocytosis sample) or phorbol-12-myristate-13-acetate (PMA) (stimulation control) – in a water bath (20 min, 37 °C). Non-stimulated cells were used in parallel as a control sample to assess spontaneous oxidization of DHR123 to rhodamine-123. After incubation, the lysis solution was added and the test tubes were gently vortexed. Then, the remaining erythrocytes were lysed for 5 min at room temperature and 3 ml of deionized water was added. The samples were incubated for another 10 min at RT and then centrifuged (400 × g, RT, 10 min). Supernatant was discarded and the pellet was resuspended in 100 μl of PBS. Intensity of DHR 123 green fluorescence on a single cell level was meas-

ured using blue (488 nm) laser excitation. The results are reported as the ratio of mean fluorescence intensity (MFI) of granulocytes stimulated by *E. coli* or PMA to the MFI in the non-stimulated sample.

Flow cytometry and sorting. FCM was run on a 3-laser 9-color CyAn (Beckman Coulter, USA) analyser and 3-laser 9-color FACSAria II (Becton Dickinson, USA) cell sorter. Excitation light scattering and 3-color surface immunophenotyping was used to identify neutrophils and determine their purity as shown in Figure 1. Major lymphocyte subsets – T-cells and B-cells – were identified using anti-CD19/PE (clone LT19, Exbio, Czech Republic), and anti-CD3/APC-H7 (clone SK7, BD Pharmingen, Germany), respectively. Monocytes were identified on the basis of their bright staining with an anti-CD14/FITC antibody (clone M5E2, BD Pharmingen, Germany). Briefly, 100 µl cell suspensions (5×10^5 cells) were incubated with the moAb cocktail for 30 min in the dark at 4 °C, washed with 3 ml of washing and staining buffer (WSB, PBS containing 0.2% gelatine from cold water fish skin and 0.1% sodium azide, all from Sigma Aldrich, Germany) and then centrifuged. The supernatant was discarded and the pellet was resuspended in 200 µl WSB. Propidium iodide (PI, Sigma Aldrich, Germany) was added just before acquisition to a final concentration of 0.1 µg/ml. Reverse gating was used to identify $SSC^{hi}CD3^-CD19^-CD14^{low+}$ granulocytes in the forward angle light scatter (FSC) versus side angle light scatter (SSC) diagram as shown in Figure 1.

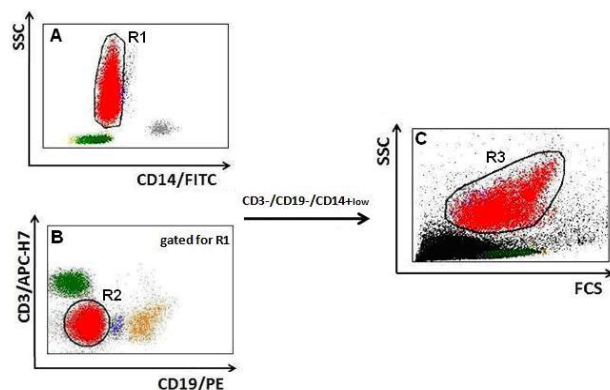Cell sorting was performed on a BD FACSAria II sorter. System performance was confirmed by

Figure 1. Granulocyte identification using immunofluorescence based gating. SSChiCD14lo+ cells (R1 in **A**) negative for CD3 and CD19 surface markers (R2 in **B**) were identified as the granulocyte population (R3 in **C**) in the FSC/SSC dot plot (C)
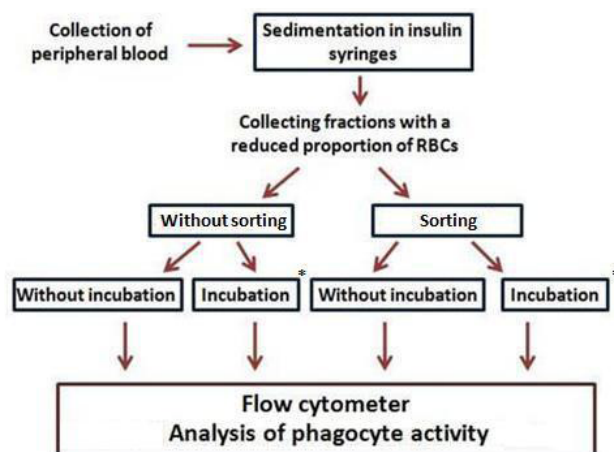

Figure 2. Workflow: Peripheral blood was treated by sedimentation in insulin syringes, and fractions with reduced proportions of RBCs were then collected. Such modified blood samples were divided into two parts: (1) non-sorted samples, and (2) samples that were sorted on the FACS Aria II sorter. Each of the samples was then subdivided into two fractions, half was analysed immediately, the other part after 20 h incubation. For all samples, the phagocytic activity of granulocytes was measured using the FagoFlowEx®Kit and the apoptosis was detected using Annexin V binding kit

the BD™ Cytometer Setup and Tracking module and the drop delay for individual nozzles was set using BD™ Accudrop Fluorescent Beads and the automatic drop delay procedure. Only leukocyte-enriched preparations using the 1 × g sedimentation method were used for sorting as the other pre-analytical techniques proved inconvenient for neutrophil isolation (see the Results section). For sorting, no surface staining was used to prevent any staining-associated interference with neutrophil function and neutrophils were identified as shown in Figure 1 using a CD3CD19-stained sample run just before sorting as the $CD3^-CD19^-SSC^{hi}$ population.

Standard (70, 85, and 100 µm) nozzles were used and 70, 40 and 20 psi internal pressure was selected. Cell preparations were filtrated using a 35 µm mesh (Falcon, BD Biosciences, USA) before placing in the sample port. The yield sorting mode was used to increase the efficacy of neutrophil collection and shorten the time of sorting. After sorting, the cells were sedimented (600 g, 4 °C), 10 min), washed in IMDM and resuspended in IMDM.

Statistical analysis. Values were expressed as the means of duplicate samples. A paired *t*-test was
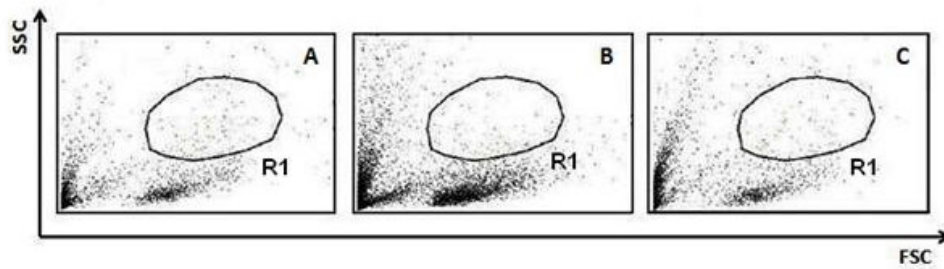
Figure 3. Representative scattergram of peripheral blood cells isolated using the modified buffy coat method. Unstimulated (**A**), PMA-stimulated (**B**) and *E. coli*-stimulated (**C**) samples are showen showing a very low proportion of cells with a high SSC parameter (neutrophils, R1)

used to compare data between control samples, sorted samples, incubated control, and incubated sorted samples for all nozzles, and to compare data between analyses using different nozzles. A probability value of $P < 0.05$ denoted statistical significance. Fit analysis was performed using statistical software (MS Office Excel 2007, Statistica 6).

RESULTS

Pre-analytical treatment, neutrophil enrichment and oxidative burst response

The optimal sample preparation technique for subsequent sorting experiments was selected based both
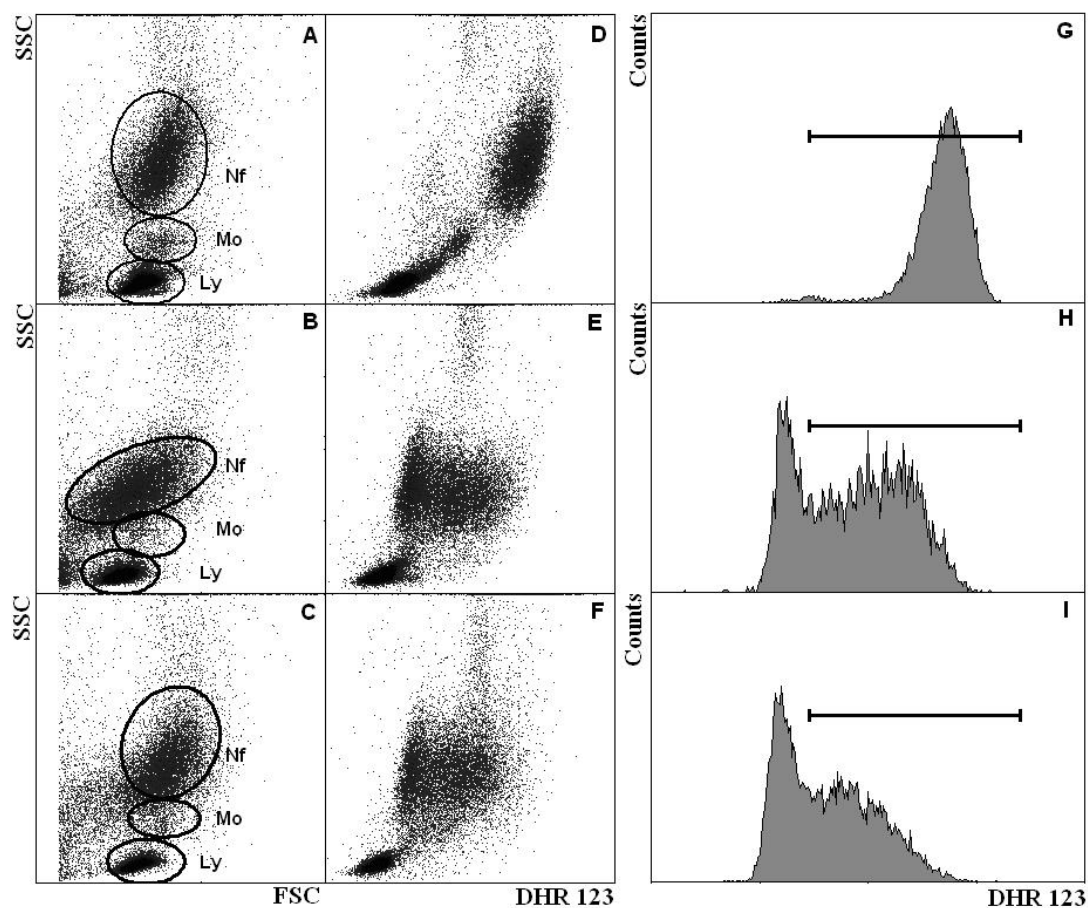

Figure 4. Example of results of the FagoFlowEx assay in full blood (**A**, **D**, **G**) and ammonium chloride (**B**, **E**, **H**) or hypotonic shock (**C**, **F**, **I**) derived leukocyte preparations. While essentially all neutrophils in full blood are able to respond to the presence of *E. coli* by reactive oxygen species production (D and G), significant populations of neutrophils that had lost such capacity was found both in EasyLyse- (E and H) and water- (F and I) treated samples. The proportions of activated cells are shown in the histograms gated for the neutrophil population (G, H, I)

on the proportion of granulocytes among leukocytes identified on a flow cytometer by scatter parameters (neutrophil yield) and the capability of isolated granulocytes of mounting oxidative burst upon specific (*E. coli*) and non-specific (PMA) stimulation (Figure 2).

The modified buffy coat method proved to be inconvenient for neutrophil enrichment as granulocyte counts were too low for subsequent processing and analysis (Figure 3). In addition, respiratory burst activity did not reach the level found in a standard, full blood preparation (data not shown).

Hypotonic red blood cell removal, either by a short exposure to water or by using the ammonium chloride-based EasyLyse resulted in much higher relative neutrophil counts in peripheral blood leukocyte preparations that were comparable to the results of the full blood FagoFlowEx assay recommended by the manufacturer. Figure 4 shows typical results of the phagocytic activity test performed with full blood and leukocytes isolated with the ammonium chloride-based solution (EasyLyse) or by hypotonic shock. Lymphocytes (Ly), monocytes (Mo) and neutrophils (Nf) were identified in the FSC versus SSC dot plot as clusters of cells with low, intermediate and high values in the SSC parameter, respectively (Figure 4A, B, C). Green fluorescence of the reduced DHR123 versus SSC parameter diagrams (Figure 4D, E, F) clearly show that, when compared to full blood preparations (Figure 4D), a significant subpopulation of neutrophils isolated by either exposure to ammonium chloride (Figure 4E) or hypotonic shock (Figure 4F) had impaired ability to

mount oxidative burst when exposed to *E. coli* cell wall components. The response to PMA was not altered in such cell preparations and the results were identical to those from full blood preparations (data not shown). Figure 4G, H and I show histograms of rhodamine-123 fluorescence intensity in neutrophils (gated for Nf gate in the scattergrams) with the positive histogram gate lower boundary set based on the basal fluorescence found in non-stimulated samples.

The relative numbers of neutrophils within the leukocyte population in the three preparations with a high neutrophil yield (all but the modified buffy coat method) and the summary of oxidative burst activation efficiency are given in Figure 5. Ammonium chloride- as well as hypotonic shock-mediated erythrocyte removal followed by *E. coli* activation had no effect on relative neutrophil counts when compared to full blood samples. Exposure to PMA generally resulted in a significant decrease in the proportion of neutrophils; such a reduction was most pronounced in water-treated samples while the difference between full blood and EasyLyse-treated samples was not statistically significant. No significant differences were found among any of the samples when the potential of mounting an oxidative burst in response to non-specific activation (PMA) was compared. However, the more physiological activation by *E. coli* cell wall components showed that, in contrast to neutrophils in heparin-treated full blood which exhibited an almost 100% prevalence of activation, ammonium chloride resulted in a significantly lower percentage of *E. coli*-reactive neutrophils (65%). Neutrophils
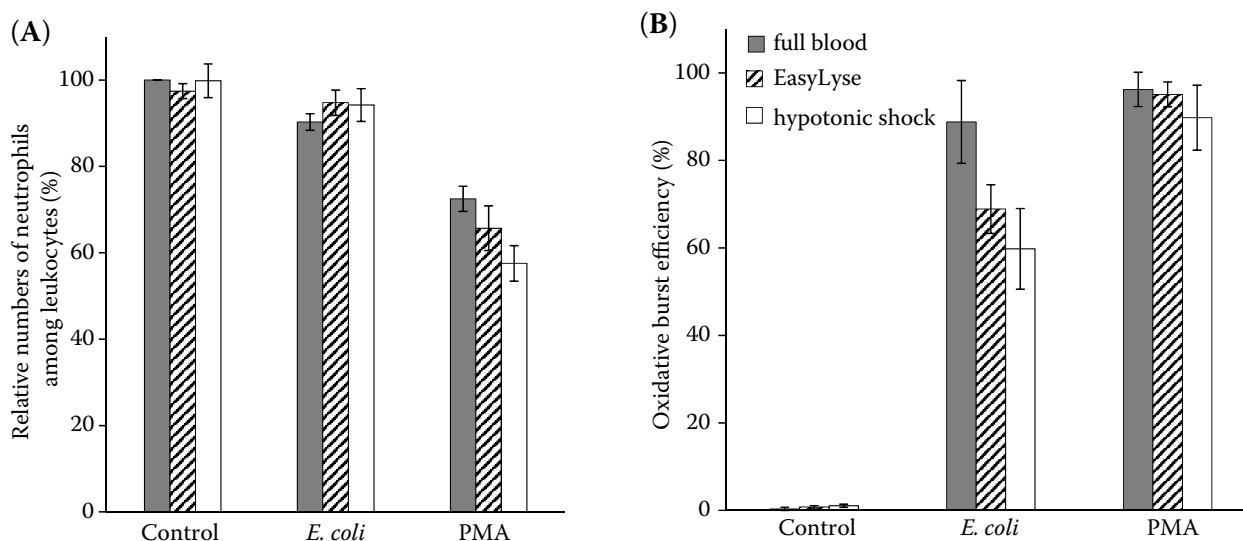

Figure 5. Relative neutrophil counts among leukocytes (**A**) and the proportion of *E. coli* and PMA-reactive neutrophils (**B**) in full blood, EasyLyse and hypotonic shock preparations subjected to the FagoFlowEx assay. Mean values ± standard deviation are given
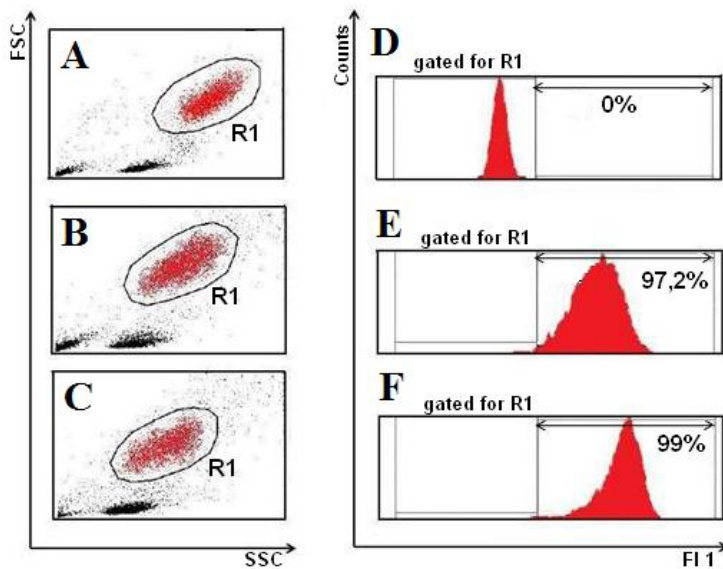
Figure 6. Representative results of oxidative burst in neutrophils (**D**, **E**, **F**) isolated from heparin-anticoagulated peripheral blood sedimented in insulin syringes at 1 g gravity. Neutrophils (R1), the numbers of which corresponded to those obtained by a standard FagoFlowEx approach in full blood (**A**, **B**, **C**) were capable of mounting a standard response both to *E. coli* (**B** and **E**) and PMA (**C** and **F**). Background fluorescence (**D**) in a non-stimulated sample (**A** and **D**) is also shown to document that no activation of neutrophils occurred by 1 g sedimentation

isolated by hypotonic shock were even more affected (52% reactive cells on average).

One g sedimentation in insulin syringes proved to be the most convenient isolation technique both in terms of neutrophil counts and function (Figure 6).

Based on the comparison of the four selected leukocyte isolation techniques, 1g sedimentation within insulin syringes, which provided high numbers of functionally intact neutrophils, was selected for experimentation on a flow sorter. The modified
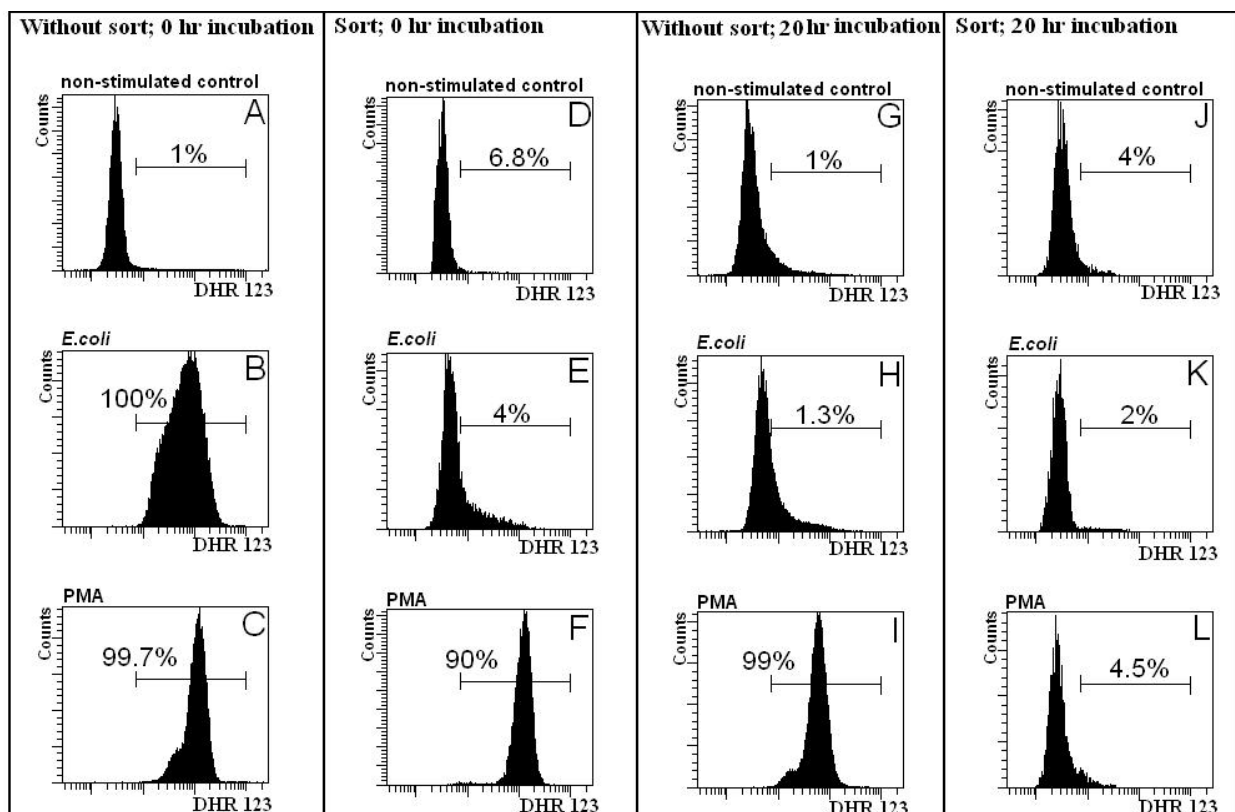

Figure 7. Rhodamine-123 fluorescence intensity of neutrophils identified by their scatter characteristics (the Nf gate in Figure 4 A–C). Cells before (**A**–**C**, **G**–**I**) or after (**D**–**F**, **J**–**L**) sorting using the 85 µm nozzle were either tested prior to (**A**–**F**) or after (**G**–**L**) overnight (20 h) incubation. The relative proportions of neutrophils with higher than background fluorescence (non-stimulated controls, **A**, **D**, **G** and **J**) are given for *E. coli*- (**B**, **E**, **H**, **K**) and PMA- (**C**, **F**, **I**, **L**) mediated stimulation. A-C correspond to D-F in Figure 6 but originate from another experimental day and donor

buffy coat method as well as leukocyte isolation using ammonium chloride or hypotonic shock were found to be unsuitable as neutrophil numbers were either significantly reduced or their function was heavily impaired by the treatment.

Cell sorting

Neutrophils were sorted from 1 g sedimentation-enriched peripheral blood leukocytes using the three available nozzle orifices of 70, 85 and 100 μm and corresponding internal flow chamber pressures of 70, 45 and 20 psi, respectively, as is recommended by the sorter manufacturer. The predefined "Enrichment" sorting mode was selected both to increase the neutrophil yield and to shorten sorting time. Only sorts with neutrophil purity higher than 90% were used for subsequent experiments. Cell functionality and viability was assayed by determining the ability of producing reactive oxygen species and by Annexin V binding. Figure 7 shows representative results of 85 μm nozzle-sorted neutrophils assayed by the FagoFlowEx either immediately after sorting or upon 20 h incubation and their comparison to a non-sorted sample as an untreated control.

Whilst essentially all neutrophils in 1g sedimentation-enriched leukocyte preparations responded both to the more physiological (*E. coli*) and chemically-induced (PMA) activation (Figure 7B–C), sorted neutrophils exhibited an intact response to PMA only (Figure 7F), and their response to *E. coli* was heavily affected (Figure 7E). An incubation of 20 h further decreased the capability of neutrophils of responding to the selected stimuli and only unsorted cells

were able to mount oxidative burst in their response to PMA (Figure 7I), while activation in response to *E. coli* was significantly decreased (Figure 7H). Sorted and incubated cells were very poor responders both *to E. coli* and PMA (Figure 7K–L).

The summary of results obtained from neutrophils isolated from all three donors, a comparison of unsorted cells and samples sorted using nozzles with different orifice sizes and the evaluation of *in vitro* incubation on neutrophil function are presented in Figure 8.

When neutrophils were tested for their oxidative burst activity immediately after sorting, no difference was observed in their response to PMA, whichever nozzle size had been used for sorting (the bar cluster in the middle of Figure 8A). All sorted preparations resembled the non-sorted preparations; thus, the high variation on the 85 μm nozzle was likely due to the random error of results from one experimental day (Figure 8A). In contrast, *E. coli* stimulation was adversely affected (see also Figure 7E for the 85 μm nozzle) but, quite surprisingly, the only statistically significant difference between the sorted samples was in the case of the 85 and 100 μm nozzle and *E. coli*-mediated stimulation. The 100 μm nozzle appeared to be more effective in reducing the *E. coli*-mediated oxidative burst (the right cluster of columns in Figure 8A) than its 85 μm counterpart. *In vitro* cultivation resulted in an impaired ability of neutrophils to mount oxidative burst upon *E. coli* stimulation (see also Figure 7H). The modest capability of reducing DHR 123, which was observable in unsorted samples, was paralleled in 85 μm and 100 μm nozzle-activated cells (the clusters of columns on
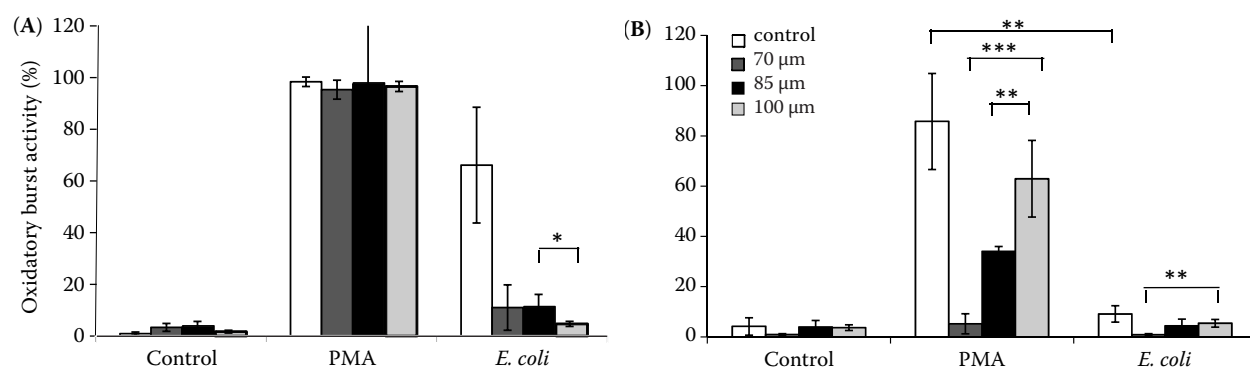

Figure 8. Oxidative burst mounting activity of neutrophils prepared by 1 g sedimentation method before (**A**) and after (**B**) 20 h *in vitro* cultivation. Unstimulated, PMA and *E. coli* stimulated neutrophils are compared for non-sorted cells and neutrophils sorted using the 70 μm, 85 μm and 100 μm nozzle. The results are given as the average percentage of bursting cells ± standard deviation from all donors run in duplicate for every single nozzle. Statistically significant differences: *$P < 0.01$, ** $P < 0.05$, ***$P < 0.005$

the right in Figure 8B), while cells sorted on the 70 μm nozzle gave similar values as non-stimulated cells. Importantly, PMA stimulation in sorted and cultivated neutrophils was the only experimental setup that clearly confirmed the hypothesis that decompression influences cell integrity – the rule "the bigger the better" (read bigger nozzle orifice resulting in smaller internal pressure with favourable results for cell integrity) was clearly confirmed in this setup. Statistically significant differences were recorded between PMA-stimulated neutrophils sorted using different nozzle orifice sizes with the 100 μm nozzle being comparable to the situation in non-sorted neutrophils. The 70 μm nozzle adversely affected the reactivity of the sorted cells to PMA.

DISCUSSION

Leukocyte isolation from blood, bone marrow, lymphatic organs, mucosa and solid tissues is one of the most frequently used laboratory techniques. Cell sorting based on the immunophenotype of the cells of interest – e.g. the expression of CD45 as the common leukocyte common (LCA) – is the technique of choice both on magnetic and flow sorters (Ibrahim and van den Engh 2003). In the case of erythrocyte-rich suspensions like peripheral blood, where the excess of red blood cells is higher than 1000×, erythrocyte removal before sorting is a prerequisite because erythrocyte mass is a general issue for the purity and/or speed of sorting (Halasa et al. 2008). Different approaches have been developed since the dawn of leukocyte biology including red blood cell lysis by hypotonic shock using short exposure either to water- or ammonium chloride-based buffers, treatment with different fixatives and permeabilising chemicals, density gradient centrifugation and spontaneous or polymer-mediated sedimentation. However, for functional assays, where a high proportion of viable cells is required, only some these approaches are convenient as many treatments affect cell viability and function, which is not always well appreciated. In addition, subsequent positive sorting (sorting of the stained cells) suffers from the fact that immunoreagent binding to the cell surface may activate or damage its target population (e.g. Kim and Kim 2014). For this reason, cell depletion (negative sorting) of stained unwanted cells has been introduced. In the case of neutrophils, the dominant granulocyte population in circulation, the most

popular gentle erythrocyte removal technique using Ficoll cushion cannot be used due to the high granulocyte density. On the other hand, neutrophils can easily be discriminated from all other leukocyte populations and erythrocytes on flow sorters by their strong scattering under higher angles relative to the incident light direction (side scatter). Partial erythrocyte removal followed by flow sorting of unstained suspensions thus appears an ideal choice for neutrophil isolation for functional studies. As neutrophils are believed to represent a relatively fragile leukocyte population, it is important to determine which of the leukocyte enrichment techniques are convenient for such an approach and what the role of flow sorter conditions is.

Our results clearly show that careful selection of the erythrocyte removal technique is the most important factor for neutrophil yield and viability. The buffy coat method proved to be unsuitable due to a very low neutrophil yield, which did not allow us to measure phagocytic activity in a reasonable manner. In contrast, 1 g sedimentation in narrow (insulin) syringes provided high yields of leukocytes with the proportion of neutrophils resembling the enrichment observed after standard erythrocyte removal using commercial fixing buffers like the BD FACS Lysing solution. Similar neutrophil enrichment has been obtained upon hypotonic erythrocyte lysis using either pure water or the ammonium chloride-based buffer EasyLyse. However, when individual leukocyte-enriched preparations were assayed for their capacity to mount oxidative burst, only 1 g sedimented neutrophils were capable of reducing DHR 123 to the same extent as untreated cells assayed in full blood. The unambiguous conclusion of this part of the study is that only neutrophils gently sedimented in insulin syringes exerted full functionality and such cell preparations were thus used in subsequent experiments on the flow sorter.

Running neutrophils through the FACSAria II had a detrimental impact on their capability of responding to a natural activator – *E. coli* cell wall components – while PMA activation remained unaffected independent of the nozzle orifice and the corresponding internal pressure of the flow sorter. To exclude the possibility that the loss of ability of responding to *E. coli* was a short-term effect the cells were incubated for 20 h after sorting. However, instead of a functional recovery in terms of *E. coli* activation, neutrophils partially lost their capacity to respond to the stronger activator PMA and this effect was dependent on the nozzle orifice and

the experienced decompression and shear force effects. As the differences were highly statistically significant we conclude that, under such conditions – sorting followed by relatively long incubation – the difference between nozzle sizes plays a role. Interestingly, although the capability of mounting respiratory burst upon *E. coli* activation remained tiny, the size of nozzle orifice had a significant effect on the ability of cells to respond to PMA.

In conclusion, our data suggest that the settings used on the flow cytometer sorter, i.e. nozzle size and internal pressure, are much less important for functional assays on relatively fragile peripheral blood leukocytes than the pre-analytical treatment of neutrophils.

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