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Flow cytometry-based characterization of isolated human neutrophils: Evaluating viability, purity and defining a reference interval for basal activation in functional assays

Hachem Bouarroudj [a], Constant Gillot [a], Jonathan Decarpentrie [a], François Mullier [b], Jonathan Douxfils [a,c,*]

[a] Clinical Pharmacology and Toxicology Research Unit, Namur Research Institute for Life Sciences, Namur Thrombosis and Hemostasis Center, University of Namur, Namur, Belgium
[b] Department of Laboratory Medicine, CHU UCL-Namur Site Godinne, Godinne, Belgium
[c] Department of Biological Hematology, Centre Hospitalier Universitaire Clermont-Ferrand, Hôpital Estaing, Clermont-Ferrand, France

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ABSTRACT

Introduction: Neutrophils are the most abundant circulating leukocytes and play a central role in innate immunity. As in vitro functional assays (NETosis, degranulation, phagocytosis, etc.) depend on cell viability, purity and basal activation, it is essential to validate neutrophil suspensions before experimental use.
Objectives: To characterize isolated neutrophils using an in-house flow cytometry workflow for assessing viability, purity, and activation, and to establish a reference interval (RI) for neutrophil basal activation.
Methods: Twenty healthy volunteers were recruited, and neutrophils were isolated from whole blood by negative selection. Cell viability was determined by propidium iodide staining. Leukocyte and neutrophil percentage were evaluated using conjugated antibodies against CD45 and CD15 respectively. Basal activation was assessed by measuring mean fluorescence intensity (MFI) using a conjugated antibody against CD66b. To validate the RI of basal activation, neutrophils were also stimulated with TNF. RI were calculated as mean ± 2 standard deviations (SD).
Results: Neutrophil suspensions displayed high quality across all criteria. Viability averaged 99.4% (SD =0.4). Leukocyte purity reached 99.3% (SD =0.4), and neutrophil purity was elevated, with a mean of 98.6% (SD = 0.5). Isolated neutrophils presented a mean CD66b MFI of 37.4 (SD =10.8), and CD66b expression increased significantly following TNF stimulation. The calculated RI was 37.4 ± 21.6.
Conclusion: This study demonstrated that neutrophils isolated by negative selection displayed high viability, purity, and minimal activation. It also allowed the definition of a RI for basal activation which could serve as objective criterion to validate neutrophil suspensions for functional assays.

1. Introduction

Neutrophils are the most abundant leukocyte population in human circulation and play a central role in innate immunity. They act as rapid first responders to infection and tissue injury through phagocytosis, degranulation, and the formation of neutrophil extracellular traps (NETs), a process known as NETosis (Brinkmann et al., 2004) (Vorobjeva and Chernyak, 2020). Neutrophils are short lived, terminally differentiated cells with a circulating lifespan reported as 7–9 h before they undergo constitutive apoptosis (Hidalgo et al., 2019). In vitro neutrophil functional assays could be used to investigate disease mechanisms and evaluate therapeutic interventions (Blanter et al., 2021).

A critical factor influencing the reliability of these assays is the quality of the neutrophils employed. During isolation from whole blood, neutrophils may undergo partial activation, loss of viability, or contamination with other leukocyte subsets (Kremer et al., 2023). Despite this, no standardized flow cytometric approach currently exists for assessing the quality of isolated neutrophil including viability, purity, and activation status prior to functional assays, owing to substantial

* Corresponding author at: Clinical Pharmacology and Toxicology Research Unit, University of Namur, rue de Bruxelles 61, B-5000 Namur, Belgium.
E-mail address: jonathan.douxfils@unamur.be (J. Douxfils).

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inter-study variability in surface marker selection, viability dyes, and gating strategies. Furthermore, no reference interval (RI) has been established for basal neutrophil activation, which could serve as an acceptability criterion. The absence of such interval could be considered as limitation for the reliable interpretation and comparison of functional assays.

The objective of this study was to characterize isolated human neutrophils using an in-house flow cytometry workflow for simultaneously assessing viability, purity, and activation and to establish a RI for basal activation which will serve to validate neutrophil preparations prior to their use in functional assays.

2. Material and methods

2.1. Blood collection and neutrophil isolation

This study was conducted in accordance with the Declaration of Helsinki, approved by the local Ethics Committee (NUB: B03920096633), and samples were collected after volunteers' written consent. Twenty healthy volunteers (10 men, 10 women, age range [21–36] years, mean age: 25 years) were selected using a screening questionnaire according to the following criteria: volunteers in good health (defined as individuals without fever, neutropenia, or a history of leukemia or systemic lupus erythematosus), with no medication that could affect the immune system such as vaccines or immunomodulatory agents. Blood samples were collected between June and September 2025, consistently at the same time of day (9,00 AM) and by the same two trained phlebotomists. Three K2EDTA tubes (Vacutainer, Becton Dickinson) were collected from each volunteer after venipuncture of the median cubital or basilic vein, with a 21 G straight needle (Vacuette, Greiner Bio-One, Kremsmünster, Austria).

Neutrophils were isolated from whole blood using the MACSxpress Whole Blood Neutrophil Isolation Kit, human (catalog no 130–104-434, Miltenyi Biotec, Bergisch Gladbach, Germany), based on negative selection with magnetic beads according to the manufacturer's protocol. Following isolation, neutrophils were resuspended in RPMI medium (catalog no 11835030, Capricorn Scientific, Ebsdorfergrund, Germany) at 2×10^6 cells/mL and cell counting was performed using Countess II FL Automated Cell Counter (Thermo Fisher Scientific, Waltham, MA, USA) with 0.4% trypan blue solution (catalog no T10282, Invitrogen, Carlsbad, CA, USA).

2.2. Surface marker selection

The choice of surface markers was based on flow cytometry literature characterizing human neutrophils. CD45 is a pan-leukocyte antigen expressed on all white blood cells. CD15 is widely recognized as a characteristic marker of mature neutrophils (Verschoor et al., 2015). Several membrane markers have been described to assess neutrophil activation, including CD11b, CD64 and CD66b. CD66b was selected as it is a glycoprotein stored in secondary granules, constitutively present at low levels on resting neutrophils and becomes increasingly expressed upon activation in certain pathological conditions such as bacterial infection, making it a reliable marker of their activation state. (Schmidt et al., 2015)

2.3. Neutrophil activation

To evaluate the activation status of isolated neutrophils and to establish a positive control for comparison, 500 µL of the neutrophil suspension prepared from each volunteer (5 men and 5 women) were incubated in a 24-well suspension culture plate (catalog no 662102, Greiner Bio-One) under gentle agitation with 500 µL of Tumor Necrosis Factor (TNF-α) solution (catalog no A42552, Invitrogen) at a final concentration of 0.5 µg/mL for 15 min at 37 °C in a humidified incubator containing 5% CO_2. Activated neutrophils were subsequently stained following the protocol described below.

2.4. Flow cytometry workflow

The flow cytometry methodology was designed and reported in accordance with the MIFlowCyt guidelines, an international standard developed by the International Society for the Advancement of Cytometry (ISAC) that defines the information required regarding experimental design, sample preparation, instrument configuration, and data analysis to ensure transparency and reproducibility of flow cytometry experiments. (Lee et al., 2008)

2.4.1. Staining protocol

Approximately one million neutrophils, either freshly isolated from each volunteer ($n = 20$) or previously activated ($n = 10$), were transferred into an Eppendorf tube and centrifuged at 300 *g* for 10 min. The supernatant was discarded and the cell pellet was resuspended in FACS buffer (PBS supplemented with BSA and EDTA) together with propidium iodide (PI) (dilution 1:100, catalog no 130–092-052, Miltenyi Biotec) for viability assessment, anti CD45-Vioblue (dilution 1:50, catalog no 130–110-775, Miltenyi Biotec), anti CD15-FITC (Fluorescein Isothiocyanate, dilution 1:50, catalog no 130–114-010, Miltenyi Biotec), and anti CD66b-APC (Allophycocyanin, dilution 1:50, catalog no 130–117-804, Miltenyi Biotec). The choice of fluorochromes was based on the manufacturer's recommendations (Miltenyi Biotec) to minimize spectral spillover from adjacent detectors and cross-laser excitation.

The mixture was incubated for 10 min at 4 °C in the dark. Following incubation, 1 mL of FACS buffer was added and the suspension was centrifuged again at 300 *g* for 10 min. The supernatant was removed, and the pellet was resuspended in 100 µL of FACS buffer for flow cytometric acquisition using MACSQuant Analyzer 10 (Miltenyi Biotec), which was calibrated prior to each acquisition using calibration beads (130–093-607, Miltenyi Biotec) according to the manufacturer's recommendations. The MACSQuant Analyzer 10 is equipped with multiple lasers (405, 488 and 640 nm) and appropriate optical filters for the detection of the selected fluorochromes. Fluorescence compensation was performed automatically using the MACSQuant Analyzer software, which applies predefined correction settings based on the spectral properties of the fluorochromes to account for signal spillover between detection channels.

2.4.2. Evaluation of antibody specificity and non-specific binding

To verify the specificity of the antibodies used for flow cytometry staining, isotype controls corresponding to each fluorochrome were included. For each fluorochrome-conjugated antibody, a corresponding isotype was selected: REA Control (*S*)-VioBlue (catalog no 130–113-442, Miltenyi Biotec), REA Control (*S*)-APC (catalog no 130–113-434, Miltenyi Biotec), and Mouse IgM-FITC (catalog no 130–093-178, Miltenyi Biotec). These recombinant antibodies are designed to minimize Fc receptor-mediated binding and allow the evaluation of residual non-specific interactions, including those related to the fluorochrome, thereby providing a reliable assessment of background signal.

Freshly isolated neutrophils from 10 volunteers (5 men and 5 women) were stained, separately, according to the manufacturer's protocol. Cell debris and dead cells were excluded from the analysis based on scatter signals and PI fluorescence.

2.4.3. Gating strategy

Flow cytometry data were analyzed using a sequential gating strategy. After debris and doublets were excluded, viable cells were selected by PI versus Forward Scatter Area (FSC-A). Leukocytes were then identified using VioBlue versus FSC-A, and neutrophils were subsequently gated based on the FITC signal versus FSC-A. Finally, CD66b expression was quantified on the neutrophil population by measuring the Mean Fluorescence Intensity (MFI) of APC.

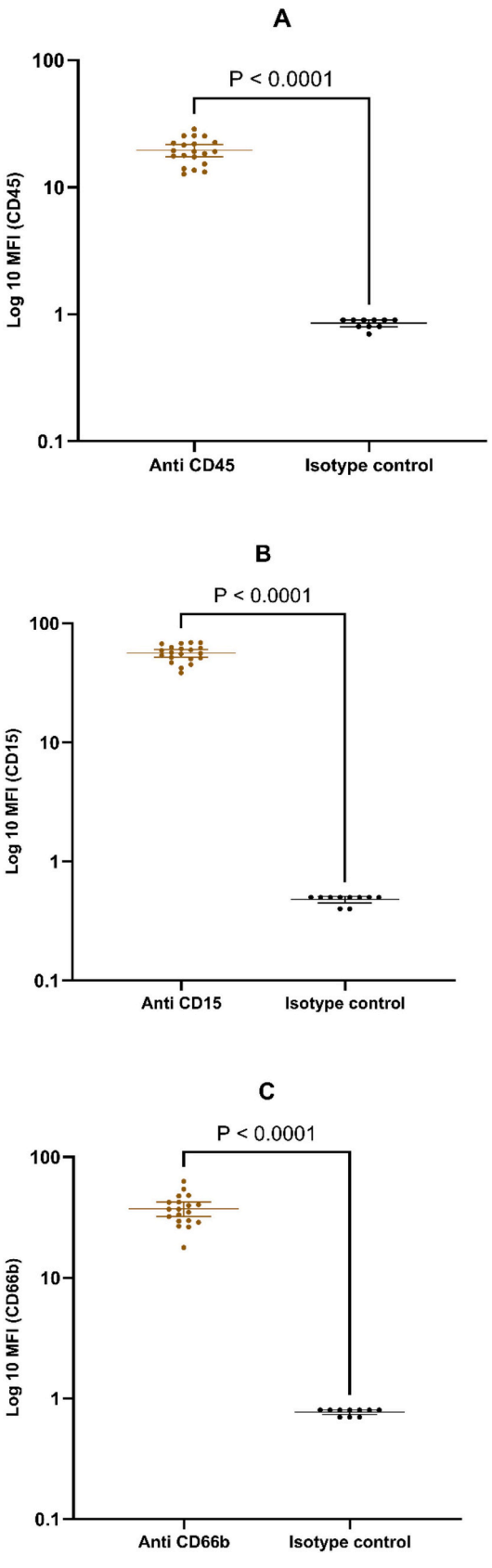

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Fig. 1. Results of the antibody specificity analysis performed using selected antibodies ($n = 20$) and their matched isotype controls ($n = 10$). Panel A shows the MFI values with 95% CI of CD45 measured with the VioBlue-conjugated antibody with its corresponding isotype control, Panel B shows the MFI values with 95% CI of CD15 measured with the FITC-conjugated antibody with its corresponding isotype control, and Panel C shows the MFI values with 95% CI of CD66b measured with the APC-conjugated antibody with its corresponding isotype control. In Panel C, one outlier from the anti-CD66b antibody group was identified and excluded from the analysis. Fluorescence intensities are displayed on a logarithmic (log10) scale. For all three markers, specific antibodies yielded significantly higher fluorescence signals compared with their respective isotype controls ($p < 0.0001$). *Abbreviations: APC: Allophycocyanin; CI: Confidence Interval; FITC: Fluorescein Isothiocyanate; MFI: Mean Fluorescence Intensity.*

2.5. Statistical analysis

Statistical analyses were performed using GraphPad Prism (Version 10.4.1, San Diego, CA, USA). Outliers were identified and excluded using the ROUT method (Q = 1%). Descriptive statistics were performed, including mean, standard deviation (SD), and 95% confidence intervals (95% IC). Data distribution was assessed using the Shapiro–Wilk test to evaluate normality. For group comparisons, Welch's *t*-test was applied to account for unequal variances and sample sizes. For all statistical tests, a *p*-value of <0.05 was significant.

The RI for basal neutrophil activation was established following the Clinical and Laboratory Standards Institute (CLSI) EP28-A3c recommendations (Yang et al., 2022). The parametric approach was applied to calculate the RI using the following formula:

$$RI = \text{mean} \pm 2\ SD$$

3. Results

3.1. Selected antibodies showed no nonspecific binding

Antibody specificity was confirmed across all fluorochromes, with each antibody showing a significantly higher MFI than its corresponding isotype control (Fig. 1). Anti-CD45 staining resulted in a mean MFI of 19.5 (95% CI [17.4–21.7]) compared with 0.9 (95% CI [0.8–0.9]) for the isotype ($p < 0.0001$). Anti CD15 presented a mean MFI of 56.3 (95% CI [52.1–60.4]) versus 0.5 (95% CI [0.4–0.5]) for the isotype ($p < 0.0001$) and anti CD66b presented a mean MFI of 37.4 (95% CI [32.2–42.6]) compared with 0.8 (95% CI [0.7–0.8]) for the isotype ($p < 0.0001$). The raw data are provided in supplementary materials (Fig. S1).

3.2. Isolated neutrophils presented high quality

The quality assessment of the isolated neutrophils indicated overall high performance across all evaluated criteria (Figs. 2 and 3). Cell viability was consistently high, approaching the theoretical maximum, with a mean of 99.4% (SD =0.4; 95% CI [99.2–99.6]). The leukocyte purity of the preparations reached 99.3% (SD =0.4; 95% CI [99.1–99.6]), and neutrophil purity was similarly elevated, with a mean of 98.6% (SD = 0.5; 95% CI [98.4–98.8]).

3.3. CD66b as a marker of neutrophil activation and establishment of the RI for basal activation

Isolated neutrophils presented a mean CD66b MFI of 37.4 (SD =10.8; 95% CI [32.2–42.6]), This baseline level of CD66b confirmed the constitutive presence of this activation marker at the neutrophil surface.

CD66b expression increased significantly following TNF stimulation, reaching a mean MFI of 106.90 (95% CI [85.79–128.10]; $p < 0.0001$). This marked upregulation confirmed the expected increase in CD66b expression upon activation and supported that CD66b is expressed at low levels under basal conditions.

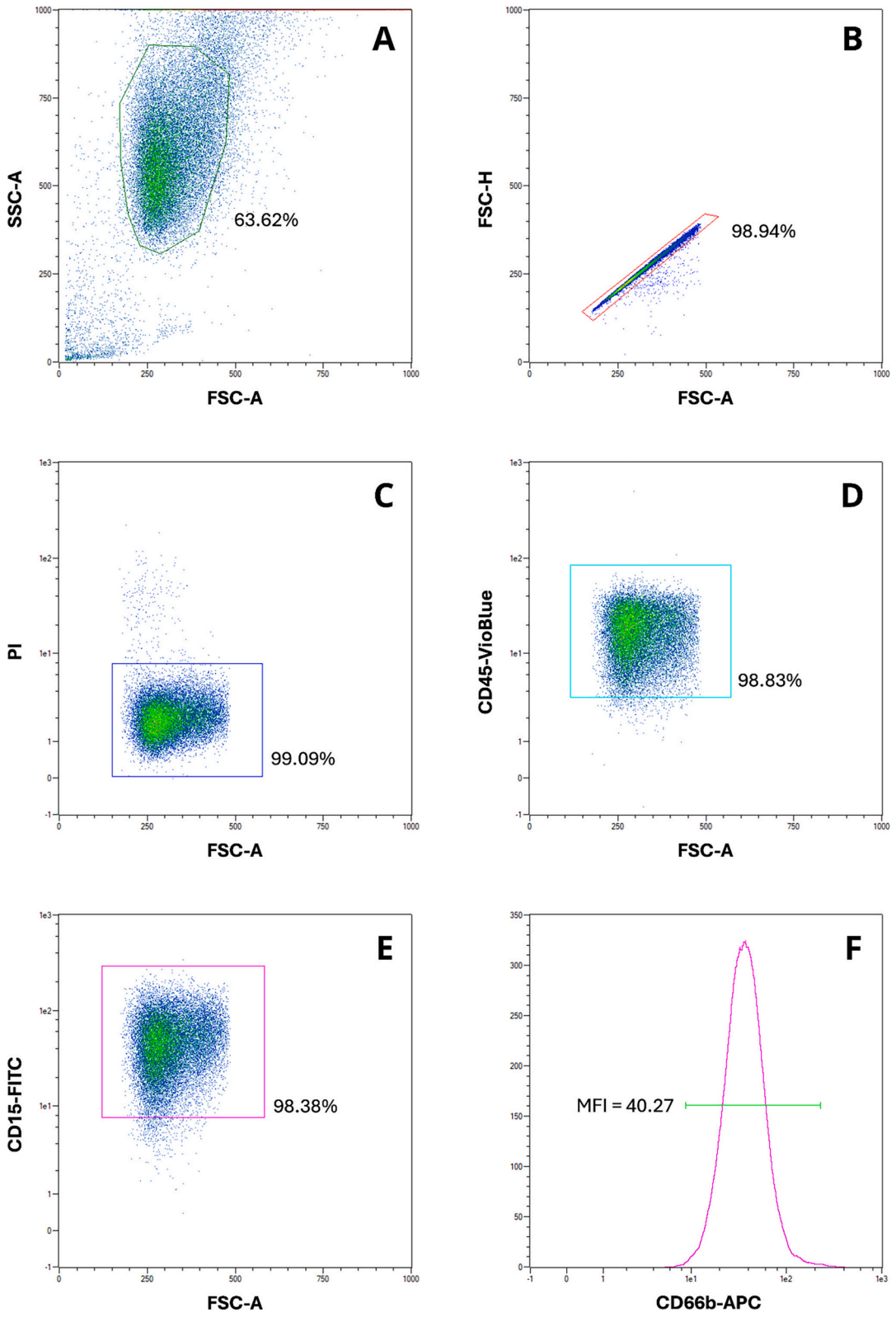

Fig. 2. Sequential gating strategy in a healthy volunteer. Gate A: Cells were first gated on FSC-A vs SSC-A to exclude debris and retain the main population based on cell size (FSC) And granularity (SSC). Gate B: Singlets were identified on FSC-H vs FSC-A by selecting events along the diagonal, allowing exclusion of doublets and aggregates. Gate C: Cell viability was assessed using PI; viable cells, which exclude the dye, show low PI signal, while dead cells with compromised membranes display high fluorescence and were excluded. Gate D: Leukocytes were identified using CD45-VioBlue; $CD45^{+}$ cells showed a strong fluorescence signal and were selected. Gate E: Neutrophils were identified using CD15-FITC; $CD15^{+}$ cells showed a strong fluorescence signal and were selected. Gate F: Neutrophil activation was assessed with CD66b-APC; the histogram shows the distribution of fluorescence intensity, and the MFI was calculated as the average signal of the gated neutrophil population. *Abbreviations: APC: Allophycocyanin; FITC: Fluorescein Isothiocyanate; FSC-A: Forward Scatter Area; FSC-H: Forward Scatter Height; MFI: Mean Fluorescence Intensity; PI: Propidium Iodide; SSC-A: Side Scatter Area.*

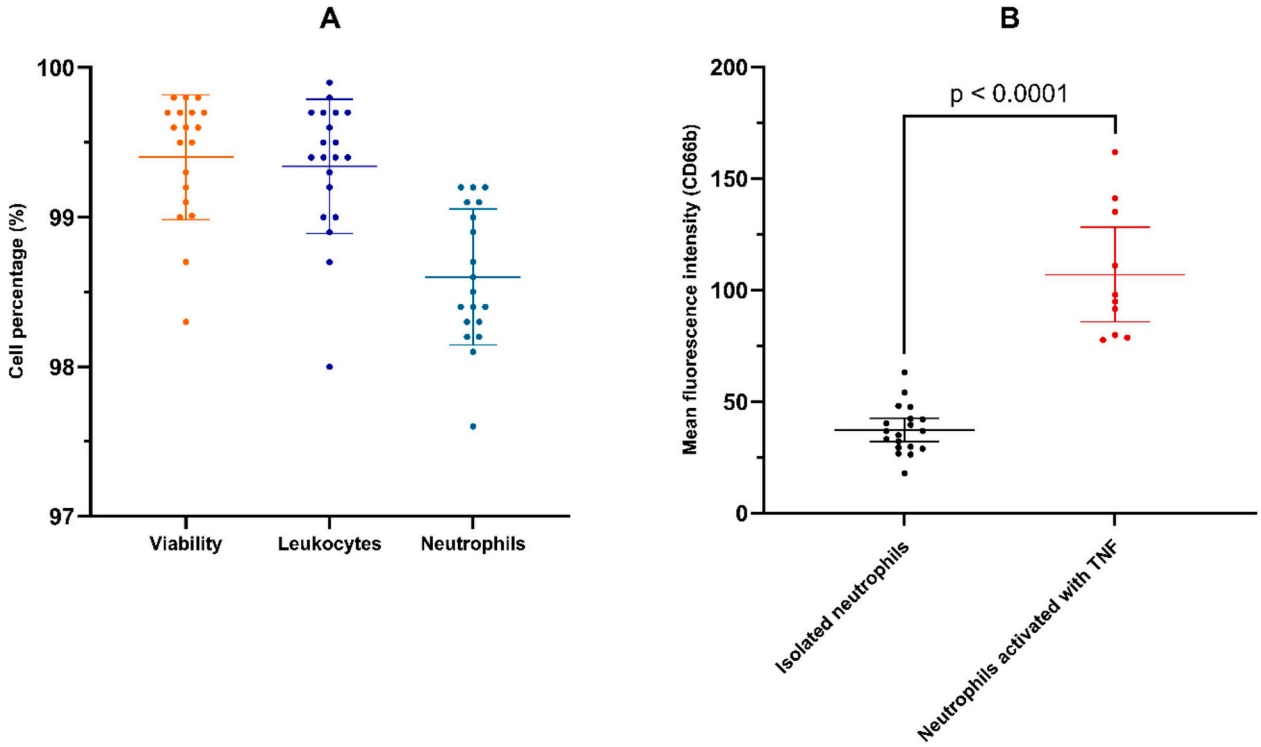

Fig. 3. Assessment of cell viability, leukocyte purity, neutrophil purity, basal activation and TNF-induced activation. Panel A shows the percentage of viable cells (n = 19; one outlier excluded), leukocytes (n = 20), and neutrophils (n = 19; one outlier excluded) obtained after isolation, presented as mean ± SD. Panel B shows the MFI values of CD66b with 95% CI for isolated neutrophils (n = 19; one outlier excluded) compared with neutrophils activated with TNF (n = 10, final concentration 0.5 μg/mL), demonstrating a significant increase in CD66b expression following activation ($p < 0.0001$). *Abbreviations: CI: Confidence Interval; MFI: Mean Fluorescence Intensity; SD: Standard Deviation; TNF: Tumor Necrosis Factor.*

For the calculation of the RI, one outlier (MFI = 80.7) was identified and excluded. After exclusion, CD66b MFI values followed a normal distribution (p = 0.78), allowing the use of the CLSI recommended parametric approach. The RI was therefore estimated at 37.4 ± 21.6, providing a quantitative threshold for assessing the acceptability of neutrophil suspensions.

4. Discussion

Flow cytometry provides a robust and sensitive approach to evaluating the quality of isolated neutrophils. It allowed simultaneous assessment of viability, purity, and basal activation using well-established markers such as CD45, CD15, and CD66b. Our results are consistent with what has been reported in the literature. Kremer et al. (Kremer et al., 2023) demonstrated that neutrophil isolation using the same Miltenyi kit yields a viability above 99.5% and a neutrophil purity of 97.91% ± 1.06% (SD). Karawajczyk et al. (Karawajczyk et al., 2021) reported a mean CD66b MFI of 34.02 (SD = 2.2) in 23 healthy volunteers.

Establishing a RI for basal neutrophil activation adds substantial value to the evaluation of cell quality. To our knowledge, no RI has previously been defined in the literature, even though isolated neutrophils are used in various functional assays, including NETosis induction, reactive oxygen species generation, degranulation, and phagocytosis assays. Different neutrophil isolation methods yield distinct quality profiles, which can influence neutrophil activation and responsiveness and, consequently, lead to variability in functional test results. (Kremer et al., 2023). MFI CD66b values exceeding this range may reflect procedure induced activation and impact functional responses. Such deviations could lead to experimental bias, misinterpretation of treatment effects, or reduced reproducibility across studies. Therefore, applying the RI as an acceptability criterion strengthens the reliability and comparability of functional assays involving neutrophils.

However, this study has some limitations. The RI we established for basal CD66b expression was derived exclusively from neutrophils isolated using the Miltenyi immunomagnetic kit, and its applicability may therefore be limited to this specific isolation method. Other commercial kits or alternative techniques may induce different degrees of handling-related activation, which could modify basal CD66b levels and compromise the use of this interval in those contexts. In addition, this study did not evaluate the effect of extreme preanalytical conditions such as prolonged processing delays, stronger or longer centrifugation, etc. which could also alter CD66b expression. Further investigations incorporating these factors are needed to confirm the robustness of the proposed RI. Finally, future studies should also investigate the relationship between basal CD66b expression and complementary neutrophil activation assays, such as the DHR oxidative burst assay, to further evaluate the biological relevance and robustness of CD66b as a quality assessment marker for isolated neutrophils.

5. Conclusion

Isolation of neutrophils using immunomagnetic kit yields cell preparations with high viability, high purity, and a preserved basal activation state. Based on these high-quality preparations, a RI for CD66b MFI was established at 37.4 ± 21.6, providing a practical indicator to determine whether isolated neutrophils remain within an acceptable range for functional assays. Although this interval is likely laboratory and kit dependent, it offers a useful first step toward improving consistency and reliability in studies relying on freshly isolated neutrophils.

CRediT authorship contribution statement

Hachem Bouarroudj: Writing – original draft, Software,

Methodology, Formal analysis, Conceptualization. **Constant Gillot:** Writing – review & editing, Methodology, Conceptualization. **Jonathan Decarpentrie:** Writing – review & editing, Methodology, Conceptualization. **François Mullier:** Writing – review & editing. **Jonathan Douxfils:** Writing – review & editing, Supervision.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2026.114104.

Data availability

Data will be made available on request.

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