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SHORT COMMUNICATION **OPEN**

Reducing neutrophil exposure to oxygen allows their basal state maintenance

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Abstract

Neutrophils are the most abundant circulating white blood cells and are the central players of the innate immune response. During their lifecycle, neutrophils mainly evolve under low oxygen conditions (0.1–4% O_2), to which they are well adapted. Neutrophils are atypical cells since they are highly glycolytic and susceptible to oxygen exposure, which induces their activation and death through mechanisms that remain currently elusive. Nevertheless, nearly all studies conducted on neutrophils are carried out under atmospheric oxygen (21%), corresponding to hyperoxia. Here, we investigated the impact of hyperoxia during neutrophil purification and culture on neutrophil viability, activation and cytosolic protein content. We demonstrate that neutrophil hyper-activation (CD62L shedding) is induced during culture under hyperoxic conditions (24 h), compared with neutrophils cultured under anoxic conditions. Spontaneous neutrophil extracellular trap (NET) formation is observed when neutrophils face hyperoxia during purification or culture. In addition, we show that maintaining neutrophils in autologous plasma is the preferred strategy to maintain their basal state. Our results show that manipulating neutrophils under hyperoxic conditions leads to the loss of 57 cytosolic proteins during purification, while it does not lead to an immediate impact on neutrophil activation ($CD11b^{high}$, $CD54^{high}$, $CD62L^{neg}$) or viability ($DAPI^{+}$). We identified two clusters of proteins belonging to cholesterol metabolism and to the complement and coagulation cascade pathways, which are highly susceptible to neutrophil oxygen exposure during neutrophil purification. In conclusion, protecting neutrophil from oxygen during their purification and culture is recommended to avoid activation and to prevent the alteration of cytosolic protein composition.

INTRODUCTION

Polymorphonuclear neutrophils (neutrophils, PMNs) are the most abundant leukocytes in the peripheral circulation ($2.5–7.5 \times 10^9$ PMNs L^{-1}). Neutrophils are atypical and fully differentiated cells, with a polylobed nucleus (3–5 lobes in human neutrophils), a granule-rich cytosol and a low mitochondrial content, producing most of their energy using glycolysis.[1] Neutrophils differentiate from hematopoietic stem cells (HSCs, $CD34^{+}$) in the bone marrow, in a hypoxic compartment (1.3–4.1% O_2).[2] Under basal conditions, 10% of the mature bone marrow

neutrophils are released into the plasma fraction of the blood, containing low amounts of dissolved oxygen ($\sim$0.1–1.2%).[3,4] In conclusion, neutrophils are adapted to low oxygen concentrations in homeostasis; they face more elevated oxygen tensions only while transmigrating into perfused tissues during inflammation or infection processes. Under basal conditions, tissues contain < 10% O_2 (reviewed by Carreau and colleagues),[5] whereas during inflammation and infection the tissues become hypoxic with < 1% O_2 (inflammatory hypoxia and infectious hypoxia).[6,7]

The impact of hypoxia on neutrophils has been well documented.[8,9] Moreover, our group and others have demonstrated that anoxic conditions (0% O_2) increase neutrophil viability,[10,11] although in these studies neutrophils were transiently exposed to atmospheric oxygen during purification prior to culture under controlled oxygen conditions. Conversely, the molecular mechanism mediating the deleterious impact of hyperoxia, corresponding to atmospheric oxygen level (21% O_2), on neutrophil physiology remains unclear.

In this report, we investigated the impact of hyperoxia (21%) during neutrophil purification and culture on their viability and activation, demonstrating the importance of protecting neutrophils from oxygen exposure for the maintenance of their basal state.

RESULTS AND DISCUSSION

Modulation of neutrophil viability upon oxygen exposure during purification

We have previously demonstrated that neutrophil viability is maintained upon subsequent culture under anoxic conditions after neutrophil purification under hyperoxia (21% O_2).[11] We therefore hypothesized that avoiding any exposure to oxygen during the purification and manipulation of neutrophils may extend the maintenance of their basal state in terms of viability and activation. Thus, an innovative method was developed consisting of purifying neutrophils from peripheral human blood samples under oxygen-free conditions (anoxia, 0% O_2).

Upon reception, human blood samples were immediately transferred into an anoxic chamber or kept under atmospheric conditions (Figure 1a). Neutrophils were purified in parallel using the same conditions on a Percoll® gradient and, as a control, a rapid purification method was used (MACSxpress® whole blood neutrophil isolation kit, Miltenyi) (Figure 1a). We demonstrated by flow cytometry that the viability of neutrophils after the purification was not significantly different between anoxic and hyperoxic purifications (Figure 1b, $P > 0.05$). However, the viability of neutrophils was slightly increased in both conditions compared with the viability of neutrophils purified with the rapid method [Figure 1b, $P < 0.001$ (hyperoxia) and $P < 0.001$ (anoxia)]. We further assessed neutrophil activation upon anoxic or hyperoxic purification and observed no significant difference using CD54, CD11b and CD62L cell-surface markers (Supplementary figure 1a–c, $P > 0.05$). It should be noticed that the purification of neutrophils using the Percoll® gradient method tends to slightly activate neutrophils (CD62L shedding) in both conditions, compared with the rapid purification method (Supplementary figure 1c, $P < 0.01$).

Combining neutrophil purification and culture under anoxic conditions maintains their viability and limits their activation

We recently determined by direct measurements that the plasma fraction of the blood contains a limited amount of dissolved oxygen (< 1.2% O_2).[4] Accordingly, we set up a new method consisting of limiting oxygen exposure during neutrophil culture and compared its impact on the phenotype of neutrophils purified in anoxia or hyperoxia (Figure 1c). This strategy allowed us to evaluate the respective impact of oxygen exposure during neutrophil purification and culture on their viability and activation. Neutrophils are short-lived cells, with an estimated life expectancy ranging from a few hours to a few days.[12,13] To observe changes in neutrophil viability and activation, anoxia/hyperoxia-purified neutrophils were cultured in anoxia or hyperoxia for 24 h in a previously optimized culture medium.[11] As a control, anoxia-purified neutrophils were cultured in autologous plasma in anoxia/hyperoxia (Figure 1c).

First, we demonstrated that neutrophil survival was increased when cultured in plasma, irrespective of their exposure to oxygen (< 10% mortality, Figure 1d and Supplementary tables 1 and 2), suggesting that in addition to preventing oxygen exposure, other plasma components may protect neutrophils from cell death. Based on this observation, further investigations will be required to identify these beneficial components.

When neutrophils were cultured in commercial medium, we confirmed an increase in mortality in hyperoxia, as observed previously,[11] regardless of the purification conditions (anoxia/hyperoxia) (Figure 1d, $P < 0.01$ and Supplementary tables 1 and 2). The corollary of this result is that no significant impact of the purification conditions (anoxia/hyperoxia) on neutrophil survival was observed, regardless of the culture conditions ($P > 0.05$, Figure 1d and Supplementary tables 1 and 2). In conclusion, culturing neutrophils under anoxic conditions is the most important parameter to maintain their viability. Above all, maintaining neutrophils in plasma is essential to maintain their quiescence and viability.

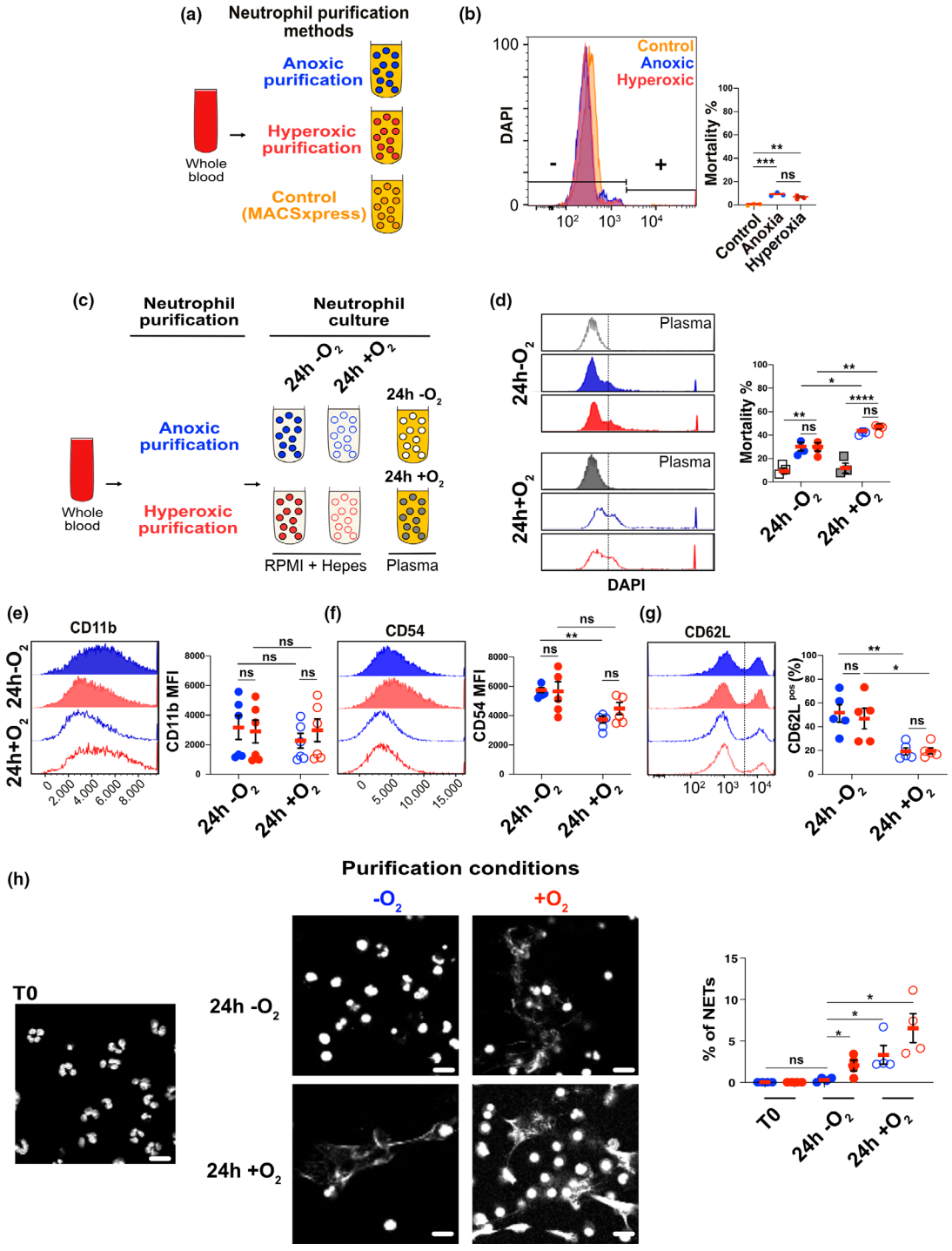


Figure 1. Respective impact of neutrophil purification and culture in anoxia on cell viability maintenance and activation limitation. **(a)** Experimental plan description: comparison of neutrophils purified in anoxia (blue), hyperoxia (red) or with a rapid-purification method MACSxpress (control; orange). **(b)** Flow cytometry assessment of neutrophil mortality ($DAPI^+$ cells, (**P = 0.0037; ***P = 0.0003), n = 3)). **(c)** Experimental plan description: comparison of neutrophils purified in anoxia or hyperoxia, exposed to anoxia or hyperoxia for 24 h in RPMI or in autologous plasma (control) (n = 3). **(d)** Assessment of $DAPI^+$ neutrophils after anoxic or hyperoxia purification and incubation (24 h) in RPMI and autologous plasma measured with flow cytometry. **P = 0.001, **P = 0.002; ***P < 0.0001; *P = 0.0263; **P = 0.0049). Assessment of **(e)** $CD11b^{high}$ **(f)** $CD54^{high}$**;** **P = 0.0072, n = 6). and **(g)** $CD62L^{pos}$ (**P = 0.0035, *P = 0.0114) neutrophils after anoxic or hyperoxia purification and incubation (24 h) in RPMI measured with flow cytometry (n = 6). **(h)** Images of detected NETs in neutrophils purified under anoxia or hyperoxia and cultures in anoxia or hyperoxia for 24 h evaluated using DAPI on fixed cells. The percentage of spontaneous NET formation in each condition (*P = 0.0268, *P = 0.0391, *P = 0.0121, n = 4). Bars are 10 µm.

We subsequently assessed neutrophil activation in all tested conditions, using cell-surface markers and the induction of spontaneous neutrophil extracellular traps (NETs). We did not observe a beneficial impact on culturing neutrophils in anoxic conditions compared with hyperoxic conditions when the cells were purified in hyperoxia in terms of CD11b (Figure 1e, ns) and CD54 (ICAM-1) (Figure 1f, $P < 0.05$) cell surface exposure. Although the difference in CD54 abundance was statistically significant, we estimate that the presence of oxygen decreases CD54 exposure rather than anoxic incubation increases its exposure, since fresh neutrophils expressed similar levels of CD54 compared with the anoxia-incubated neutrophils (Supplementary figure 1a). However, we demonstrated that neutrophil culture under anoxia significantly limits CD62L shedding in both purification conditions (anoxia/hyperoxia) (Figure 1g, Supplementary tables 1 and 2, $P < 0.05$ and $P < 0.01$ respectively), which is in accordance with previous studies on neutrophils from mice and rabbits.[14–16] In addition to cell surface markers, we assessed whether oxygen exposure would lead to the generation of spontaneous NETs. Interestingly, we observed the formation of NETs only when neutrophils were exposed to hyperoxia either during purification or during culture for 24 h, with a cumulative effect. Culturing neutrophils in anoxia did not reverse the impact of oxygen exposure during purification (Figure 1h). Therefore, maintaining neutrophils under anoxic conditions prevents the spontaneous formation of NETs, suggesting that other alternative activation mechanisms may be stimulated due to oxygen exposure, not detectable with classic surface markers (CD11b, CD54, CD62L).

In conclusion, we demonstrate here that neutrophil culture under anoxia is crucial to maintain their viability and limit their CD62L-dependent activation *in vitro*, even if oxygen exposure during purification did not lead to significant changes in these two parameters (Figure 1d–g). In addition, we demonstrated that purifying and culturing neutrophils in anoxia limits spontaneous NET induction, suggesting that oxygen exposure during purification may be sufficient to interrupt the basal state maintenance of neutrophils.

Beyond neutrophil activation and viability, we investigated the impact of oxygen exposure during purification on the cytosolic protein content of neutrophils.

Neutrophil purification under hyperoxic conditions (21% O_2) leads to the loss of 57 cytosolic proteins

We hypothesized that the stability of neutrophil cytosolic oxidation-sensitive proteins may be affected by a transient exposure to oxygen (hyperoxia) of neutrophils during purification. This hypothesis was validated using a proteomic approach, revealing that the cytosolic protein content of neutrophils purified under anoxia or hyperoxia was largely different. Mass-spectrometry sample quality control using principal component analysis (PCA) indicated very little variation between samples enabling the study of the majority of cytosolic proteins (Figure 2a, b, $n = 3$). Indeed, most of the cytosolic proteins identified by mass spectrometry were present in all replicates (Figure 2a, 1741 proteins upon anoxic purification and 1940 proteins upon hyperoxic purification). Strikingly, a large set of proteins (148 proteins) was more abundant in the cytosol of neutrophils purified under anoxia, while 56 proteins were significantly more abundant in the cytosol of neutrophils purified under hyperoxia (Figure 2c and Supplementary tables 3 and 4). More importantly, only 4 proteins were uniquely present in the cytosol of neutrophils purified under hyperoxic conditions (Supplementary table 3), while 57 proteins were uniquely present in the cytosol of neutrophils purified under anoxic conditions (Supplementary table 4). We performed a pathway enrichment analysis of uniquely abundant proteins using Database for Annotation, Visualization, and Integrated Discovery (DAVID) software v6.8.[17] The enrichment analysis did not reveal any metabolic pathways specifically expressed in neutrophils purified under hyperoxic conditions (Figure 2d and Supplementary table 3), while the lipid and cholesterol metabolism pathway (GO0043691, GO0001523, GO0042157, GO0008203; composed of HPX, APOB, IGJ, AGT, APOE, APOA1, APOA2, APOA4 and APOC3) and the complement activation and coagulation cascades [GO0043691/*hsa*04610; composed of A2 M, C4A, IGHM, CFB, IGHV4-34, SERPING1, IGHV1D-33, PLG, IGHV3-7, IGKV3-20, IGKV1-5, F2, SERPIND1, CFH, IGHV3-23, SERPINF2, C4BPA, IGKV3-11, C1S, IGLV3-25, SERPINF1, IGKV4-1, C7, C1QB, 3IGKVID-33 and IGHV3-23) were uniquely enriched when neutrophils were purified under anoxic conditions (Figure 2e and Supplementary table 4)]. We hypothesize that adverse protein oxidation may occur upon neutrophil oxygen exposure, leading to their subsequent degradation by proteasome and autophagic proteolytic systems (as previously reported for ApoB-100,[18] and reviewed[19]). Further investigations are required to address this question, which may lead to an improved understanding of neutrophil physiology under basal conditions and the identification of novel signaling pathways involved in neutrophil activation. Since neutrophils are well adapted to low oxygen availability, we hypothesize that the difference in cytosolic proteins is due to a loss of proteins in hyperoxia, rather than a gain in anoxia. Moreover, the uniquely abundant proteins detected in

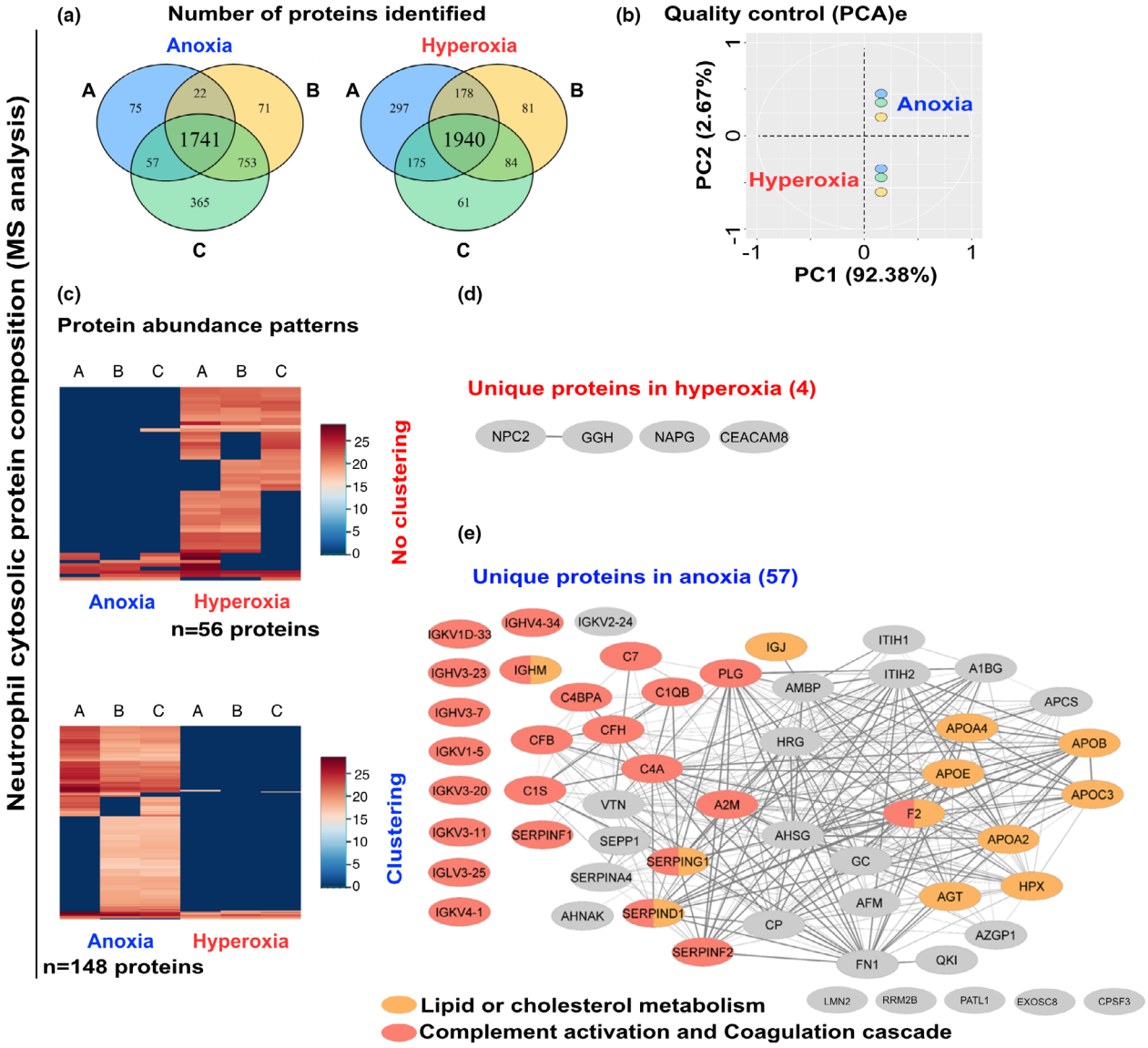


Figure 2. Neutrophil cytosolic protein composition is altered during their purification in hyperoxia, compared with anoxia. **(a)** Commonly identified proteins in all three samples **(a–c)** in anoxia and hyperoxia. **(b)** Data quality control assessment using principal component analysis (PCA) of cytosolic protein samples of neutrophils purified in anoxia or hyperoxia after mass-spectrometry analysis (n = 3). **(c)** Heat map representing differential protein abundance between anoxia- and hyperoxia-purified human neutrophils. **(d)** Pathway enrichment analysis of uniquely identified proteins in hyperoxic conditions using Database for Annotation, Visualization, and Integrated Discovery (DAVID) and Cytoscape for network visualization. **(e)** Enrichment of the lipid and cholesterol metabolism (GO0043691, GO0001523, GO0042157, GO0008203) and the complement and coagulation cascade (GO0006958/hsa04610) pathways identified with DAVID and visualized with Cytoscape in anoxia-purified neutrophils. Enrichment *P*-values for the GO terms and *hsa* pathways are *P* (GO0043691; Reverse cholesterol transport) = 9.10×10^{-6}, *P* (GO0001523; Retinoid metabolic process) = 1.77×10^{-5}, *P* (GO0042157; Lipoprotein metabolic processes) = 2.6×10^{-2}, *P* (GO0008203; Cholesterol metabolic process) = 3.76×10^{-4}, *P* (GO0006958; Complement activation, classic pathway) = 3.87×10^{-22}, *P* (*hsa*04610; Complement and coagulation cascade) = 5.57×10^{-12} (n = 3).

anoxia have not been associated with low oxygen availability, suggesting again that exposing neutrophils to hyperoxia leads to the loss of a large set of cytosolic proteins.

In conclusion, preserving neutrophils from hyperoxia during their purification, manipulation and physiological study is a critical parameter to maintain cells in a basal state. Our results confirm the beneficial effect of

neutrophil purification and culture under anoxic conditions. New metabolic and signaling pathways linked to differential oxygen exposure were revealed in this study, opening new insights into the physiology of neutrophils.

METHODS

Neutrophil purification

All participants gave written, informed consent in accordance with the Declaration of Helsinki principles. Peripheral human blood was collected from healthy patients at the ICAReB service of the Pasteur Institute (authorization DC No. 2008-68) and from the Etablissement Français du Sang (EFS) de Strasbourg (authorization no ALC/PIL/DIR/AJR/FO/606). Human blood samples were collected from the antecubital vein into tubes or blood collection bags containing sodium citrate (3.8% final) as an anticoagulant.

For Percoll gradient human neutrophil purification, whole blood samples were centrifuged at 594 *g* for 20 min without a break. Platelet-rich plasma was collected and centrifuged at 594 *g* for 20 min to form platelet-poor plasma. Blood cells were resuspended in NaCl 0.9% and dextran sulfate (0.72% final). After 30 min sedimentation, the leukocyte-containing upper layer was centrifuged at 300 × *g* for 10 min. The resuspended pellet was separated on a 42% Percoll-plasma (GE Healthcare) gradient by centrifugation at 800 × *g* for 20 min. Neutrophils were collected from the pellet with remaining red blood cells, which were removed using CD235a (glycophorin) microbeads (negative selection, Miltenyi Biotec, Germany). Anoxic purification steps were performed in the anoxic chamber using oxygen-free media.

For MACSxpress purification of human neutrophils we used MACSxpress whole blood neutrophil isolation kit, human (Miltenyi Biotec), following the manufacturer's procedure. The remaining red blood cells were removed using CD235a (glycophorin) microbeads (Miltenyi Biotec).

Neutrophil culture

Purified neutrophils were centrifuged for 10 min at 300 × *g* and resuspended at 1.10^6 cells mL^{-1} in RPMI1640 medium (Gibco, US) supplemented with 10 mM HEPES buffer (Thermo Fisher, USA) and 10% autologous plasma. Neutrophils were plated into a 24-well plate at 1 mL per well in the presence or absence of oxygen (21% and 0% O_2).

Flow cytometry

Cell viability and activation were measured by flow cytometry. Cells in culture were resuspended and centrifuged for 10 min at 300 × *g*. Cell-containing pellets were subsequently resuspended in 300 µL PBS + 2 mM EDTA and incubated for 15 min at RT in the presence of the following fluorescent markers (1/100 dilution), as indicated: 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich), CD11b-PE; CD54-APC, CD62L-FITC (Abcam). Labeled cells were analyzed with an 8-color cell analyzer BD FACSCanto™ (BD Biosciences, USA).

Data mining was achieved with the FlowJo software (FlowJo, LLC, USA). Data were further analyzed using Prism 8.0 software (GraphPad, USA) for statistical analyses.

NET detection

Purified neutrophils were centrifuged for 10 min 300 × *g* and resuspended at 1×10^6 cells mL^{-1} in RPMI1640 medium (Gibco) supplemented with 10 mM HEPES buffer (Thermo Fisher). Neutrophils were plated into a 24-well plate at 1 mL per well in the presence or absence of oxygen (21% and 0% O_2). Cells were fixed at T0 and after 24 h culture with 3.2% paraformaldehyde for 30 min and washed with DPBS. The cells were penetrated with 0.1% Tween-100 and stained with DAPI. Coverslips were mounted with ProLong Gold (Invitrogen, USA). Cells were visualized under a confocal microscope (Leica SP8, Germany) with 40 × oil-immersed objectives.

Mass spectrometry

Cell extracts preparation: Human neutrophils purified under hyperoxic or anoxic conditions were pelleted and resuspended in ice-cold PBS at 27×10^6 cells mL^{-1} containing diisopropylfluorophosphate (DFP; Sigma-Aldrich, Germany) (0.5 µL in 1 mL) and incubated on ice for 15 min. The cells were centrifuged at 300 × *g* for 10 min at 4°C. Pellets were resuspended at 20×10^6 cells mL^{-1} in relaxation buffer 1x containing 3 mM PMSF, 1 mM orthovanadate, 400 µM pepstatin, 400 µM leupeptin, 1 mM ATP, 1 mM EDTA. Resuspended cells were introduced into a cavitation bomb chamber. Pressure was stabilized at 350 psi for 20 min with nitrogen. Cell membranes were disrupted by slow gas expansion. Cell lysates were collected in tubes containing 1.25 mM EGTA.[20,21]

Neutrophil cytosolic fractions were isolated by centrifugation. First, nuclei and remaining cells were removed by centrifuging samples at 500 × *g* for 15 min at 4°C. Then, neutrophil granules and organites were removed by centrifuging samples at 37000 × *g* for 1.5 h at 4°C (11 × 32 mm PC tubes ref 343778, TLA102 rotor, TL100 ultracentrifuge or equivalent; Beckman Coulter, USA).

The protein concentration was determined in each sample and 5 µg of protein was run on 12% SDS-PAGE to select homogeneous samples. Proteins (45 µg) were digested for 14 h at 37°C with 1 µg trypsin (Promega, USA), and were fractionated by strong cationic exchange (SCX) StageTips. Mass spectrometry analyses were performed on a U3000 RSLC nano-LC system coupled to an LTQ Orbitrap-Velos mass spectrometer (Thermo Fisher). The data were analyzed using MaxQuant version 1.5.2.8. The database used was a concatenation of human sequences from the UniProt–Swiss-Prot database (UniProt, release 2015-02) and a list of contaminant sequences from MaxQuant. The identification

false discovery rate (FDR) was kept below 1% on both peptides and proteins. The quantification of each identified protein was performed by using the MaxLFQ algorithm available in MaxQuant.[22] Label-free protein quantification (LFQ) was carried out using both unique and razor peptides. At least two such peptides were required for LFQ quantification of a protein.

Bioinformatic analysis of proteomics data

Proteins identified in the reverse and contaminant databases and proteins "only identified by site" (with an identification score too low – not exceeding the 1% FDR threshold) were first discarded. Then, proteins exhibiting fewer than 2 LFQ intensities in at least one condition (either anoxia or hyperoxia) were discarded from the list to ensure a minimum of replicability in quantified values.

After $\log_2$ transformation of the LFQ intensities of the leftover proteins, PCA was performed from the proteins without missing values in all the samples (856 proteins). Logged LFQ intensities in each sample were centered and scaled before analysis. The variables factor map (Figure 2b) represents each sample projected on the first and second component of the PCA. It allows visualizing clusters of correlated samples. Of note, the LFQ intensities of samples are globally strongly correlated whatever the condition, such that the first principal component explains most of the variance (92%), while the second component distinguishes anoxic samples from hyperoxic samples (2.6% of explained variance). This shows good reproducibility of the quantified values.

The differential analysis highlighting more (148 proteins) or less (56 proteins) abundant proteins in anoxia than in hyperoxia was carried out using an in-house R analysis pipeline based on the R packages DAPAR,[23] imp4p,[24] limma[25] and cp4p.[26] Statistical enrichment tests were performed from these proteins to highlight major gene ontology terms or KEGG pathways that may be affected using DAVID software v6.8[17] by selecting the set of quantified proteins among all samples as background. The protein–protein interaction graph was determined from STRING v11[27] and visualized using Cytoscape v3.8.0[28] with the plug-ins (Cytoscape) stringApp[27] and Omics Visualizer.[29]Only "exclusive" proteins with unique peptides identified in a condition (anoxia or hyperoxia) and no peptide identified in the other condition, were represented. The widths of the edges correspond to the combined score of STRING reflecting the confidence placed in each interaction. The proteins highlighted as "Lipid or Cholesterol metabolism" and "Complement activation and coagulation cascades" were determined from enriched annotation clusters using the fuzzy heuristic clustering method of DAVID v6.8.[17]

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CONFLICT OF INTEREST

All authors declare no conflicts of interest.

AUTHOR CONTRIBUTION

Louise Injarabian: Formal analysis; Investigation; Writing-original draft. **Jurate Skerniskyte:** Investigation; Writing-review & editing. **Quentin G Gianetto:** Formal analysis; Software. **Veronique Witko-Sarsat:** Formal analysis; Investigation; Methodology. **Benoit S Marteyn:** Conceptualization; Funding acquisition; Investigation; Methodology; Project administration; Supervision; Validation; Writing-original draft.

REFERENCES

1. Maianski NA, Geissler J, Srinivasula SM, *et al.* Functional characterization of mitochondria in neutrophils: a role restricted to apoptosis. *Cell Death Differ* 2004; **11**: 143–153.
2. Spencer JA, Ferraro F, Roussakis E, *et al.* Direct measurement of local oxygen concentration in the bone marrow of live animals. *Nature* 2014; **508**: 269–273.
3. Pittman RN. Regulation of Tissue Oxygenation. Morgan & Claypool Life Sciences: San Rafael (CA), 2011.
4. Injarabian L, Scherlinger M, Devin A, *et al.* Ascorbate maintains a low plasma oxygen level. *Sci Rep* 2020; **10**: 10659.
5. Carreau A, El Hafny-Rahbi B, Matejuk A, Grillon C, Kieda C. Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia. *J Cell Mol Med* 2011; **15**: 1239–1253.
6. Karhausen J, Furuta GT, Tomaszewski JE, *et al.* Epithelial hypoxia-inducible factor-1 is protective in murine experimental colitis. *J Clin Invest* 2004; **114**: 1098–1106.
7. Tinevez J-Y, Arena ET, Anderson M, *et al. Shigella*-mediated oxygen depletion is essential for intestinal mucosa colonization. *Nat Microbiol* 2019; **4**: 2001–2009.
8. Taylor CT, Doherty G, Fallon PG, Cummins EP. Hypoxia-dependent regulation of inflammatory pathways in immune cells. *J Clin Invest* 2016; **126**: 3716–3724.
9. Egners A, Erdem M, Cramer T. The Response of Macrophages and Neutrophils to Hypoxia in the Context of Cancer and Other Inflammatory Diseases. *Mediators Inflamm* 2016; **2016**: 2053646.
10. Walmsley SR, Print C, Farahi N, *et al.* Hypoxia-induced neutrophil survival is mediated by HIF-1α-dependent NF-κB activity. *J Exp Med* 2005; **201**: 105–115.
11. Monceaux V, Chiche-Lapierre C, Chaput C, *et al.* Anoxia and glucose supplementation preserve neutrophil viability and function. *Blood* 2016; **128**: 993–1002.
12. Athens JW, Haab OP, Raab SO, *et al.* Leukokinetic studies. IV. The total blood, circulating and marginal granulocyte pools and the granulocyte turnover rate in normal subjects. *J Clin Invest* 1961; **40**: 989–995.

13. Pillay J, den Braber I, Vrisekoop N, *et al*. *In vivo* labeling with $^{2}H_2O$ reveals a human neutrophil lifespan of 5.4 days. *Blood* 2010; **116**: 625–627.
14. Casanova-Acebes M, Pitaval C, Weiss LA, *et al*. Rhythmic modulation of the hematopoietic niche through neutrophil clearance. *Cell* 2013; **153**: 1025–1035.
15. Matsuba KT, Van Eeden SF, Bicknell SG, *et al*. Apoptosis in circulating PMN: increased susceptibility in L-selectin-deficient PMN. *Am J Physiol* 1997; **272**: H2852–H2858.
16. Zhang D, Chen G, Manwani D, *et al*. Neutrophil ageing is regulated by the microbiome. *Nature* 2015; **525**: 528–532.
17. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 2009; **4**: 44–57.
18. Edelstein C, Nakajima K, Pfaffinger D, Scanu AM. Oxidative events cause degradation of apoB-100 but not of apo[a] and facilitate enzymatic cleavage of both proteins. *J Lipid Res* 2001; **42**: 1664–1670.
19. Jung T, Höhn A, Grune T. The proteasome and the degradation of oxidized proteins: Part II - protein oxidation and proteasomal degradation. *Redox Biol* 2014; **2**: 99–104.
20. Borregaard N, Heiple JM, Simons ER, Clark RA. Subcellular localization of the b-cytochrome component of the human neutrophil microbicidal oxidase: translocation during activation. *J Cell Biol* 1983; **97**: 52–61.
21. Witko-Sarsat V, Cramer EM, Hieblot C, *et al*. Presence of proteinase 3 in secretory vesicles: evidence of a novel, highly mobilizable intracellular pool distinct from azurophil granules. *Blood* 1999; **94**: 2487–2496.
22. Cox J, Hein MY, Luber CA, *et al*. Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. *Mol Cell Proteomics MCP* 2014; **13**: 2513–2526.
23. Wieczorek S, Combes F, Lazar C, *et al*. DAPAR & ProStaR: software to perform statistical analyses in quantitative discovery proteomics. *Bioinforma Oxf Engl* 2017; **33**: 135–136.
24. Gianetto G, Woeczorek S, Couté Y, Burger T. A peptide-level multiple imputation strategy accounting for the different natures of missing values in proteomics data. *bioRxiv*. https://doi.org/10.1101/2020.05.29.122770v1
25. Ritchie ME, Phipson B, Wu D, *et al*. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 2015; **43**: e47.
26. Gianetto QG, Combes F, Ramus C, *et al*. Calibration plot for proteomics: a graphical tool to visually check the assumptions underlying FDR control in quantitative experiments. *Proteomics* 2016; **16**: 29–32.
27. Szklarczyk D, Morris JH, Cook H, *et al*. The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible. *Nucleic Acids Res* 2017; **45**: D362–D368.
28. Shannon P, Markiel A, Ozier O, *et al*. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res* 2003; **13**: 2498–2504.
29. Legeay M, Doncheva NT, Morris JH, Jensen LJ. Visualize omics data on networks with Omics Visualizer, a Cytoscape App. *F1000Research* 2020; **9**: 157.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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