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Research article

Clustering analyses of murine bone marrow-derived neutrophils reveal a phenotypic heterogeneity that can respond differentially to stimulation

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A B S T R A C T

Neutrophils are granulocytic cells produced in the bone marrow from a granulocytic progenitor cell. During infection, the production of chemokines and cytokines induces the recruitment of neutrophils to the infected tissue to promote the clearance of microbial pathogens. Several studies have shown that different subpopulations of neutrophils can be identified during infection. However, no previous studies evaluated subpopulations of neutrophils purified from the bone marrow (BM), which are typically used to study the biology of these cells based on the assumption that the neutrophil population is homogeneous. In the present study, responses of purified BM-derived neutrophils to various stimuli such as PMA, LPS, and *Streptococcus pneumoniae* were evaluated using flow cytometry and bh-SNE analyses. Further, neutrophil population heterogeneity was assessed by clustering analyses. Our data suggest that purified BM-derived neutrophils were not a homogeneous cell population and were clustered into 12 subsets, each displaying a unique marker profile, where CD11b and CD62L emerged as pivotal markers for neutrophil function. Importantly, the subsets responded differentially to each stimulus, suggesting a nuanced activation pattern. Changes in biomarker expression were analyzed via Ingenuity Pathway Analysis (IPA) to unravel functional implications of the identified clusters, revealing subsets associated with different neutrophil functions, such as "Migration of neutrophils" or "Phagocytosis in neutrophils". This study contributes to understanding the diversity of purified BM-derived neutrophils and the implications of using these cellular preparations to raise conclusions about the functionality of these cells in various infection models.

1. Introduction

Neutrophils are innate granulocytic short-life cells produced in the bone marrow from a granulocytic progenitor cell [1]. They are critical innate cells that detect and destroy pathogenic microbes and tumoral cells, also having an important pathological role during autoimmune diseases [1]. During infection, chemokines and cytokines released locally by injured tissues recruit neutrophils from the bone marrow and bloodstream to infected tissues [1,2]. Neutrophil recruitment to infected tissues involves the extravasation from the

Abbreviations: bh-SNE, Barnes-hut distributed stochastic neighbor embedding; BM, bone marrow; EMGM, EM algorithm for Gaussian mixtures; IL-6, Interleukin-6; IPA, Ingenuity Pathway Analysis; LPS, Lipopolysaccharide; PMA, phorbol 12-Myristate 13-Acetate-dependent; PBS, Phosphate buffer saline; PCA, Principal component analysis; Spn, *Streptococcus pneumoniae* D39.

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endothelium, a process that is mediated by the interaction of molecules expressed on the surface of endothelial cells with selectins expressed in the neutrophil membrane, such as L-selectin and integrins like CD11b [1,2,3]. Following extravasation, neutrophils recognize the pathogen microorganism and employ a vast arsenal of mechanisms to clear infecting microbes, including phagocytosis, the release of the protein content stored in intracellular granules known as degranulation, and the release of DNA extracellular traps (NETs) composed by chromatin embedded with histones and antimicrobial proteins, a process known as NETosis [1].

Neutrophils develop in the bone marrow and are later released to the bloodstream at different maturation states. Although classical neutrophils are described as pro-inflammatory cells, neutrophils with other phenotypes have been widely identified and, up to date, different neutrophil subsets have been described in mice and humans in normal and pathological conditions, being well established that neutrophils are indeed highly heterogeneous cells [4–9]. In humans, neutrophils CD62L^{dim} have been related to suppressive function because of their difficulty responding to lipopolysaccharide (LPS) and because their numbers increase in breast cancer tissues, probably preventing inflammation [10–12]. In other studies, a subset of neutrophils that express olfatoctomedin4 (OLFM4) have been described and associated with worse outcomes of sepsis in a context where their presence is possibly related to NET formation [13–15]. Granulocytic myeloid-derived suppressor cells (G-MDSC) are immunosuppressive cells that have been considered another neutrophil subpopulation since they are morphologically identical and present the same surface markers as classical neutrophils. However, these cells can express different molecules, such as arginase-1, inducible nitric oxide synthetase (iNOS), and IL-10, that inhibit the action of other immune cells [16,17]. Phenotypically, neutrophil heterogeneity has also been defined by unique transcriptomic profiles characteristic of different stages of neutrophil development [18,19] which is consistent with neutrophil heterogeneity at a functional level.

In vitro and in vivo studies have contributed to the understanding and dissection of interactions between neutrophils and microbes during health and disease and the mechanisms used by neutrophils involved in host defense during infections. However, due to the lack of immortalized cell lines that fully recapitulate neutrophil biology and physiology, in vitro studies of neutrophil biology involve isolating primary cells from the bloodstream in humans and the bone marrow or the peritoneal cavity of mice. The use of mouse bone marrow-isolated neutrophils is a gold standard methodology to study neutrophil biology in vitro due to the possibility of isolating fully mature neutrophils that have not undergone activation processes [20–22]. Neutrophil isolation from bone marrow progenitors through biotinylated antibody-mediated separation (negative selection) usually renders a purity between 95 and 98 % [23] and results in a population of cells characterized by high expression levels of the neutrophil-specific surface markers CD11b and Ly6G [23–25], which in theory constitutes a homogeneous population of mature classical neutrophils.

In the present study, we studied the heterogeneity of BM-neutrophils isolated through biotinylated antibody separation and their response after being stimulated with phosphate-buffered saline (PBS), phorbol 12-Myristate 13-Acetate-dependent (PMA), bacterial lipopolysaccharide (LPS), and *Streptococcus pneumoniae* D39 strain (Spn). After stimulation, the expression patterns of CD11b, Ly6G, CD44, CD64, CD62L, and CD16/32 were evaluated by flow cytometry and Barnes-Hut-Stochastic Neighbor Embedding (bh-SNE), in addition to unbiased EM algorithm for the Gaussian mixtures (EMGM) cluster analyses. These analyses show that bone marrow isolated neutrophils are also phenotypically heterogeneous cells that differentially respond against specific stimuli.

2. Materials and methods

2.1. Ethics statement

Mouse handling and experimental protocols were approved by the Scientific Ethics Committee (Animal and Environment Care and Research Biosafety) of the Pontificia Universidad Católica de Chile (identification number 210820005).

2.2. Primary bone marrow neutrophil isolation

Bone marrow was harvested from femurs of 6–8 weeks old male C57BL/6 mice. Then, cells were centrifuged at 1500 rpm for 10 min and resuspended in ACK buffer for 5 min to eliminate red blood cells. Finally, cells were resuspended in 500 L of PBS containing 0.5 % bovine serum albumin (BSA) and 2 mM EDTA. Neutrophils were isolated using a Neutrophil isolation kit, mouse (Miltenyi Biotech), and LS-column (Miltenyi Biotech) according to the manufacturer's instructions.

2.3. Bacterial growth and dose preparation

Streptococcus pneumoniae strain D39 (Culture collections, UK Health Security Agency) was grown in Todd Hewitt broth (Sigma) supplemented with 0.5 % yeast extract (Sigma) at 37 °C, 5 % CO_2 without agitation until OD_{600} 0.5, and frozen at −80 °C in the same medium with 10 % glycerol until the day of the experiment. Next, bacteria were thawed and diluted to reach the desired concentration.

2.4. Neutrophil stimulation

Isolated neutrophils were resuspended in HBSS medium (Gibco ref. 14025–076) with 0.1 % of fish gelatin (Sigma) at a concentration of 2.5×10^5 cells/mL. Then, 2.5×10^5 cells were treated with vehicle (PBS), 100 nM of phorbol 12-myristate 13-acetate (PMA) (Sigma), 100 ng/mL of LPS from *Escherichia coli* (Sigma), and *Streptococcus pneumoniae* strain D39 (Spn, Multiplicity of Infection MOI = 1) for 1 h in 2 mL tubes at 37 °C with 5 % CO_2 in rotation. After 1 h, cells were centrifuged (1400 rpm at 4 °C), resuspended in PBS, and stained for flow cytometry.

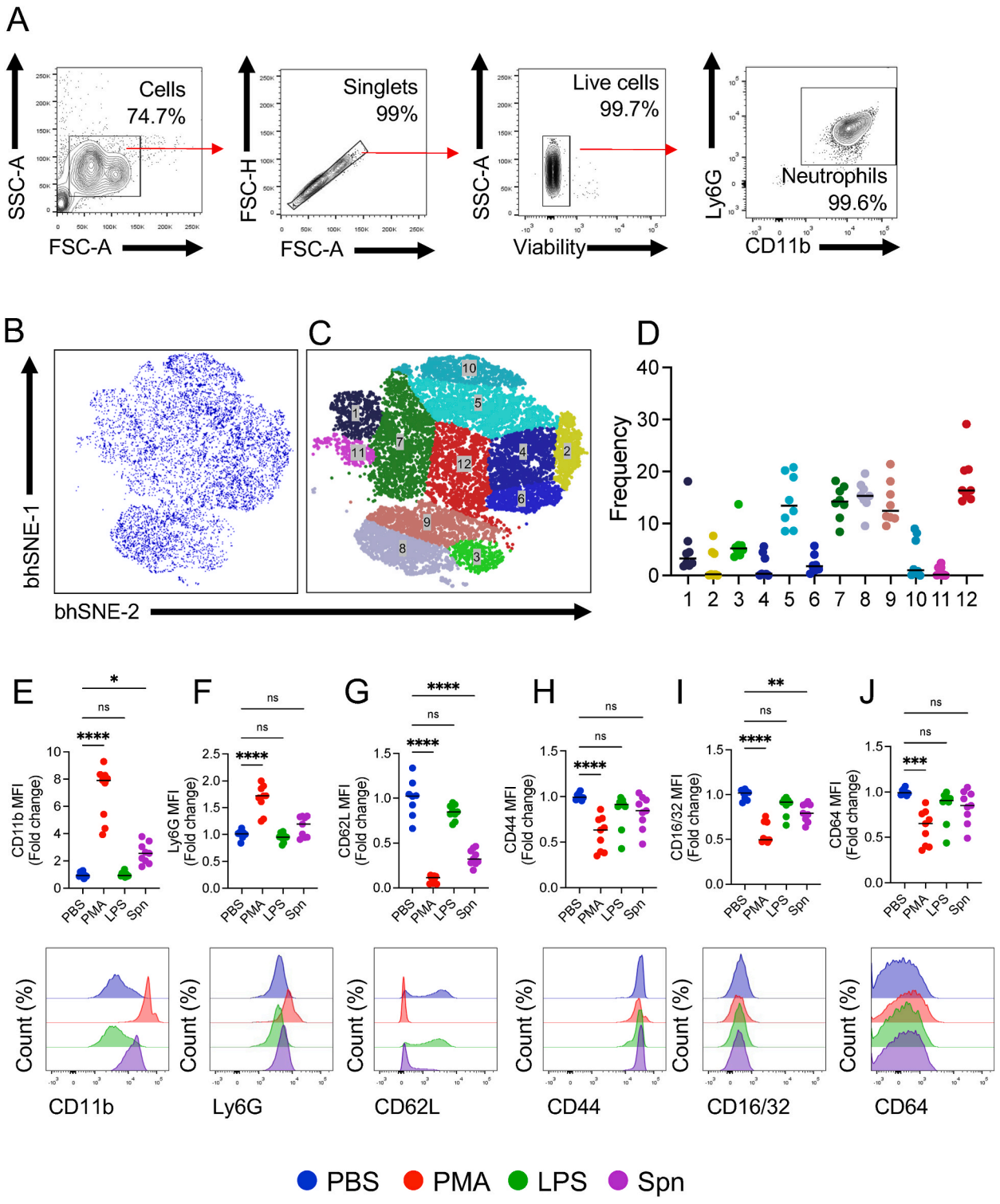

Fig. 1. Bone marrow isolated neutrophils change the expression of membrane markers in response to activation stimulus. 2.5×10^5 cells were treated with vehicle (PBS 1X), PMA 100 nM, LPS from *Escherichia coli* 100 ng/mL and *Streptococcus pneumoniae* strain D39 (MOI = 1) for 1 h. (A) Gating strategy of purified neutrophils. (B) bh-SNE distribution of gated neutrophils after PBS treatment. (C) Clustering analysis of total data obtained from bh-SNE. (D) Frequency of neutrophil clusters. (E–J) Relative expression of MFI (Geometric mean) and histograms of CD11b, Ly6G, CD62L, CD44, CD16/32, and CD64 in neutrophils after PBS, PMA, LPS and Spn treatment. One-way ANOVA followed by Sidak's post-test was performed to analyze the data of 3 independent experiments with 3 replicates each.

2.5. Raw 264.7 cells treatment

Raw 264.7 cells were seeded in a 24-well plate at a concentration of 2.5×10^5 cell/mL. Then, cells were treated with vehicle (PBS), 100 ng/mL, or 1000 ng/mL of LPS from *Escherichia coli* (Sigma) for 1 h or 24 h. Supernatants were recovered, and interleukin-6 (IL-6) was measured using an ELISA kit (BD Biosciences ref. 555240) according to manufacturer instructions. ELISA plates were read in an 800 TS microplate reader (Biotek) at 540 nm.

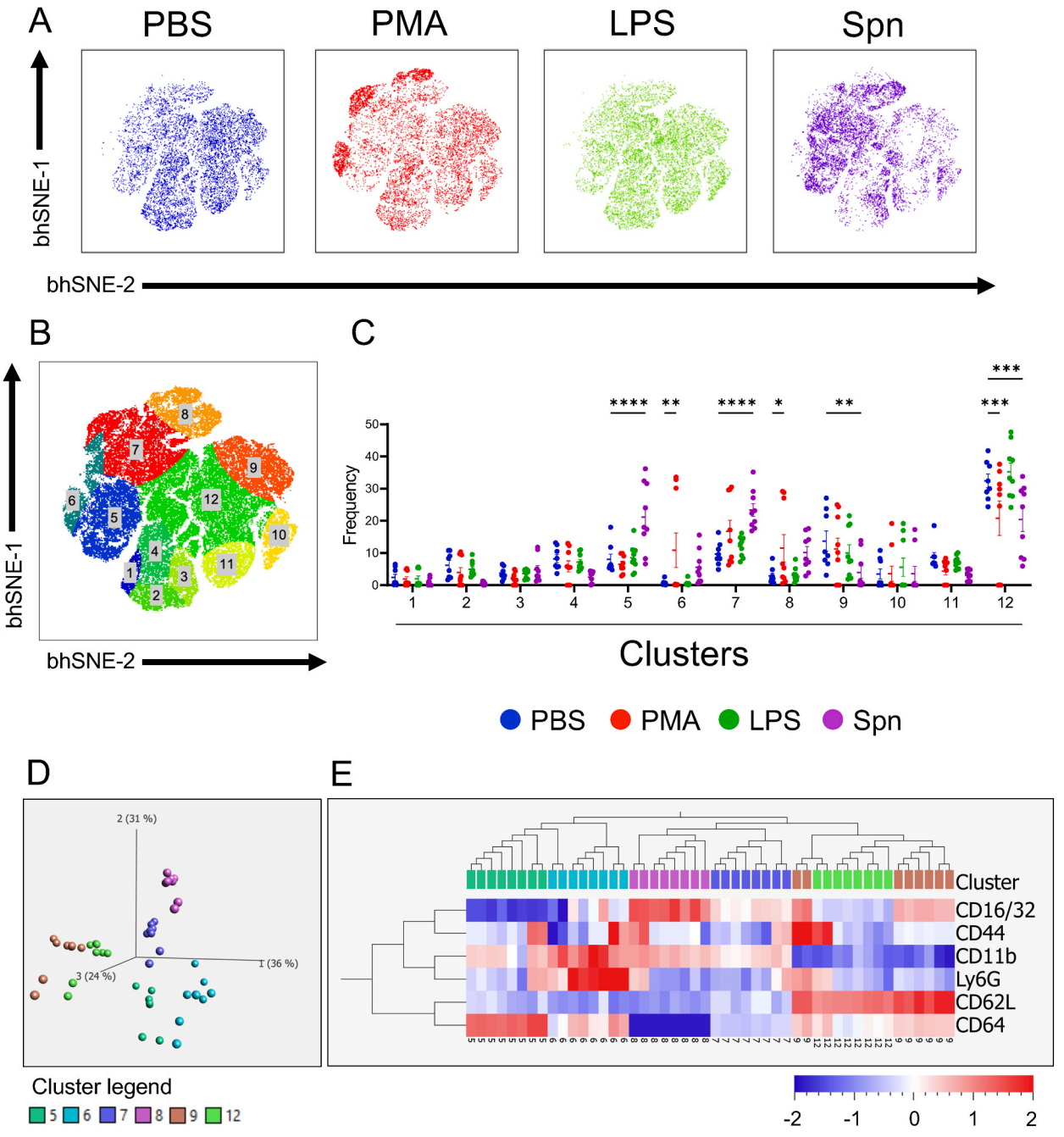

Fig. 2. Clustering analysis of MFI shows high heterogeneity in purified neutrophils with and without stimulus. Barnes-hut distributed stochastic neighbor embedding (bh-SNE) was performed in the gated neutrophil population, followed by clustering analysis. (A) bh-SNE distribution of gated neutrophils according to treatment. (B) Clustering analysis of total data obtained from bh-SNE. (C) Frequency of neutrophil clusters. (D–E) PCA plot and heatmap of clusters 5, 6, 7, 8, 9, and 12 were done according to the data of normalized MFI from PBS-treated neutrophils in Qlucore Omics Explorer software.

2.6. Flow cytometry analyses

Neutrophils were resuspended in PBS with viability dye BV510 1:1000 (BD Biosciences) for 30 min at 4 °C, then cells were washed 3 times with PBS and resuspended in staining buffer (PBS 2 mM EDTA 0.5 % BSA) with anti-CD11b APC-Cy7 (BD Biosciences, clone: M1/70) (1:1000), anti-Ly6G AF700 (BioLegend, clone: 1A8) (1:1000), anti-CD62L PE (BD Biosciences, clone: MEL-14) (1:1000), anti-CD16/32 PerCP cy5.5 (1:1000), anti-CD64 AF647 (1: 1000) and anti-CD44 BV605 (BD biosciences clone IM7) (1:1000) for 30 min at 4 °C. Then, cells were washed 3 times with PBS and fixed in PFA 2 % for 15 min at 4 °C. Finally, cells were washed 3 times with PBS and resuspended in 150 μL of PBS. Data was acquired in an LSRFortessa X2-0 cytometer (BD Biosciences) and analyzed using FlowJo v10.0.7 software (BD Biosciences). $CD11b^{+}Ly6G^{+}$ cells were considered as neutrophils.

2.7. Barnes-hut distributed stochastic neighbor embedding (bh-SNE) analyses

Gated neutrophil data acquired in Flowjo were exported as CSV files. Obtained CSV files were used to create new FSC files to generate Barnes-hut distributed stochastic neighbor embedding (bh-SNE) analyses in Cyt (SightOf) version 2.0 [26] run in MatLab R2022b. For bh-SNE analyses, samples were subsampled to 2000 events, and bh-SNE analyses were completed considering FSC-A, SSC-A, CD11b, Ly6G, CD62L, CD16/32, CD64 and CD44. Clustering analyses (12 clusters) were completed in Cyt using the EMGM algorithm. Samples were concatenated by stimulus and exported to a new FSC file to be individually analyzed in Flowjo (10.9.0).

2.8. Prediction of neutrophil functions

The list of biomarkers (CD11b, CD62L, Ly6G, CD44, CD16, CD32, and CD64) and the log2ratio values calculated from their changes in expression between clusters of PBS-treated neutrophils and stimulated neutrophils were exported to IPA bioinformatic software (QIAGEN) to build networks where each node is a biomarker. The relationships are represented as arrows and edges. Networks were grown based on publications associated with "neutrophil functions", where predictions for the activity of these functions were performed according to the data showed in Principal Components Analysis (PCA) and heatmap (Fig. 2D and E), where PCA plot showed a high relevance of CD62L and CD11b. For the prediction, clusters 9 and 12 were grouped as they exhibit similar CD62L and CD11b patterns, as indicated by the heatmap. The same grouping approach was applied to clusters 5, 6, 7, and 8.

2.9. Statistical analyses

One-way ANOVA followed by Sidak's post-test was performed to compare the normalized MFI of markers. Two-way ANOVA followed by Dunnett's post-test was conducted to compare the frequency of clusters per treatment and the normalized MFI of markers in the clusters per treatment. All comparisons were performed in GRAPHPAD PRISM V10.0.2 software for Macintosh. PCA plot and marker expression heatmap per cluster were created using Normalized MFI data from bh-SNE in Qlucore Omics Explorer software version 3.9.11.

3. Results and discussion

Murine BM-neutrophils were isolated through magnetic negative selection, reaching an average viability of 97.2 % percent and an average purity of 98.4 % (Fig. 1A, Supplementary Fig. 1), and the expression of CD11b, Ly6G, CD62L, CD16/32, CD64 and CD44 was measured by flow cytometry. Through bh-SNE analysis, we evaluated the distribution of BM-neutrophils treated with PBS depending upon the expression of membrane markers (Fig. 1B), where our data shows that these neutrophils belong to a highly heterogeneous population even when cells were purified from BM by magnetic beads. To further characterize them, clustering analyses were performed using the EMGM algorithm, where cells were grouped in 12 clusters (Fig. 1C) with a cell frequency specific for each cluster (Fig. 1D). This frequency pattern is probably related to neutrophil functionality in vivo and could change in response to stimulus, as seen in other studies [27].

Then, we evaluated the surface expression of membrane markers in purified neutrophils stimulated for 1 h with PMA (100 nM), LPS (100 ng/mL), and Spn (MOI = 1). First, we evaluated the surface expression of CD11b, which is a beta-integrin constitutively expressed in the surface of neutrophils that are involved in cell migration and endothelium adhesion and overexpressed in the surface of neutrophils following degranulation of tertiary granules [3]. As expected, PMA and Spn stimulation increased CD11b expression in isolated neutrophils, but no changes in surface CD11b expression were observed in response to LPS (Fig. 1E). We also evaluated Ly6G, a surface protein related to maturity or neutrophil migration [28]. We observed that Ly6G levels increased following stimulation with PMA but not in response to infection with Spn or LPS (Fig. 1F). In the case of CD62L (also known as L-selectin), a glycoprotein related to the attachment of neutrophils to epithelial cells [29], our data shows that its expression decreased in response to PMA and Spn (Fig. 1G). Interestingly, some studies have suggested that activated neutrophils shed CD62L, probably to reduce their aggregation in affected tissues [29,30]. In contrast, CD44, another glycoprotein related to the attachment of neutrophils to epithelial [31] decreased its expression only after PMA stimulation (Fig. 1H). We also evaluated CD64 and CD16/32, also known as Fc-gamma receptors I and II/III, respectively, because these receptors can elicit neutrophil recruitment and mediate the phagocytosis of opsonized microbes [32]. In our analyses, CD16/32 expression significantly decreased in response to PMA and Spn (Fig. 1I), while CD64 expression decreased exclusively after PMA stimulation (Fig. 1J). Previous studies showed that in vitro activation of human neutrophils with cryoglobulin complexes increased the expression of Fc-gamma receptor I on the surface, and at the same time, it decreased the surface expression of

Fc-gamma receptors II and III [33]. On the other hand, other studies showed that CD64 expression in neutrophils is used as an infection biomarker in peripheral blood and other biological fluids [34,35]. These responses are probably due to tissue-specific expression on the neutrophil membrane, but more studies are required to evaluate this hypothesis. Interestingly, our data shows that LPS did not affect the expression of each marker analyzed in BM-neutrophils. Therefore, we speculated that our experimental setting (100 ng/mL and 1 h of treatment) may not be enough to induce detectable changes in the expression of the analyzed markers. To evaluate this hypothesis, we treated Raw 264.7 cells, a murine macrophage cell line, with PBS, LPS 100 ng/mL, and 1000 ng/mL for 1 and 24 h, and we evaluated the levels of IL-6 in the supernatant by ELISA. Our data shows that after 1 h of treatment with LPS, the levels of IL-6 were similar to those of the PBS-treated cells, but these levels increased significantly after 24 h of treatment (Supplementary Fig. 1C). This data suggests that 1 h of treatment with LPS (100 ng/mL) is insufficient to induce robust myeloid cell activation in vitro.

Clustering analyses were performed using the EMGM algorithm to characterize the heterogeneous response of neutrophils against different stimuli. Stimulated BM-Neutrophils were grouped in 12 clusters (Fig. 2A and B). While BM-neutrophil distribution in LPS-stimulated cells was highly similar to PBS-treated neutrophils, significant differences in neutrophil distribution were observed in PMA- and Spn-stimulated BM-neutrophils (Fig. 2A). Frequency analyses, used to characterize the specific response of BM-neutrophils

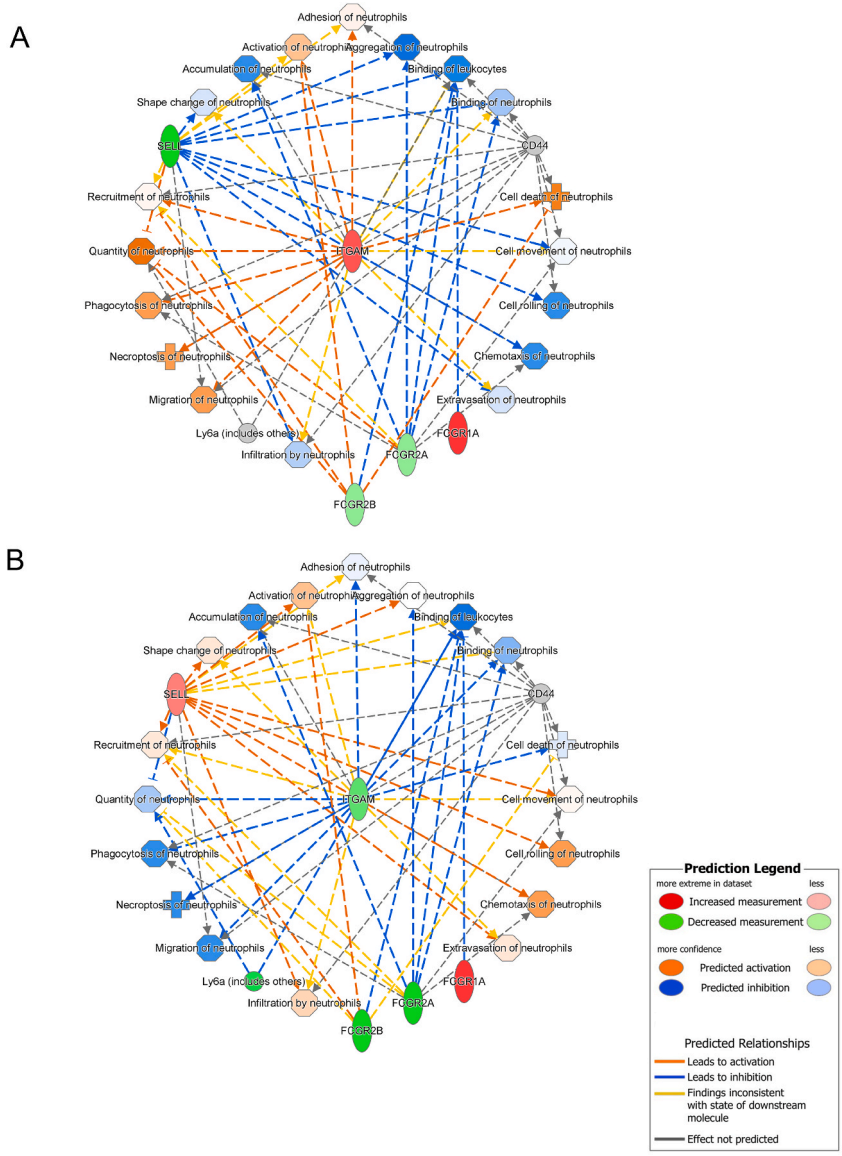

Fig. 3. Neutrophil marker patterns are related to specific neutrophil functions. (A–B) Neutrophil function network prediction via IPA software (QIAGEN) using biomarkers expression from Flow Cytometry assay. considering high CD11b and low CD62L expression (A), and low CD11b and high CD62L expression (B). The following name equivalents are drawn in the network. ITGAM: CD11b, SELL: CD62L, Ly6a (includes others): Ly6G, FCGR1A: CD64, FCGR2A: CD16 and FCGR2B: CD32. The meaning of colored symbols and lines is depicted in the insert.

against PMA and Spn, showed that clusters 5 and 7 significantly increased their frequency in response to Spn. In contrast, clusters 6 and 8 increased their frequencies only after PMA stimulation (Fig. 2C). Additionally, whereas the frequency of cluster 9 decreased following stimulation with Spn (Fig. 2C), cluster 12 showed a reduction in frequency following stimulation with both PMA and Spn (Fig. 2C).

A PCA plot was applied to neutrophil clusters that significantly changed their frequency after treatment with each stimulus (5, 6, 7, 8, 9, and 12). As expected, neutrophils from the same cluster grouped, indicating equivalent expression marker dynamics. Moreover, the PCA analysis showed that 3 components explain the 91 % of the variation in the plot, which are CD62L (36 %), CD64 (31 %), and CD11b (24 %), while the rest of the variation is explained by CD16/32 (6 %), Ly6G (2 %) and CD44 (1 %) (Fig. 2D). Therefore, CD62L, CD64, and CD11b are mainly responsible for the differences between these clusters, but more importantly, these markers might be related to specific functions within the identified clusters. Similarly, a heatmap followed by a hierarchical order was performed to visualize and compare the patterns of marker expression in the evaluated clusters (Fig. 2E). Interestingly, CD62L and CD11b expression patterns can be detected in two clusters. CD62L is increased in clusters 9 and 12 but decreased in clusters 5 to 8, and the opposite occurs once comparing CD11b (Fig. 2E). In addition, clusters did not alter marker expression in response to the stimulus, except for an increased CD11b expression in clusters 5, 8, 9, and 12 upon exposure to Spn (Supplementary Figs. 2A–B). This data suggests that each cluster of neutrophils may differentially respond to a specific stimulus, where a specific stimulus (here, Spn or PMA) promotes the enrichment of a specific cluster. Given the neutrophil frequency and the expression patterns of the analyzed markers, we hypothesize that clusters 5, 6, 7, 8, 9, and 12 represent active neutrophil subsets among total BM-neutrophils accomplishing specific functions, and their proportions may undergo differential changes in response to Spn or PMA. Clusters 1, 2, 3, 4, 10, and 11 did not change their frequency with any stimulus, and it is unknown whether the unresponsive clusters have different undetected phenotypes during infection like anti-inflammatory or suppressive phenotypes described in other studies [24] or if they are activated in response to other pathogens or stimuli.

To characterize the potential function of identified clusters, we conducted a comprehensive pathway and network analysis to explore biological functions associated with the expression of analyzed surface neutrophil markers, employing the IPA software. IPA facilitates the visualization of experimental data, its connections to other pertinent molecules and biological functions, and the predictive activity of those derived from the original data measurements. The biomarkers measured through flow cytometry were seamlessly integrated into IPA, leading to the development of a network comprising neutrophil functions. As we found that patterns of CD11b (represented as ITGAM) and CD62L (Represented as SELL) can be responsible for clustering significance (Fig. 2D and E), we used these membrane markers as nucleation points for the classification of neutrophil functions (Fig. 3A and B). As shown in Fig. 1, neutrophils stimulated with PMA or Spn show high expression of CD11b and low expression of CD62L that is predicted to be related to an inflammatory phenotype with an increase of several functions including “Quantity of neutrophils”, “Phagocytosis in neutrophils” or “Migration of neutrophils” (Fig. 3A), where these functions could be associated with a pro-inflammatory phenotype. On the other hand, a hypothetical low expression of CD11b and high expression of CD62L is not related to an inflammatory response but with potential mobilization from the bloodstream to infected tissues, as “cell movement of neutrophils”, “chemotaxis in neutrophils”, “extravasation in neutrophils” and “cell rolling in neutrophils” pathways are increased (Fig. 3B). Ly6G (represented as Ly6a (including others) is not predicted to have a significant role in neutrophil function, possibly due to the unknown function of this protein in neutrophil response [28]. Also, expression of CD44, CD16/32 (FCGR2A & FCGR2B, respectively), and CD64 (FCGR1A) seem to be involved in specific effects of neutrophil functions such as “aggregation of neutrophils”, “binding of leukocytes”, and “accumulation of neutrophils”, which could be related to the fine regulation of these cells depending on the biological context [27,36].

Differential expression of Ly6G and CD62L have been found in mature and immature neutrophils in mice and humans respectively. Both markers are highly expressed in mature neutrophils in circulation, but less expressed in early released neutrophils during inflammatory processes [28,37,38]. In our dataset, we observed differential expression of CD62L among different clusters, these findings suggest a potential correlation between the described clusters and the maturity state of the neutrophils within each cluster. In other studies, a subpopulation of neutrophils has been described, where immature and mature groups of neutrophils are in circulation. The proportion of both populations changes during an inflammatory stimulus in a similar way as has been described here [27]. Therefore, an increase or decrease in the proportion of clusters could occur during an inflammatory process depending on the physiological conditions and requirements to increase the availability of mature and immature neutrophils.

Our data show the existence of high phenotypic and possibly functional heterogeneity in purified BM-neutrophils that differentially respond to stimuli. Even though neutrophils were initially thought to be phenotypically and functionally homogeneous cells, in the last two decades, several data have consistently shown that these cells are heterogeneous from phenotypic and functional standpoints [19,24,27,39–41]. In the specific context of infectious diseases, previous work from our lab has shown that different phenotypic subsets of neutrophils are found in the lungs of mice during acute pulmonary infection caused by Spn [24,39]. These cells were shown to have differences in size, granularity, activation potential, and IL-10 production in vivo. Moreover, differential subsets of neutrophils have been found in other diseases, including COVID-19, where suppressive neutrophils have been identified and associated with lymphopenia and severe disease [42]. This study analyzes the heterogeneity of a highly purified population of cells obtained through a routinary technique such as magnetic/antibody-mediated negative selection. It is also likely that precursor cells of neutrophils from bone marrow are absent in the analysis. Here, we are adding further data to demonstrate that even purified BM-derived neutrophils are highly heterogeneous cells, and this information should be taken into account when raising conclusions on pathological models studied in vitro.

4. Conclusion

Human and mouse neutrophils are heterogeneous cells with highly diverse phenotypes and functions [19]. Due to the difficulties in studying neutrophil biology in vitro, studies focused on identifying neutrophil subsets during inflammatory conditions in mice have been done mostly in vivo [39,40,43]. Our data shows that murine BM-neutrophils isolated by a common technique, such as magnetic/antibody-mediated negative selection, are not a homogeneous population but highly heterogeneous. More importantly, in the present report, we show that neutrophil subsets differentially change their proportions in response to specific stimuli. Future approaches designed to complement multiparametric flow cytometry data with high-throughput technologies, such as single-cell transcriptomics and others, constitute new opportunities to study whether the heterogeneity of neutrophils observed after magnetic negative selection in the bone marrow replicates in different tissues during infection, as well as other inflammatory disorders such as autoimmunity and cancer.

CRediT authorship contribution statement

Pedro H. Silva: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Hernán F. Peñaloza:** Writing – review & editing, Supervision, Formal analysis, Conceptualization. **José Cordero:** Writing – review & editing, Formal analysis. **Alexis M. Kalergis:** Writing – review & editing, Resources. **Nelson P. Barrera:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Susan M. Bueno:** Writing – review & editing, Funding acquisition, Conceptualization.

Data and code availability statement

Data will be made available on request. For requesting data, please write to the corresponding author.

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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.heliyon.2025.e42227.

References

[1] P.X. Liew, P. Kubes, The Neutrophil’s role during health and disease, Physiol. Rev. 99 (2) (2019), https://doi.org/10.1152/physrev.00012.2018.
[2] N. Borregaard, Neutrophils, from Marrow to Microbes, 2010, https://doi.org/10.1016/j.immuni.2010.11.011.
[3] G.D. Ross, Role of the Lectin Domain of Mac-1/CR3 (CD11b/CD18) in Regulating Intercellular Adhesion, 2002, https://doi.org/10.1385/IR:25:3:219.
[4] L. Grassi, et al., Dynamics of transcription regulation in human bone marrow myeloid differentiation to mature blood neutrophils, Cell Rep. 24 (10) (2018), https://doi.org/10.1016/j.celrep.2018.08.018.
[5] S.N. Clemmensen, et al., Olfactomedin 4 defines a subset of human neutrophils, J. Leukoc. Biol. 91 (3) (2011), https://doi.org/10.1189/jlb.0811417.
[6] G. Zhou, et al., CD177+ neutrophils suppress epithelial cell tumourigenesis in colitis-associated cancer and predict good prognosis in colorectal cancer, Carcinogenesis 39 (2) (2018), https://doi.org/10.1093/carcin/bgx142.
[7] S. Massena, et al., Identification and characterization of VEGF-A-responsive neutrophils expressing CD49d, VEGFR1, and CXCR4 in mice and humans, Blood 126 (17) (2015), https://doi.org/10.1182/blood-2015-03-631572.
[8] B. Becher, et al., High-dimensional analysis of the murine myeloid cell system, Nat. Immunol. 15 (12) (2014), https://doi.org/10.1038/ni.3006.
[9] K. Ella, R. Csépányi-Kömi, K. Káldi, Circadian regulation of human peripheral neutrophils, Brain Behav. Immun. 57 (2016), https://doi.org/10.1016/j.bbi.2016.04.016.
[10] T. Tak, et al., Human CD62Ldim neutrophils identified as a separate subset by proteome profiling and in vivo pulse-chase labeling, Blood 129 (26) (2017), https://doi.org/10.1182/blood-2016-07-727669.
[11] Z. Wang, et al., CD62Ldim neutrophils specifically migrate to the lung and participate in the formation of the pre-metastatic niche of breast cancer, Front. Oncol. 10 (2020), https://doi.org/10.3389/fonc.2020.540484.
[12] J. Valdés-Ferrada, et al., Peripheral blood classical monocytes and plasma interleukin 10 are associated to neoadjuvant chemotherapy response in breast cancer patients, Front. Immunol. 11 (2020), https://doi.org/10.3389/fimmu.2020.01413.
[13] M.N. Alder, J. Mallela, A.M. Opoka, P. Lahni, D.A. Hildeman, H.R. Wong, Olfactomedin 4 marks a subset of neutrophils in mice, Innate Immun. 25 (1) (2019), https://doi.org/10.1177/1753425918817611.

[14] A. Welin, et al., The human neutrophil subsets defined by the presence or absence of OLFM4 both transmigrate into tissue in vivo and give rise to distinct NETs in vitro, PLoS One 8 (7) (2013), https://doi.org/10.1371/journal.pone.0069575.
[15] S.N. Clemmensen, et al., Olfactomedin 4 defines a subset of human neutrophils, J. Leukoc. Biol. 91 (3) (2011), https://doi.org/10.1189/jlb.0811417.
[16] N. Tumino, et al., Granulocytic myeloid-derived suppressor cells increased in early phases of primary HIV infection depending on TRAIL plasma level. Journal of Acquired Immune Deficiency Syndromes, 2017, https://doi.org/10.1097/QAI.0000000000001283.
[17] Y. Zhang, et al., Granulocytic myeloid-derived suppressor cells inhibit T follicular helper cells during experimental Schistosoma japonicum infection, Parasites Vectors 14 (1) (2021), https://doi.org/10.1186/s13071-021-05006-8.
[18] M. Garley, E. Jabłońska, Heterogeneity Among Neutrophils, 2018, https://doi.org/10.1007/s00005-017-0476-4.
[19] C. Silvestre-Roig, Z.G. Fridlender, M. Glogauer, P. Scapini, Neutrophil Diversity in Health and Disease, 2019, https://doi.org/10.1016/j.it.2019.04.012.
[20] H.F. Peñaloza, D. Ahn, B.M. Schultz, A. Piña-Iturbe, L.A. González, S.M. Bueno, L-arginine enhances intracellular killing of carbapenem-resistant Klebsiella pneumoniae ST258 by murine neutrophils, Front. Cell. Infect. Microbiol. 10 (2020), https://doi.org/10.3389/fcimb.2020.571771.
[21] R.J. Lu, et al., Multi-omic profiling of primary mouse neutrophils predicts a pattern of sex- and age-related functional regulation, Nat Aging 1 (8) (2021), https://doi.org/10.1038/s43587-021-00086-8.
[22] C.J. McGill, R.J. Lu, B.A. Benayoun, Protocol for analysis of mouse neutrophil NETosis by flow cytometry, STAR Protoc 2 (4) (2021), https://doi.org/10.1016/j.xpro.2021.100948.
[23] M. Swamydas, Y. Luo, M.E. Dorf, M.S. Lionakis, Isolation of mouse neutrophils, Curr. Protoc. Im. 2015 (2015), https://doi.org/10.1002/0471142735.im0320s110.
[24] L.A. González, et al., Characterization of the anti-inflammatory capacity of IL-10-producing neutrophils in response to Streptococcus pneumoniae infection, Front. Immunol. 12 (2021), https://doi.org/10.3389/fimmu.2021.638917.
[25] J. Li, et al., TGFβ1+CCR5+ neutrophil subset increases in bone marrow and causes age-related osteoporosis in male mice, Nat. Commun. 14 (1) (2023), https://doi.org/10.1038/s41467-023-35801-z.
[26] E.A.D. Amir, et al., ViSNE enables visualization of high dimensional single-cell data and reveals phenotypic heterogeneity of leukemia, Nat. Biotechnol. 31 (6) (2013), https://doi.org/10.1038/nbt.2594.
[27] X. Xie, et al., Single-cell transcriptome profiling reveals neutrophil heterogeneity in homeostasis and infection, Nat. Immunol. 21 (9) (2020), https://doi.org/10.1038/s41590-020-0736-z.
[28] P.Y. Lee, J.-X. Wang, E. Parisini, C.C. Dascher, P.A. Nigrovic, Ly6 family proteins in neutrophil biology, J. Leukoc. Biol. 94 (4) (2013), https://doi.org/10.1189/jlb.0113014.
[29] A. Ivetic, H.L.H. Green, S.J. Hart, L-Selectin: A Major Regulator of Leukocyte Adhesion, Migration and Signaling, 2019, https://doi.org/10.3389/fimmu.2019.01068.
[30] M. Berg, S. James, Human neutrophils release the Leu-8 lymph node homing receptor during cell activation, Blood 76 (11) (1990), https://doi.org/10.1182/blood.v76.11.2381.bloodjournal76112381.
[31] Z. Hasan, K. Palani, M. Rahman, H. Thorlacius, Targeting CD44 expressed on neutrophils inhibits lung damage in abdominal sepsis, Shock 35 (6) (2011), https://doi.org/10.1097/SHK.0b013e3182144935.
[32] N. Tsuboi, K. Asano, M. Lauterbach, T.N. Mayadas, Human neutrophil fcγ receptors initiate and play specialized nonredundant roles in antibody-mediated inflammatory diseases, Immunity 28 (6) (2008), https://doi.org/10.1016/j.immuni.2008.04.013.
[33] M. Hundt, M. Zielinska-Skowronek, R.E. Schmidt, Fc Gamma Receptor Activation of Neutrophils in Cryoglobulin-Induced Leukocytoclastic Vasculitis, 1993, https://doi.org/10.1002/art.1780360715.
[34] E. González-López, et al., CD64 expression on neutrophils as a potential biomarker for bacterial infection in ascitic fluid of cirrhotic patients, Inf. Disp. 55 (9) (2023), https://doi.org/10.1080/23744235.2023.2223294.
[35] S. Li, et al., Neutrophil CD64 Expression as a Biomarker in the Early Diagnosis of Bacterial Infection: A Meta-Analysis, 2013, https://doi.org/10.1016/j.ijid.2012.07.017.
[36] M. Metzemaekers, M. Gouwy, P. Proost, Neutrophil Chemoattractant Receptors in Health and Disease: Double-Edged Swords, 2020, https://doi.org/10.1038/s41423-020-0412-0.
[37] S.H. Bongers, et al., Kinetics of neutrophil subsets in acute, subacute, and chronic inflammation, Front. Immunol. 12 (2021), https://doi.org/10.3389/fimmu.2021.674079.
[38] Y. Ito, F. Nakahara, Y. Kagoya, M. Kurokawa, CD62L expression level determines the cell fate of myeloid progenitors, Stem Cell Rep. 16 (12) (2021), https://doi.org/10.1016/j.stemcr.2021.10.012.
[39] H.F. Peñaloza, F.J. Salazar-Echegarai, S.M. Bueno, Interleukin 10 modulation of neutrophil subsets infiltrating lungs during Streptococcus pneumoniae infection, Biochem Biophys Rep 13 (2018), https://doi.org/10.1016/j.bbrep.2017.11.004.
[40] Z.G. Fridlender, et al., Polarization of tumor-associated neutrophil phenotype by TGF-β: 'N1' versus 'N2' TAN, Cancer Cell 16 (3) (2009), https://doi.org/10.1016/j.ccr.2009.06.017.
[41] P. Scapini, O. Marini, C. Tecchio, M.A. Cassatella, Human Neutrophils in the Saga of Cellular Heterogeneity: Insights and Open Questions, 2016, https://doi.org/10.1111/imr.12448.
[42] H.F. Peñaloza, J.S. Lee, P. Ray, Neutrophils and Lymphopenia, an Unknown axis in Severe COVID-19 Disease, 2021, https://doi.org/10.1371/journal.ppat.1009850.
[43] S. Takizawa, A. Murao, M. Ochani, M. Aziz, P. Wang, Frontline Science: extracellular CIRP generates a proinflammatory Ly6G+CD11bhi subset of low-density neutrophils in sepsis, J. Leukoc. Biol. 109 (6) (2021), https://doi.org/10.1002/JLB.3HI0620-416R.