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TITLE:

Proteomic Analysis of Human Macrophage Polarization Under a Low Oxygen Environment

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SUMMARY:

We present a protocol to obtain proteomic signatures of human macrophages and apply this to determination of the impact of a low oxygen environment on macrophage polarization.

ABSTRACT:

Macrophages are innate immune cells involved in a number of physiological functions ranging from responses to infectious pathogens to tissue homeostasis. The various functions of these cells are related to their activation states, which is also called polarization. The precise molecular description of these various polarizations is a priority in the field of macrophage biology. It is currently acknowledged that a multidimensional approach is necessary to describe how polarization is controlled by environmental signals. In this report, we describe a protocol designed to obtain the proteomic signature of various polarizations in human macrophages. This protocol is based on a label-free quantification of macrophage protein expression obtained from in-gel fractionated and Lys C/trypsin-digested cellular lysis content. We also provide a protocol based on in-solution digestion and isoelectric focusing fractionation to use as an alternative. Because oxygen concentration is a relevant environmental parameter in tissues, we use this protocol to explore how atmospheric composition or a low oxygen environment affects the classification of macrophage polarization.

INTRODUCTION:

Macrophages are innate immune cells involved in a number of physiological functions ranging from responses to infectious pathogens to tissue homeostasis, including removal of apoptotic cells and remodelling of the extracellular matrix[1]. These cells are characterized by a strong phenotypic plasticity[2] that translates into a many possible activation states, which are also called

polarizations. The precise molecular description of these various polarizations is a priority in the field of macrophage biology[3]. It has been proposed to classify these polarizations using the so-called M1/M2 dichotomy, in which M1 represents pro-inflammatory and M2 represents anti-inflammatory macrophages. This model fits well in various pathological situations like acute infections, allergy, and obesity[4]. However, in chronically inflamed tissues and cancer, it has been demonstrated that this classification is unable to grasp the broad phenotypic repertoire that macrophages present in certain cellular environments[5–7]. The current consensus is that macrophage polarization is better described using a multidimensional model to integrate specific microenvironmental signals[8]. This conclusion has been confirmed through transcriptomic analysis of human macrophages showing that the M1/M2 model is inefficient in describing the obtained polarizations[9].

The study presented aims to provide a protocol to obtain proteomic signatures of various polarizations in human macrophages. We describe how to differentiate human macrophages in environments of various oxygen levels and obtain peptides from the whole macrophage proteome to perform a label-free quantification. This quantification allows the comparison of expression levels of various proteins. As research on stem cells has revealed the importance of oxygen as an environmental key parameter[10], we seek to understand how this tissue parameter can influence macrophage polarization in humans. The partial pressure of oxygen has been found to range from 3 to 20% (of total atmospheric pressure) in the human body, where 20% corresponds roughly to what is commonly used in a cell culture incubator (the exact value is around 18.6% while taking the presence of water into account).

Previous work has shown that alveolar differ from interstitial macrophages from functional and morphological point of views[11] and that these differences are probably partially due to the different oxygen levels to which they are exposed[12]. Furthermore, bone marrow-derived macrophages show an increased ability to phagocytize bacteria when exposed to a low oxygen environment[12]. The opposite effect has been found for THP1-differentiated human macrophages[13], but these results support the idea that oxygen is a regulator of macrophage biology and that it is necessary to clarify this role at the molecular level in human macrophages. In a previous study, we have applied a proteomics approach to address these issues. By measuring expression levels for thousands of proteins simultaneously, we highlighted the impact of oxygen on polarization and provided a list of new molecular markers. We were also able to relate these findings to some macrophages functions. Notably, we found that the rate of phagocytosis of apoptotic cells was increased in IL4/IL13-polarized macrophages, which was linked to the upregulation of ALOX15 as revealed by the proteomic analysis[14]. In the present study, we describe how to perform such an analysis.

PROTOCOL:

Human blood samples (LRSC) from healthy, de-identified donors were obtained from EFS (French National Blood Service) as part of an authorized protocol (CODECOH DC-2018–3114). Donors gave signed consent for the use of blood.

1. Media and Buffer Preparation

1.1. Prepare the macrophage medium [RPMI glutamax + 10 mM HEPES + 1x non-essential amino acids (NEAA)] and warm it to 37 °C.

1.2. Prepare the macrophage medium + 10% human serum from AB plasma (SAB), filter it (0.22 µm filter), then warm it to 37 °C (referred to as macrophage medium + 10% SAB hereafter).

1.3. Prepare the sorting buffer [1x phosphate buffered saline (PBS) + 0.5% bovine serum albumin (BSA) + 2 mM ethylenediaminetetraacetic acid (EDTA)], filter it (0.22 µm filter), and maintain it at 4 °C.

2. Isolation of Peripheral Blood Mononuclear Cells (PBMCs) from Leukoreduction System Chamber (LRSC)

2.1. Put 15 mL of density gradient cell separation solution (see **Table of Materials**) in a 50 mL centrifugation tube so it can warm to room temperature (RT) before receiving the LRSC.

NOTE: Density depends on temperature. As this product is stored at 4 °C, this step must be done in advance so it can equilibrate to RT.

2.2. Empty the LRSC into a 50 mL centrifugation tube, add up to 50 mL of 1x PBS, and mix. Very slowly, add 25 mL of the mix prepared during step 2.2 on top of 15 mL of density gradient solution warmed up during step 2.1.

NOTE: Be careful not to mix the phases during this step. The blood must be added on the density gradient solution without any disturbance of this phase.

2.3. Centrifuge both centrifugation tubes for 25 min at 700 x g without breaks.

NOTE: At the end of the density gradient centrifugation, the layers from bottom to top are: the erythrocytes and granulocytes forming the pellet, density gradient solution phase, layer of PBMCs, and plasma.

2.4. With a pipet, pass through the plasma phase without aspirating it and collect the PBMC layer into a new 50 mL centrifugation tube. Add up to 50 mL of 1x PBS to the PBMCs as a washing step and centrifuge for 10 min at 300 x g.

2.5. Aspirate the supernatant and resuspend the pellet in 40 mL of macrophage medium.

3. Magnetic Labeling and Isolation of $CD14^+$ Cells (Monocytes)

3.1. Count the PBMCs in a Malassez chamber. Withdraw the amount of PBMCs necessary for conducting the experiment (typically 100 to 300 x 10^6 cells), place them in a centrifugation tube, and centrifuge for 10 min at 300 x g.

3.2. Aspirate the supernatant and resuspend the pellet in 80 µL of the sorting buffer prepared during step 1.3 per 10^7 PBMCs. Add 20 µL of CD14 microbeads per 10^7 PBMCs. Mix well and incubate for 15 min at 4 °C under constant agitation.

3.3. Add 1 mL of sorting buffer per 10^7 PBMCs as a washing step and centrifuge for 10 min at 300 x g. Aspirate the supernatant and resuspend the pellet in 500 µL of sorting buffer per 10^8 PBMCs.

3.4. Place a column in the magnetic field of the separator. Prepare the column by rinsing it with 3 mL of sorting buffer.

3.5. Apply the cell suspension onto the column. The column depends on the number of cells to be isolated (here, LS columns for up to 10^9 PBMCs are used). Collect flow-through containing unlabeled cells.

NOTE: Starting at this step, all the tubes (negative and positive selections) are kept for later checking of the different steps by flow cytometry.

3.6. Wash the column with 3 x 3 mL of sorting buffer. Collect unlabeled cells passing through the same tube from step 3.12. Perform washing steps by adding sorting buffer only when the column reservoir is empty. Place a collection tube under the column and remove it from the separator.

3.7. Pipette 5 mL of sorting buffer into the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column. To increase purity of the $CD14^+$ cells, the eluted fraction is enriched over a second column.

3.8. Repeat steps 3.4 to 3.7 with a new column.

4. Plating of Monocytes

4.1. Count the monocytes in a Malassez chamber. Check the purity of the $CD14^+$ cells by flow cytometry. Withdraw the amount of monocytes necessary for the experiment and place them in a centrifugation tube.

4.2. Centrifuge for 10 min at 300 x g. Aspirate the supernatant and resuspend the monocyte pellet in macrophage medium. Plate the cells and let them settle for 50 min to 1 h. Aspirate the medium and replace it with macrophage medium + 10% SAB + 25 ng/mL macrophage colony stimulating factor (M-CSF) to induce differentiation.

5. Polarization of Macrophages at Day 6

5.1. Aspirate the medium. Replace it with macrophage medium + 10% SAB with various stimuli. For example, add 10 ng/mL interferon gamma (INFγ) + 1 ng/mL lipopolysaccharide (LPS) to obtain M1 polarization, or 20 ng/mL interleukin 4 (IL4) + 20 ng/mL interleukin 13 (IL13) for M2 polarization.

NOTE: The stimulation can be performed between 24 and 48 h before proceeding to other tests.

5.2. Harvest cells using a detaching solution or a cell scraper.

6. Cell Culture Under Low Oxygen Conditions

6.1. Starting from step 4, maintain the monocytes and then macrophages in an oxygen-controlled environment to perform hypoxic condition analysis. Use a hypoxia working station in order to maintain cells under the desired oxygen partial pressure during the experiment.

NOTE: When working under low oxygen pressure, it is important to prepare all media and washing buffers under the station and wait sufficiently to obtain the correct partial pressure in the liquid. For example, 10 mL of PBS in a 60 mm Petri dish requires roughly 2 h to reach 25 mmHg for O_2 partial pressure starting from atmospheric pressures (as we have measured it using a fiber-optic oxygen sensor). In many hypoxic stations or incubators, the oxygen pressure is set as a percentage of the atmospheric pressure. If precise measurements are necessary, it is better to use a station authorizing to directly set the oxygen pressure in mmHg.

7. Lysis and In-Gel Digestion (Protocol 1)

NOTE: In this and the following sections, two protocols used to obtain peptides and perform LC-MS/MS analysis are described. Protocol 1 describes cell lysis and in-gel fractionation and digestion, and protocol 2 describes in-solution cell lysis followed by in-solution digestion and fractionation using an isoelectric focusing method.

7.1. Perform cell lysis in Laemmli buffer [234 mM Tris-HCL (pH 6.8), 7.5% SDS, 37% glycerol, 33.3% (v/v) β-mercaptoethanol, bromophenol blue 0.2% w/v]. Load the protein equivalent of 300,000 cells for each sample on 4-12% bis-Tris acrylamide gels.

7.2. Control the duration of the electrophoretic migration to allow each protein sample to be split into 6 gel bands as exemplified in **Figure 3**.

7.3. Fix the gel with a fixing solution (30% ethanol + 7.5% acetic acid for 20 min), then add the staining solution (R-250 Coomassie blue for 45 min). Add the destaining solution (30% ethanol + 7.5% acetic until bands appear) before excising the protein bands with a clean scalpel.

7.4. Dice each excised band before introduction in 500 µL tubes. A clean glass surface is warranted to avoid contamination with keratins (5% SDS solution in deionized water can be used to clean surfaces).

7.5. Wash the gel slices 3 times in 200 µL of 25 mM ammonium bicarbonate for 20 min at 37 °C, followed by one wash in 25 mM ammonium bicarbonate and acetonitrile (50% v/v). Dehydrate the gel pieces with 200 µL of 100% acetonitrile for 10 min.

7.6. Incubate each gel piece with 10 mM DTT (dithiothreitol) in 25 mM ammonium bicarbonate for 45 min at 56 °C (200 µL), followed by 55 mM iodoacetamide in 25 mM ammonium bicarbonate (200 µL) for 35 min in the dark at RT.

7.7. To stop alkylation, incubate each gel piece with 200 µL of 10 mM DTT in 25 mM ammonium bicarbonate for 10 min at RT. Wash the gel pieces in 200 µL of 25 mM ammonium bicarbonate, then dehydrate with 200 µL of 100% acetonitrile for 10 min.

7.8. Digest the proteins overnight at 37 °C with Trypsin/Lys-C mix according to the manufacturer's instructions.

7.9. Extract the resulting peptides from gel pieces by adding 50 µL of 50% acetonitrile for 15 min, then 50 µL of 5% formic acid for 15 min, and finally, 50 µL of 100% acetonitrile for 15 min. Pool and dry each fraction in low-absorption tubes to limit adsorption of peptides and sample loss. Store the samples at -80 °C until further analysis.

8. Protein Extraction and In-Solution Digestion (Protocol 2)

8.1. Perform cell lysis (2 x 10^6 cells) with 150 µL of the following lysis buffer:

8.1.1. 7 M urea, 2 M thiourea, 40 mM Tris, and 4% CHAPS, supplemented with protease inhibitors (complete mini, EDTA-free protease inhibitor cocktail).

8.2. Homogenize the solutions for 30 min at RT with a thermoshaker. Centrifuge at 13,800 x g for 20 min RT and keep the supernatant.

8.3. Remove contaminants with a 2D clean-up kit:

8.3.1. The kit contains precipitant solution, co-precipitant solution, wash buffer, and wash additive.

8.3.2. Add 300 µL of precipitant solution and mix well. Incubate on ice for 15 min. Add 300 µL of co-precipitant solution. Centrifuge the tubes (at least) at 12,000 x g for 5 min. A small pellet should be visible. Proceed rapidly to the next step to avoid resuspension or dispersion of the pellet. Remove the supernatant without disturbing the pellet.

8.3.3. Centrifuge the tubes again with the cap-hinge and pellet facing outward to bring any remaining liquid to the bottom of the tube. A brief pulse is sufficient. There should be no visible liquid remaining in the tubes.

8.3.4. Without disturbing the pellet, add 40 µL of co-precipitant solution. Let the tube sit on ice for 5 min. Centrifuge for 5 min, then remove and discard the wash. Add 25 µL of de-ionized water. Vortex each tube for 5-10 s. The pellet should disperse but not dissolve in the water.

8.3.5. Add 1 mL of wash buffer (pre-chilled for at least 1 h at -20°C) and 5 µL of wash additive. Vortex until the pellet is fully dispersed. Incubate the tubes at -20°C for at least 30 min. Vortex for 20-30 s every 10 min

NOTE: The tubes can be stored at -20°C for up to 1 week with minimal protein degradation or modification.

8.3.6. Centrifuge the tubes (at least) at 12,000 × g for 5 min. Carefully remove and discard the supernatant. A white pellet should be visible. Allow the pellet to airdry for no more than 5 min (if the pellet is too dry, it will be difficult to resuspend).

8.4. Resuspend the protein pellet in 300 µL of 8 M urea and 0.1 M ammonium bicarbonate. Vortex strongly for 1 min. Determine the protein concentration using a colorimetric assay.

9. **In-Solution Digestion (Protocol 2)**

9.1. Reduce disulfide bridges by adding 5.1 µL of a 700 mM DTT solution (final concentration 12.5 mM) to the resuspended proteins from step 8.4 and incubate at 37 °C for 30 min with a thermoshaker. Alkylate cysteine residues by adding 20.3 µL of a 700 mM iodoacetamide solution (final concentration 40 mM) and incubating at 25°C for 30 min in the dark with a thermoshaker.

9.2. Add 990 µL of 0.1 M ammonium bicarbonate to the sample. Add a corresponding volume of Trypsin/Lys-C mix (enzyme:substrate ratio 1:100 w/w). Incubate at 37°C overnight with a thermoshaker.

10. **Clean-up Cartridge (Protocol 2)**

10.1. Wet a cartridge with 1 column-volume (1 mL) of methanol. Clean the cartridge with 1 column-volume (1 mL) of 80% acetonitrile/HPLC-grade water and discard the flow-through. Equilibrate the cartridge with 4 column-volumes (4 mL) of 0.1% formic acid/HPLC-grade water and discard the flow-through.

10.2. Acidify samples with 90 µL of 10% formic acid or water to pH 2-3 (check the pH with a pH indicator). Load the acidified samples and collect the flow-through. Reload the flow-through (containing the not-retained peptides). Wash the cartridge with 6 column-volume (6 mL) of 0.1% formic acid/HPLC-grade water.

10.3. Elute peptides from the cartridge with 1 column-volumes (1 mL) of 0.1% formic acid/50% acetonitrile/HPLC-grade water. Transfer to a 1.5 mL microcentrifuge tube. Concentrate the sample using a vacuum concentrator (150 x g, vacuum at 160 mBar).

11. Fractionation by Isoelectric Focusing (Protocol 2)

NOTE: Peptides are separated according to their isoelectric points using an off-gel fractionator

on a 13 cm strip covering a pH range from 3 to 10. We used the following protocol provided by the supplier (summarized below):

11.1. Prepare the following solutions: solution A (600 µL of glycerol solution, 60 µL of OFFGEL buffer, 4.34 mL of ultrapure water) and solution B (1.776 mL of solution A and 444 µL of ultrapure water).

11.2. Assemble the IPG strips, frames, and electrodes according to the manufacturer's instructions.

11.3. Resuspend the sample with 1.8 mL of solution B. Add 40 µL of solution B into each well. Load 150 µL of sample into each well.

11.4. Select the default method for peptides: OG12PE00 (OFFGEL default method for peptides for use with a 3100 OFFGEL Low Res Kit, pH 3-10, 12-well frames. Wait until this method has been completed (~20 h). Collect the fractions in properly labeled tubes.

12. Clean-up Harvard Apparatus Column Reverse C18 Post-IEF (Protocol 2)

12.1. Progressively add a few µL at a time of 1% TFA in de-ionized water to each fraction to acidify the sample. Check using pH paper that the pH is about 3 or below.

12.2. Prepare the following solutions: solution 1 (5 mL of acetonitrile, 10 µL of formic acid, 4.99 mL of ultrapure water) and solution 2 (0.5 mL of acetonitrile, 10 µL of formic acid, 9.49 mL of ultrapure water).

12.3. Pre-wet the spin column with 150 µL of solution 1. Centrifuge for 90 s at 750 x g and discard the flow-through. Wash the spin column with 150 µL of solution 2. Centrifuge for 90 s at 750 x g and discard the flow-through.

12.4. Pass the fraction through the column. Centrifuge for 90 s at 750 x g and discard the flow-through. Wash with 150 µL of solution 2. Centrifuge for 90 s at 750 x g and discard the flow-through.

12.5. Elute the fraction with 50 µL of solution 1. Centrifuge for 90 s at 750 x g. Repeat these steps once more.

12.6. Dry-fraction using a vacuum concentrator (150 x g, vacuum 160 mBar) and store at -80°C

13. Analysis of Proteomic Data and Bioinformatics[18]

13.1. Analyze data obtained by a nano-LC MS/MS mass spectrometer using quantification software such as MaxQuant (version 1.5.2.8) and the Andromeda search engine.

13.2. Set false discovery rate (FDR) to 1% for both proteins and peptides and a minimum length of 7 amino acids. Set enzyme specificity as C-terminal to Arg and Lys. Allow 2 missed cleavages at proline bonds. Select carbamidomethylation of cysteine as a fixed modification and N-terminal protein acetylation and methionine oxidation as variable modifications.

13.3. Further analyze the data with statistical analysis software. Perform a functional enrichment analysis using FunRich software (www.funrich.org/). Perform a gene ontology enrichment analysis using DAVID software (https://david.ncifcrf.gov/).

REPRESENTATIVE RESULTS:

Starting from peripheral blood mononuclear cells (PBMCs) obtained by differential centrifugation, the protocol permits the obtaining of a population of $CD14^+$ monocytes with an assessed purity of more than 98% by flow cytometry (**Figure 1**). These monocytes are secondarily differentiated toward various polarizations (**Figure 2**). When a fractionation on gel is chosen, the migration on SDS-page gels is adapted to obtain the number of desired bands, and the bands are excised (**Figure 3**). The digestion is secondarily performed in the excised bands of the gel, then the peptides are extracted. The peptides are analyzed using a nano-LC (liquid chromatography)-MS/MS mass spectrometer. MS/MS spectra give the identity of various proteins according to the annotation of spectra obtained for known peptides (**Figure 4A**). The quantification of the abundance of a protein is then calculated in connection with the quantity of identified peptides coming from the protein using published software and databases[15,16]. This protocol with in-gel digestion gives approximately 4000 identified proteins, and the dynamic range has been found to cover 5 logarithmic scale units (**Figure 4B**). Analysis of the differential expression of these identified proteins can be used to determine the clustering of various polarizations under different oxygen environments.

With this method, we can also recognize clusters of proteins that are up-regulated when exposed to a low oxygen concentration of 3% (**Figure 5, Table 1**). To assess efficiency of the digestion, which is not possible when an in-gel protocol is used, we proposed an in-solution digestion method that has been adapted to human macrophages (**Figure 6A**). With this method, we can easily obtain (after in-solution digestion) identification of 3600 proteins without fractionation, meaning that fractionation with IEF will sensibly increase this number (**Figure 6B**).

FIGURE AND TABLE LEGENDS:

Figure 1: Flow cytometry analysis of CD14 expression of PBMC before sorting (left panel) and after sorting (right panel) showing the obtained purity after magnetic beads selection.

Figure 2: Phase-contrast images of differentiated human macrophages showing heterogeneity of the obtained morphologies for two different polarizations. Scale bar represents 50 µm.

Figure 3: Imaging of Coomassie blue stained gel showing the various bands that will be excised [here, 6 bands in M(Ø) macrophages] for 5 polarizations of macrophages exposed to a low oxygen environment. IC = immune complexes, DXM = dexamethasone.

Figure 4: MS/MS spectrum and quantification. (A) An example of an MS/MS spectrum. Shown here is the CID (collision-induced dissociation) spectrum of a peptide found at *m/z* 597.29 on the MS spectrum with an electric charge of +2. The corresponding sequence was determined from this spectrum as Val-Ala-Glu-Leu-Glu-Asn-Ser-Glu-Phe-Arg from the protein CD58. (B) Rank ordered label-free quantification for each of the identified proteins (log_{10} LFQ).

Figure 5: Heat map representing the hierarchical clustering of all polarization states using differentially expressed proteins. Analysis reveals a cluster of proteins overexpressed in all polarizations in the 3% O_2 condition (red rectangle). The color scale represents z-scores (log2 intensity). Each row is a protein and each column is a sample. This figure originated from a previous publication[14].

Figure 6: SDS-PAGE and chromatogram. (A) Silver-stained SDS-PAGE gels with protein from cell lysis and after in-solution digestion showing the absence of degradation during lysis and efficiency of the digestion. (B) Chromatogram obtained from after in-solution digestion without fractionation.

Table 1: List of over-expressed proteins for human macrophages common to each polarization under low oxygen tension.

DISCUSSION:

Because proteomics is a powerful tool to study the expression of different proteins from a whole cell or subcellular compartments, optimization of the cell lysis protocol and digestion of proteins has been addressed by a number of studies. There are three main classes of methods, which include in-gel digestion (digestion of proteins in polyacrylamide gel matrix)[17], digestion in solution[18] and filter-aided sample preparation[19]. This last method, at first described as universal, has been reported to exhibit low reproducibility and possible loss of proteins on the filter[20]. In-gel digestion is a robust method that can be time-consuming and disadvantageous in that assessing the efficiency of digestion is not easy, if possible. In-solution digestion offers this possibility but requires the cleaning of samples after digestion and IEF. When these two methods are compared between the same sample, in-solution digestion with IEF fractionation protocol yields a higher number of identified proteins (with the same number of fractions) than in-gel digestion[21].

Despite this advantage, it is necessary to consider the possible protein degradation during in-solution lysis due to intracellular proteases (especially in myeloid cells). It is also important to bear in mind that these techniques are based on protein digestion and only able to analyze proteins presenting trypsin specific cleavage sites. It is possible to use a top-down proteomic approach that relieves this digestion constraint but adds data analysis steps and bioinformatics ressources[22]. The solubilization of proteins from various cellular compartments can also be difficult to obtain, especially from plasma membranes, leading to an uncontrolled sampling of cellular proteome. In order to proceed with a nano-LC-MS/MS mass spectrometer analysis of the samples, it is important to obtain a sufficient quantity of peptides, which can depend on the mass spectrometer used (usually, starting total protein should be at least 1 µg for a condition, and it is

implied to increase this quantity according to the number of fraction used with IEF). This constraint may be a drawback if the cell population being studied is scarce, which differentiates proteomic from genomic techniques in which amplification of raw material is possible.

Even after the seminal works of Richer and colleagues[23] and Packer and Fuehr[24], the importance of oxygen in cell cultures has been insufficiently recognized. We now know that culturing cells under low oxygen concentrations favors adhesion, lifespan, and division. It is recognized that this is of utmost importance in stem cell research[25]. The main technical issue for cell cultures under controlled oxygen conditions is related to maintenance of the desired oxygen concentration during the entire experiment. This requires pre-incubation of all media to prevent release of dissolved oxygen and use of hypoxic working stations to permit the manipulation of cells under low oxygen (processing chamber with glove box) and prevent transient exposition to high oxygen conditions.

The described protocol was used to obtain the molecular signatures of various polarizations of human monocyte-derived macrophages and study the effects of oxygen modulation on these signatures. This study has given insight on the description of those polarizations and has revealed some functional consequences. For example, we found that many proteins involved in efferocytosis were modulated by a low oxygen environment. This proteomic approach, based on the described protocol, presents the opportunity to explore how environmental parameters modify macrophage functions and how these signals can be used to design new therapeutic approaches[14].

The proteomic approach described in this work is complementary to genomic approaches that have been used during recent years in the field of human macrophage polarization studies. Proteomics offer the advantage of protein quantification, which may present a different expression than their corresponding mRNAs due to post-translational modifications and lead to the discovery of new biomarkers. Despite this advantage, proteomic data is usually difficult to interpret, in part due to the high sensitivity of mass spectrometry, leading to very complex MS spectra and false positive detection of peptides. Recently, analysis software has gained efficiency in order to prevent this. Even if it is a changing situation, proteomics also faces lower reproducibility than genomics[26] and is associated with validation steps using other techniques (flow cytometry, immunoblotting) to confirm quantitative modifications of protein expression levels.

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DISCLOSURES:
The authors declare no conflicts of interest.

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Figure

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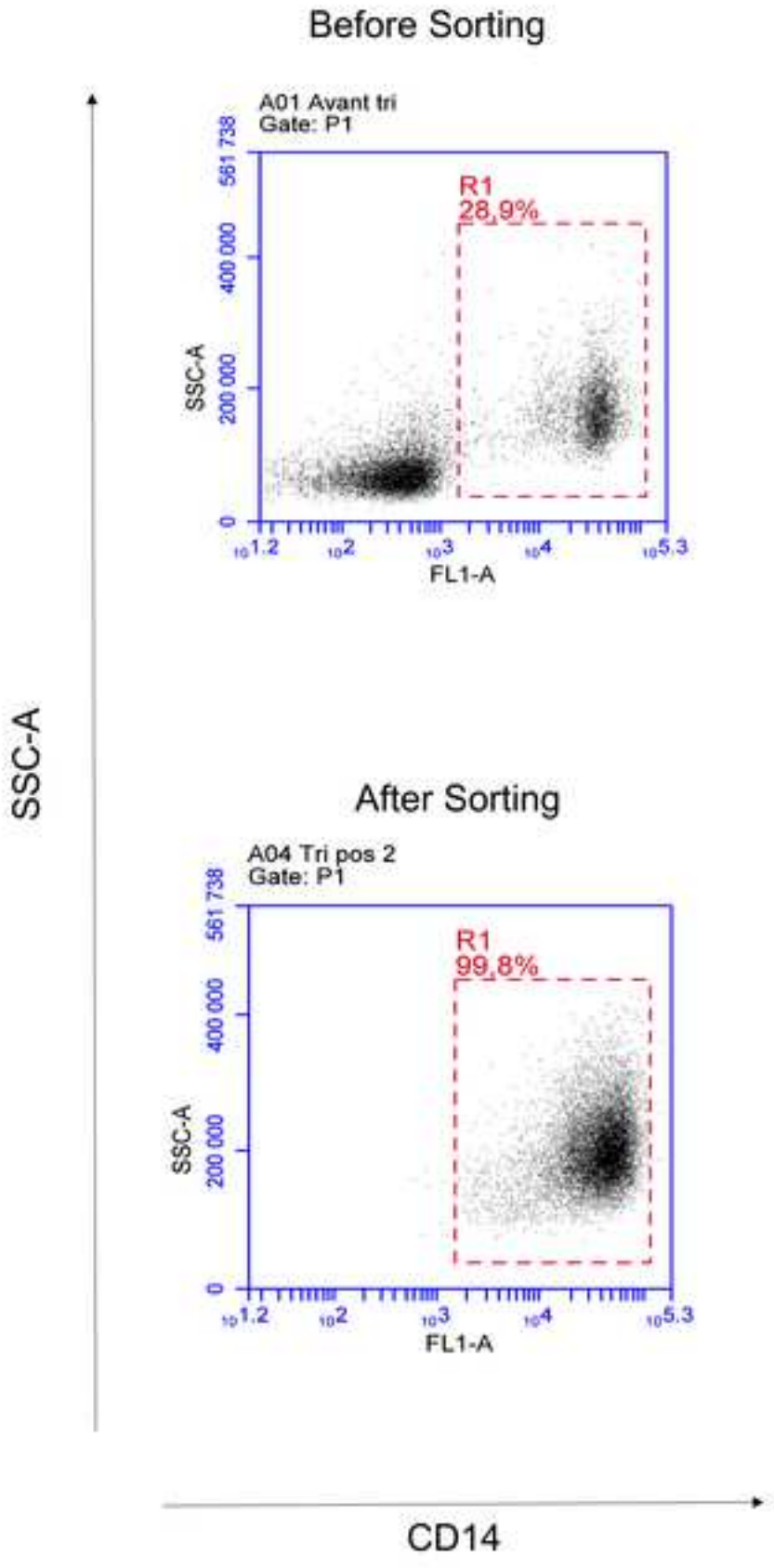

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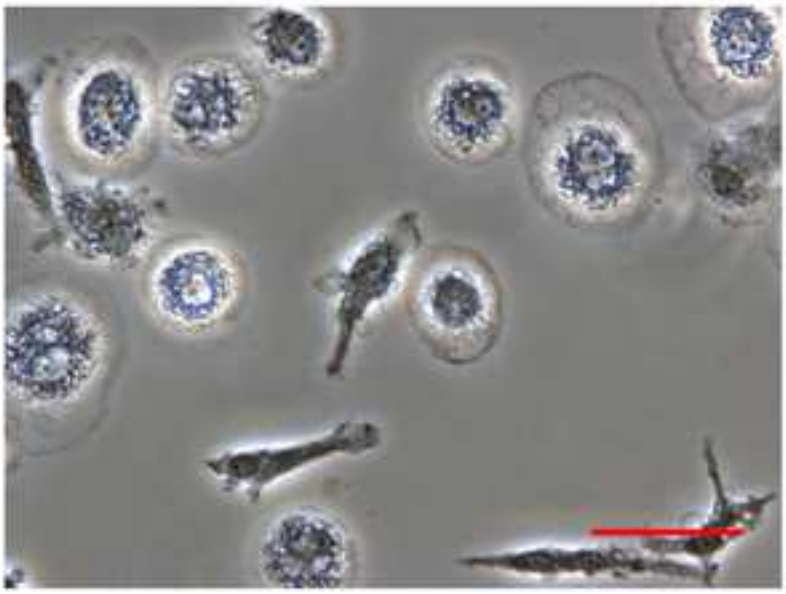

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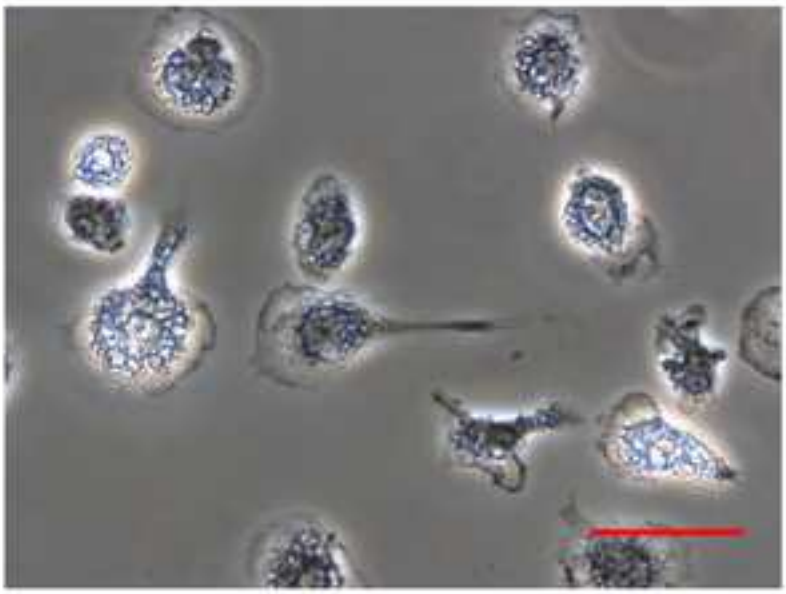

Figure

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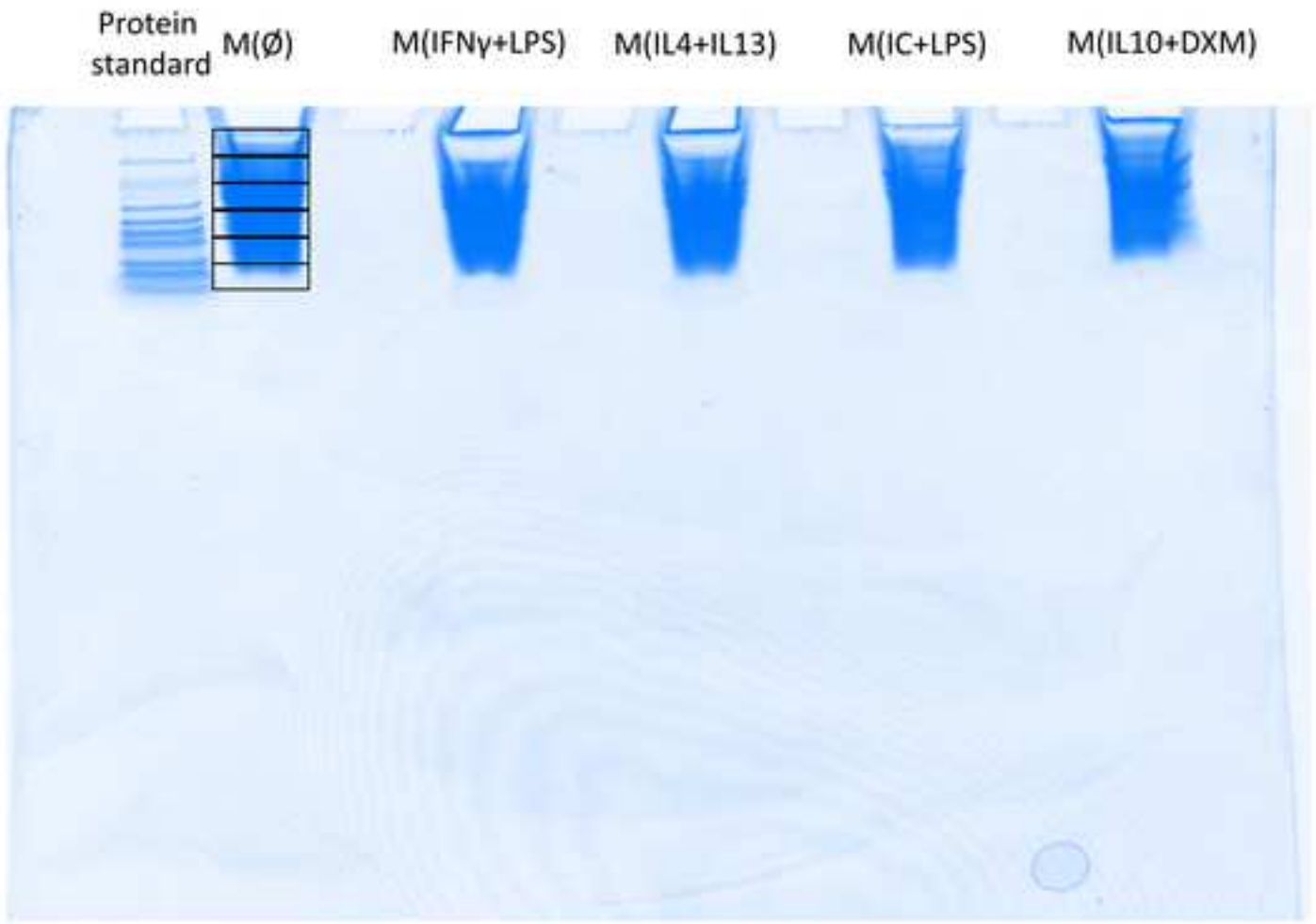

Figure

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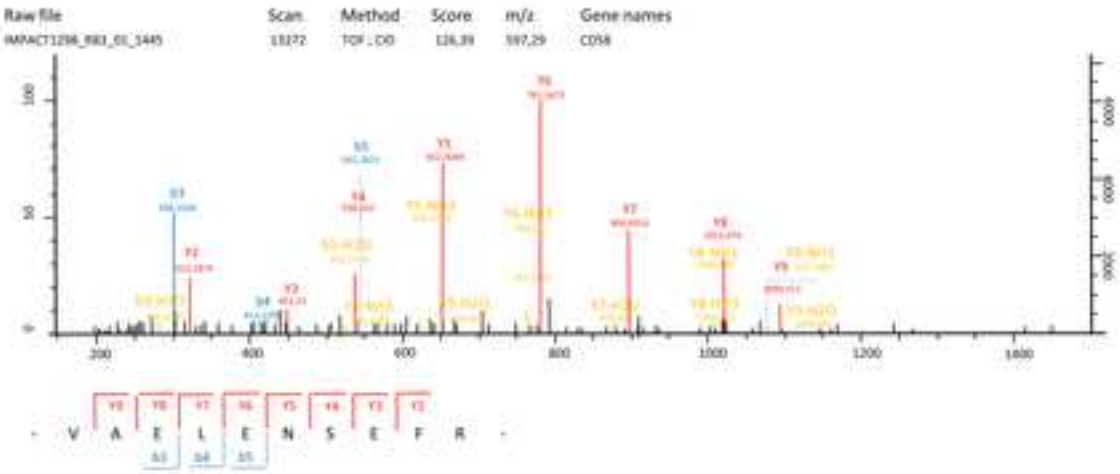

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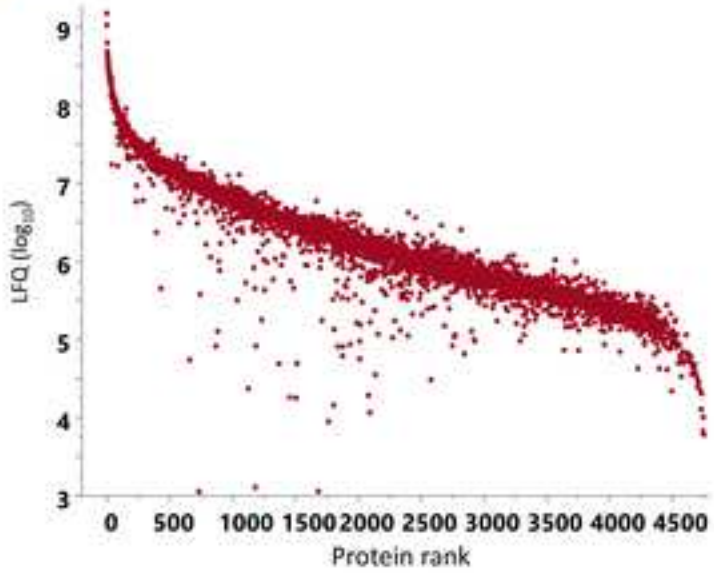

Figure

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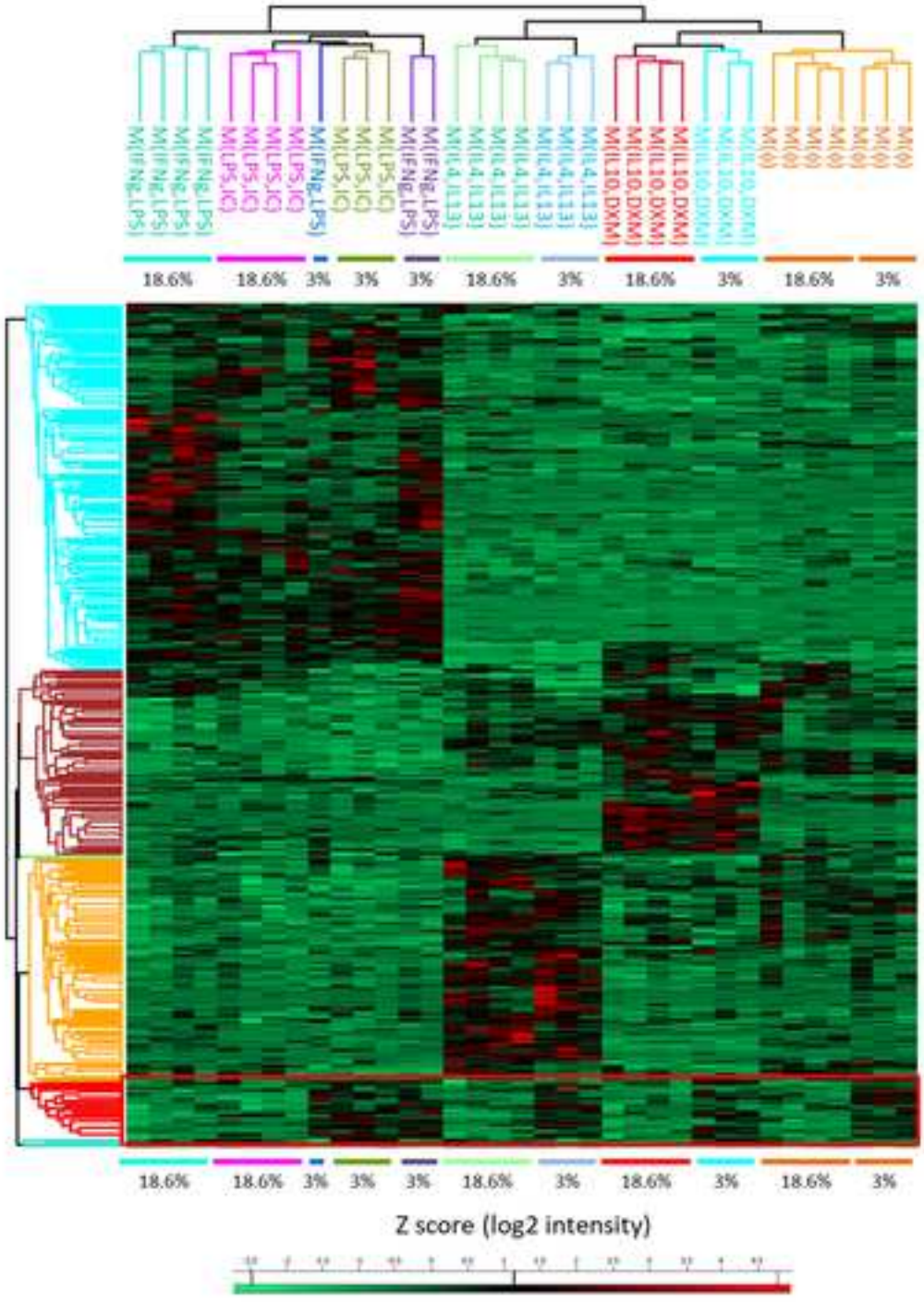

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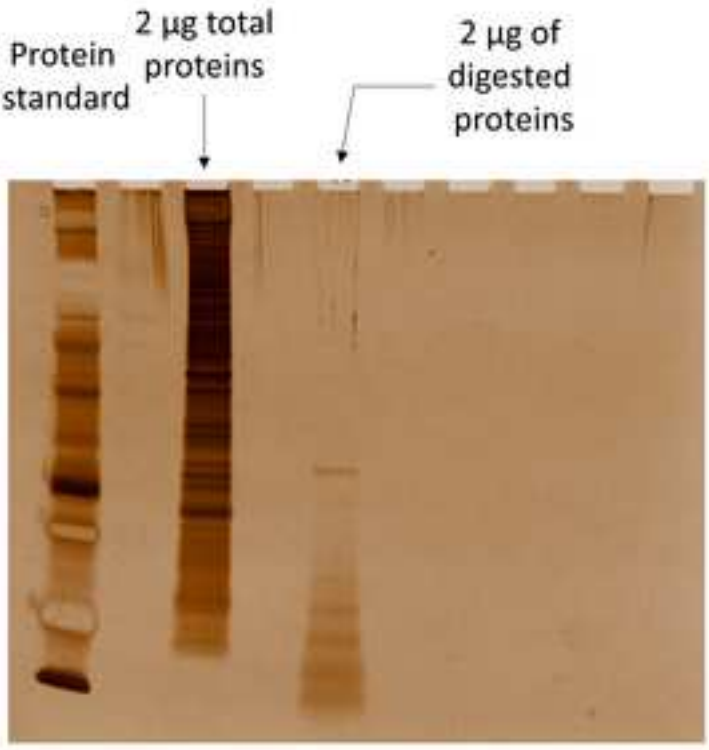

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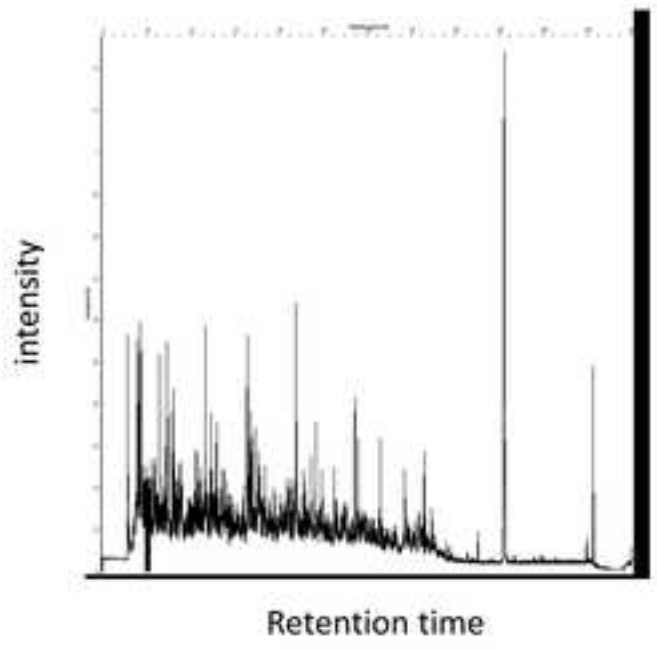

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Red	Q8WWQ0
Red	E5RHK8
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Red	O75489
Red	P21912
Red	A0A024R1Y7
Red	E5KRK5
Red	A0A024QZ30
Red	A0A024R2F9
Red	A0A024R5K3
Red	O76003
Red	Q1HBJ4
Red	Q13151
Red	V9HWN7
Red	B4DVJ0
Red	V9HWB9
Red	P13674
Red	Q6FHV6
Red	Q99798
Red	P17858
Red	A0A024R872
Red	A0A024RC61
Red	V9HWF4
Red	Q12882
Red	B4DEQ0
Red	D9UAX9
Red	V9HWK1
Red	Q96HE7
Red	P14868
Red	P36871

Protein names	Gene names
Heat shock 70 kDa protein 1B	HSPA1A
Sodium/potassium-transporting ATPase subunit beta-3	ATP1B3
Beta-mannosidase	MANBA
NADH dehydrogenase [ubiquinone] iron-sulfur protein 7, mitochondrial	NDUFS7
PH-interacting protein	PHIP
Dynamin-3	DNM3
Fructose-bisphosphate aldolase C	ALDOC
NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial	NDUFS3
Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial	SDHB
GH3 domain-containing protein	LGP1;GHDC
NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	NDUFS1
Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	SDHA
Transmembrane protein 43	TMEM43
NADH dehydrogenase [ubiquinone] iron-sulfur protein 8, mitochondrial	NDUFS8
Glutaredoxin-3	GLRX3
Mitogen-activated protein kinase;Mitogen-activated protein kinase 1	MAPK1
Heterogeneous nuclear ribonucleoprotein A0	HNRNPA0
Fructose-bisphosphate aldolase A	ALDOA
Glucose-6-phosphate isomerase	GPI
L-lactate dehydrogenase;L-lactate dehydrogenase A chain	LDHA
Prolyl 4-hydroxylase subunit alpha-1	P4HA1
Gamma-enolase;Enolase	ENO2
Aconitate hydratase, mitochondrial	ACO2
ATP-dependent 6-phosphofructokinase, liver type	PFKL
Niban-like protein 1	FAM129B
Aminopeptidase N	ANPEP
Phosphoglycerate kinase;Phosphoglycerate kinase 1	PGK1
Dihydropyrimidine dehydrogenase [NADP(+)]	DPYD
Electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial	ETFDH
MHC class I antigen	HLA-B
Triosephosphate isomerase	TPI1
ERO1-like protein alpha	ERO1L
Aspartate--tRNA ligase, cytoplasmic	DARS
Phosphoglucomutase-1	PGM1

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4	4	4
5	5	4
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Fasta headers

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>sp|O00462|MANBA_HUMAN Beta-mannosidase OS=Homo sapiens GN=MANBA PE=2 SV=3;>tr|A7LFP5|A7LFP5_HU
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>tr|E5KRK5|E5KRK5_HUMAN Mitochondrial NADH-ubiquinone oxidoreductase 75 kDa subunit OS=Homo sapiens GN=
>tr|A0A024QZ30|A0A024QZ30_HUMAN Succinate dehydrogenase complex, subunit A, flavoprotein (Fp), isoform CRA
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>sp|Q99798|ACON_HUMAN Aconitate hydratase, mitochondrial OS=Homo sapiens GN=ACO2 PE=1 SV=2;>tr|B4DZ08
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>tr|A0A024R872|A0A024R872_HUMAN Chromosome 9 open reading frame 88, isoform CRA_a OS=Homo sapiens GN
>tr|A0A024RC61|A0A024RC61_HUMAN Alanyl (Membrane) aminopeptidase (Aminopeptidase N, aminopeptidase M,
>tr|V9HWF4|V9HWF4_HUMAN Phosphoglycerate kinase OS=Homo sapiens GN=HEL-S-68p PE=2 SV=1;>sp|P00558|P
>sp|Q12882|DPYD_HUMAN Dihydropyrimidine dehydrogenase [NADP(+)] OS=Homo sapiens GN=DPYD PE=1 SV=2
>tr|B4DEQ0|B4DEQ0_HUMAN cDNA FLJ59482, highly similar to Electron transfer flavoprotein-ubiquinone oxidoredu
>tr|D9UAX9|D9UAX9_HUMAN MHC class I antigen (Fragment) OS=Homo sapiens GN=HLA-B PE=3 SV=1
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>sp|Q96HE7|ERO1A_HUMAN ERO1-like protein alpha OS=Homo sapiens GN=ERO1L PE=1 SV=2
>sp|P14868|SYDC_HUMAN Aspartate--tRNA ligase, cytoplasmic OS=Homo sapiens GN=DARS PE=1 SV=2;>tr|Q53T60|
>sp|P36871|PGM1_HUMAN Phosphoglucomutase-1 OS=Homo sapiens GN=PGM1 PE=1 SV=3;>tr|B4DFP1|B4DFP1_H

sequence coverage
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3.1
16.9
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42.4
36.4
14
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73.6
40.3
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57.9
49.1
53.1
52.7
80.8
41.8
19.6
50.9
92
66.9
60.5
42.3

Table of Materials

Click here to access/download;Table of Materials;Table of Material and Equipment.xlsx

Product/Material	Company
Hypoxia Working Station	Oxford Optronix
C6 Flow cytometer	BD
Urea	Agilent Technologies
Formic acid (FA)	ARISTAR
R-250 Coomassie blue	Biorad
Lipopolysaccharide, *E.Coli* (LPS)	Calbiochem
2D clean-up kit	GE Healthcare
RPMI 1640 medium, glutamax supplement	Gibco
HEPES 1 M	Gibco
MEM Non-Essential Amino Acids (NEAA) Solution 100X	Gibco
Phosphate Buffered Saline (PBS) 1X	Gibco
Harvard Apparatus column Reverse C18 micro spin column	Harvard Apparatus
EDTA 0.5 M, pH 8.0	Invitrogen
NuPAGE Bis-Tris 4-12%	Life Technologies SAS
CD14 Microbeads human	Miltenyi Biotec
MACS separation column LS	Miltenyi Biotec
Macrophage colony-stimulating factor (M-CSF)	Miltenyi Biotec
Interleukin 4 (IL4)	Miltenyi Biotec
Interleukin 13 (IL13)	Miltenyi Biotec
Interferon gamma (INFγ)	Miltenyi Biotec
CD14-FITC (clone TÜK4)	Miltenyi Biotec
MACSmix Tube Rotator	Miltenyi Biotec
Trifluoroacetic Acid (TFA)	Pierce
Trypsin/Lys-C Mix	PROMEGA
Complete Mini, EDTA-free Protease Inhibitor cocktail	Roche
Density Gradient Solution (Histopaque 1077)	Sigma Aldrich
Accumax	Sigma Aldrich
Human Serum from human male AB plasma (SAB)	Sigma Aldrich
Bovine Serum Albumin (BSA) solution 30%	Sigma Aldrich
Trisma-base	Sigma Aldrich
Glycerol	Sigma Aldrich
β-Mercaptoethanol	Sigma Aldrich
Bromophenol blue	Sigma Aldrich
Sodium Dodecyl Sulfate (SDS) 20%	Sigma Aldrich
Ammonium bicarbonate	Sigma Aldrich
Acetonitrile	Sigma Aldrich
Dithiothreitol	Sigma Aldrich
Iodoacetamide	Sigma Aldrich
Thiourea	Sigma Aldrich
CHAPS	Sigma Aldrich
Micro BCA Assay Kit	ThermoFisher
5 mL sterile plastic pipette	VWR
Thermomixer C Eppendorf	VWR

Sep-Pak tC18 reverse phase cartridges, 100 mg	Waters

Reference	Notes
Hypoxylab	
Accuri C6	
5188-6435	
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Title of Article: Proteomic analysis of human macrophage polarizations under low O_2 environment

Author(s): Pascale COURT, Marie ALLIER, Arnaud JILLET

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CORRESPONDING AUTHOR:

Name: MILLET

Department: Tissue environment, cell plasticity and signalling

Institution: Institute for Advanced Biosciences - Inserm

Article Title: Proteomic analysis of human macrophage polarization under low O2 environment

Signature: [signature] Date: 07/03/2018

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All modifications appear in red in the revised manuscript. In the following letter we answer every point raised by the editor.

Editorial comments:

1. The language is still unclear in places; please thoroughly proofread, ideally by a fluent English speaker.

The text has been proofread by a fluent English speaker and modifications have been done in the text and appear in red in the revised manuscript.

2. 1.1/1.3: What do you mean by 'later called'?

We have clarified this point and changed 'later called' by 'referred to as macrophage medium throughout the protocol'

3. 2.1: Please provide the density gradient solution in the Table of Materials.

We have added the density gradient solution in the Table of Materials.

4. 2.2: Where does the LRSC sample come from? Please clarify. Also, if this is from human blood, as seems to be the case, please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

We have added the following sentence:
Human blood samples (LRSC) from healthy de-identified donors were obtained from EFS (French national blood service) as part of an authorized protocol (CODECOH DC-2018–3114). Donors gave signed consent for use of their blood.

5. 2.4: I'm not sure what a 'ring' is here-do you mean 'layer'?

We have changed 'ring' to 'layer'

6. 3.1: What is a typical amount of PBMCs used?

We have precised the amount of PBMC: "typically 100-300 10^6 cells"

7. 3.7: Steps 3.8-3.13 do not exist.

This was a mistake the steps are "3.4 to 3.7"

8. 6.1: Please include the working station in the Table of Materials.

We have added the working station in the Table of Materials.

9. 7.1: What is a ‘pinch’ of bromophenol blue?

We have precised the amount of bromophenol blue: 0.2 % w/v

10. 11/12: Please remove the embedded tables and incorporate into the text.

This has been modified in the revised manuscript.

11. All the figures are rather low-resolution and pixelated (not just in the pdf); please provide higher-resolution ones (at least 300 dpi). Additionally, please remove ‘Figure 1/2/etc.’ from the Figures themselves, and remove all unnecessary whitespace.

We have performed the required modifications and are now in position to provide figures of more than 600 dpi.

12. Figure 4A: Why is this sideways? Please rotate.

We have modified the picture accordingly.

13. Table 1 is not present in the current submission.

This was an omission we provide the table in the new version.