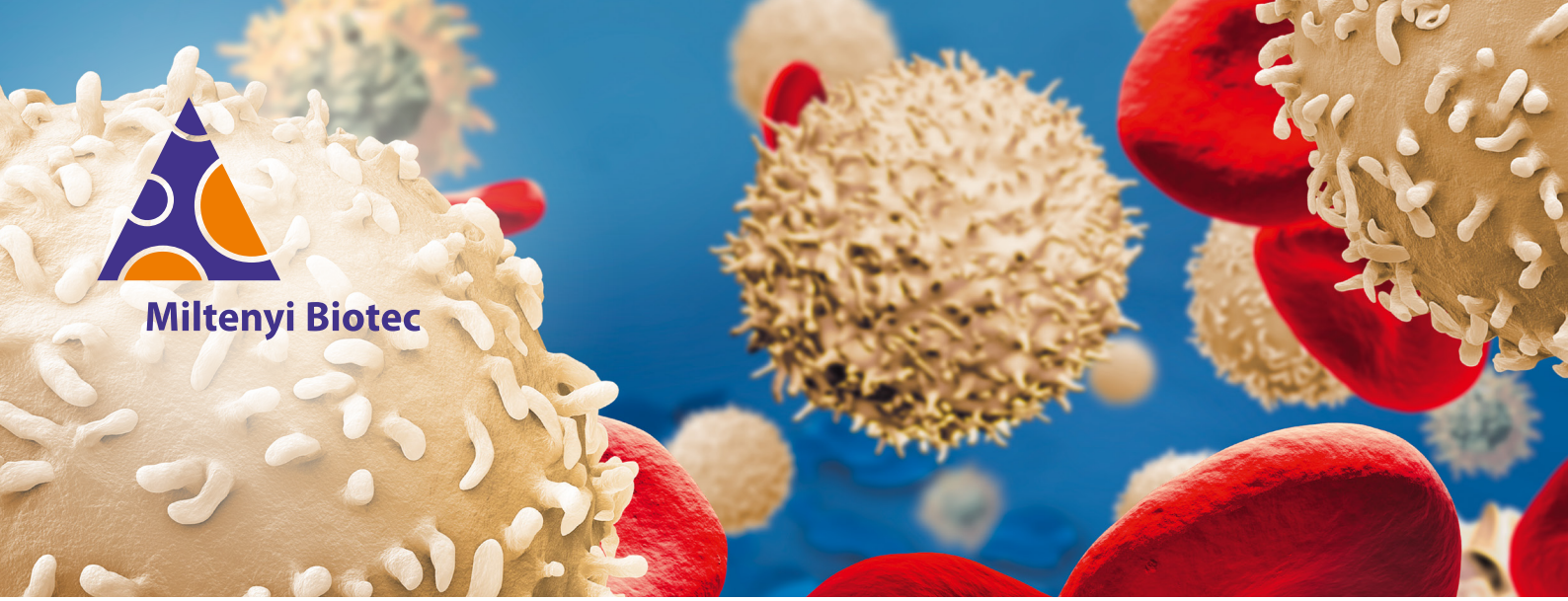

Enabling in depth omics investigation

Automated isolation of highly pure CD14+ monocytes from human blood

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Background

Immunometabolism is an expanding field with the underlying principle being that cellular metabolism underpins immune cell function[1]. Monocytes are cells whose own adaptations and contribution to tissue macrophage pools are implicated in a wide variety of altered states, such as pregnancy[2] and obesity[3,4]. As a blood immune cell, monocytes are relatively readily available for analysis in human populations, including healthy volunteers, clinical, or other groups of interest. Peripheral blood mononuclear cells (PBMCs) yield a heterogenous mix of which monocytes are only a minor cell population. Therefore, detailed downstream analyses of their function requires further isolation of highly pure monocytes. Here we show a method in which highly pure CD14+ monocytes can be isolated from human peripheral blood with an autoMACS® Pro Separator (fig. 1) and used for immunometabolic analysis. Flow cytometric analysis done in parallel monitors purity and analysis of nutrient transporters and uptake. Monocyte immunometabolism[7] is successfully analyzed as well as a further detailed analysis including metabolic capability to use specific types of fuels – carbohydrates, amino acids, and fatty acids – and targeted transcriptomics.

Figure 1: autoMACS® Pro Separator. Cells of interest are labeled with superparamagnetic nano-sized beads and then passed through a MACS® Column. When a magnetic field is applied the labeled cells are retained within the column, allowing the unlabeled fraction to be eluted. Once the negative fraction has been eluted, the magnetic field is removed, allowing the labeled cells to be collected in the positive fraction tube.

Materials

Cell isolation

- CD14 MicroBeads, human
- autoMACS® Pro Separator

Flow cytometry

- Anti-CD14 antibody, Pacific blue
- CD16 Antibody, anti-human, VioBlue®
- Anti-CD98 antibody, VioBright™ FITC

Bioenergetic analysis

- BPTES
- Etomoxir
- UK-5099
- Oligomycin
- FCCP
- Antimycin A
- Rotenone
- Seahorse XFe96 Extracellular Flux Analyzer

NanoString

- nCounter® Metabolic Pathways Panel
- nCounter® SPRINT Profiler

Methods

PBMCs were prepared via standard density centrifugation techniques from human peripheral blood obtained with healthy volunteer consent in anticoagulant containing blood collection tubes.

Magnetic cell isolation

- Cells were isolated with an autoMACS Pro Separator using a program optimized for purity.
- The positively selected $CD14^+$ monocytes were monitored for purity using flow cytometry and then used for bioenergetic analysis and metabolism focused targeted transcriptomics.

Flow cytometric analysis

- 0.1×10^6 monocytes were stained with anti-CD14 (pacific blue) for analysis of purity using standard techniques ahead of acquisition and analysis.

Bioenergetics analysis with the Agilent Fuels Assay

- $CD14^+$ monocytes were centrifuged for 300×g for 10 min before resuspension in Seahorse media
- To prevent cell motility during the assay, monocytes were seeded onto a Cell-Tak (22.4 µL/mL) coated 96-well Seahorse plate at 0.2×10^6/100 µL/well with Seahorse media.
- The plate layout was arranged with a minimum of three replicates and allowed for injection of up to four different treatments, such as cell stimuli or biochemical inhibitors of metabolic pathways of interest. Blank wells had to be included and these were filled with Seahorse media only.
- The plate was centrifuged at 200×g with no brake and 1 acceleration to allow the monocytes to gently adhere as a monolayer. 50 µL media/well was then added to the wells resulting in a final volume of 150 µL and the plate was incubated at 37 °C in an oven (not an incubator) for 45 min.

NanoString nCounter metabolism panel

- 1×10^6 monocytes were pelleted at 8,000×g for 4 min, and all media removed. Monocytes were resuspended in RLT buffer diluted 1/3 with RNase free water at a concentration of 10,000 cells/µL and stored at –80 °C until analysis.
- The monocytes were hybridized with the reporter and capture probes of the metabolic pathways panel and run on the SPRINT Profiler as per manufacturer's guidance.

Injection	Full name	Role
BPTES	Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide	Inhibitor of glutaminase (GLS1)
Etomoxir	-	Inhibitor of carnitine palmitoyltransferase-1 (CPT1)
UK-5099	2-Cyano-3-(1-phenyl-1H-indol-3-yl)-2-propenoic acid	Inhibitor of mitochondrial pyruvate carrier (MPC1)
Oligomycin	-	Inhibitor of ATP synthase
FCCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone	Uncoupler of mitochondrial oxidative phosphorylation
Antimycin A	-	Inhibitor of complex III
Rotenone	-	Inhibitor of complex I

Table 1: Injections used for the Agilent Seahorse Fuel Flex Assay.

Results

Isolation of CD14⁺ monocytes was optimized by using positive selection MicroBeads with two column separations for maximal purity (>90%). Isolating CD14⁺ monocytes allowed for a deeper investigation of their metabolic properties. The expression of nutrient transports on different monocyte subsets was explored in parallel using flow cytometry. Flow cytometric analysis also provided a platform for confirming purity of the isolated CD14⁺ cells. The use of anti-CD16 antibodies allowed for the monitoring and analysis of monocyte subsets: CD14⁺CD16⁻ classical and CD14⁺CD16⁺ non-classical monocytes with addition of antibodies of interest with the example of CD98, one of the components that form functional long chain neutral amino acid transporter (LAT), shown in figure 2. Monocytes then can be used with the Extracellular Flux Analyzer to determine their oxidative phosphorylation (oxygen consumption rate; OCR) and glycolytic capabilities (extracellular acidification rate; ECAR). The Agilent Seahorse Fuels Flex Assay is an adaptation of the Agilent Seahorse MitoStress™ assay that makes it possible to analyze the reliance of the monocytes on specific types of fuel, by inhibiting glutaminase 1 (indicator of glutamine usage), carnitine palmitoyltransferase 1A (long chain fatty acid usage), and mitochondrial pyruvate carrier (pyruvate usage). Figure 3 shows that the monocytes are mostly reliant on the import of pyruvate for oxidative phosphorylation, as it has the biggest decrease in maximal respiration in comparison to the control, followed by long chain fatty acids and they are the least reliant on glutamine. Targeted transcriptomics of the monocytes can be visualized using novel NanoString technologies, which can investigate up to 770 genes in one sample simultaneously. For this investigation into immunometabolism, the metabolic pathways panel was deemed most prudent. For visualization purposes only and to highlight the type of data which is possible to extract, monocytes from 6 healthy donors are shown in figure 4.

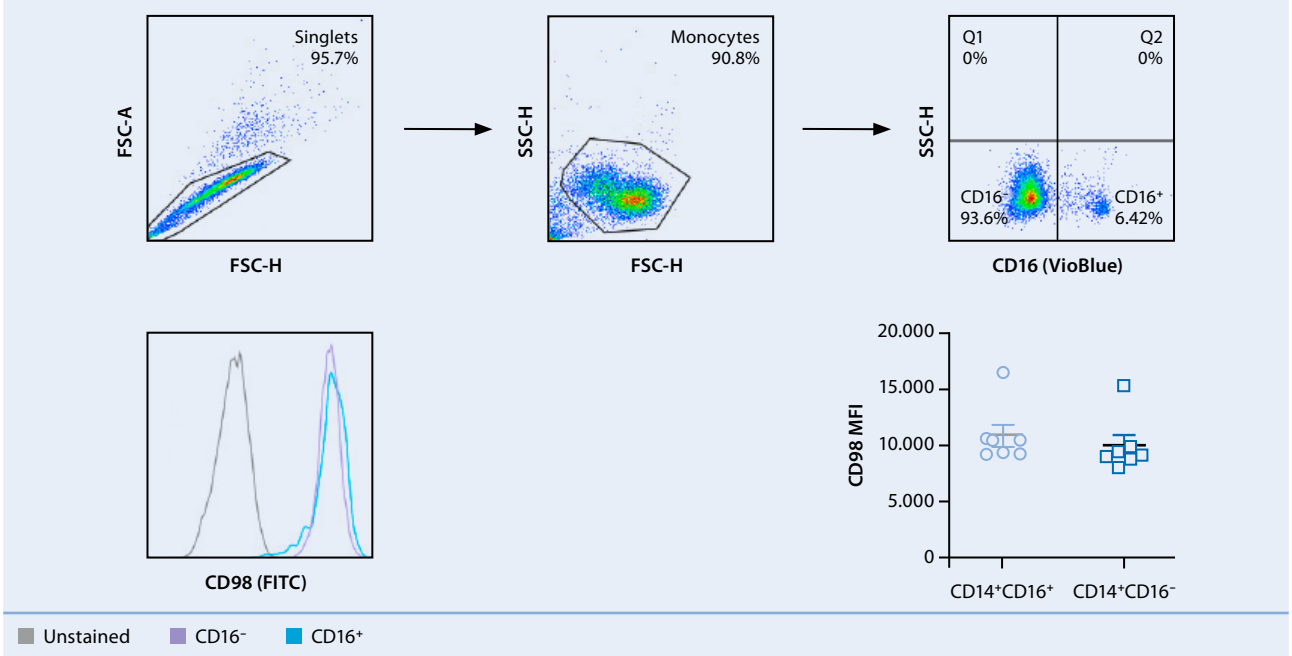

Figure 2: The identification of monocyte subsets in humans. Doublets are excluded with a forward scatter (FSC) height (H) versus area (A) plot. Isolated CD14⁺ monocytes are selected based on their size and granularity (FSC-H versus side-scatter [SSC-H]). Monocyte subsets were identified: CD16⁻ classical and CD16⁺ non-classical. Their CD98 expression is then observed as a histogram, with the MFI of n=12 shown.

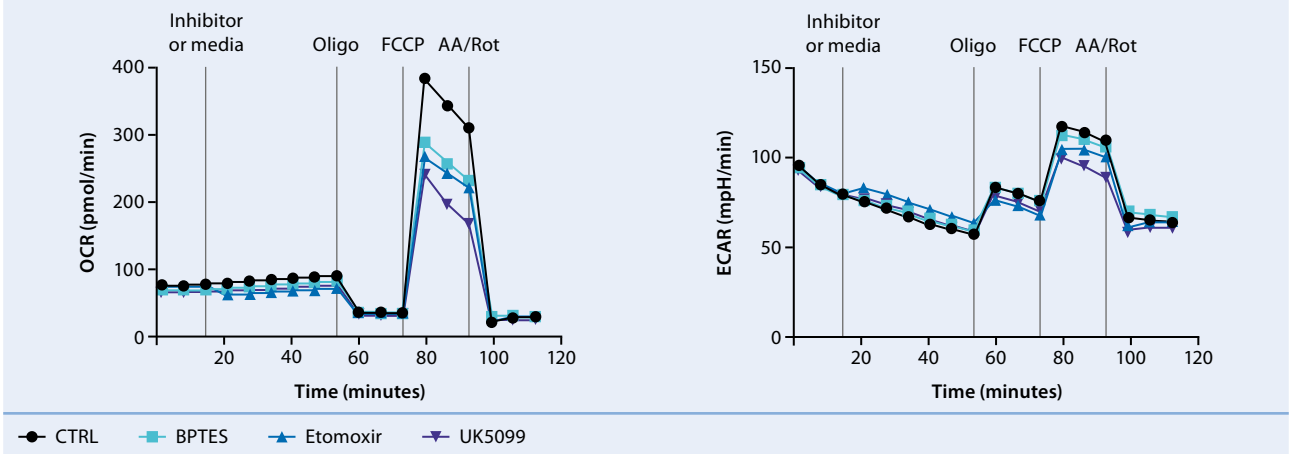

Figure 3: The effect of inhibitors of specific fuel utilisation pathways on monocyte metabolism. The fuels assay includes an injection with media or an inhibitor, followed by oligomycin, FCCP and antimycin A and rotenone. Maximal respiration occurs after the FCCP injection. OCR is indicative of the oxidative phosphorylation of the cells, whereas ECAR is indicative of the glycosis.

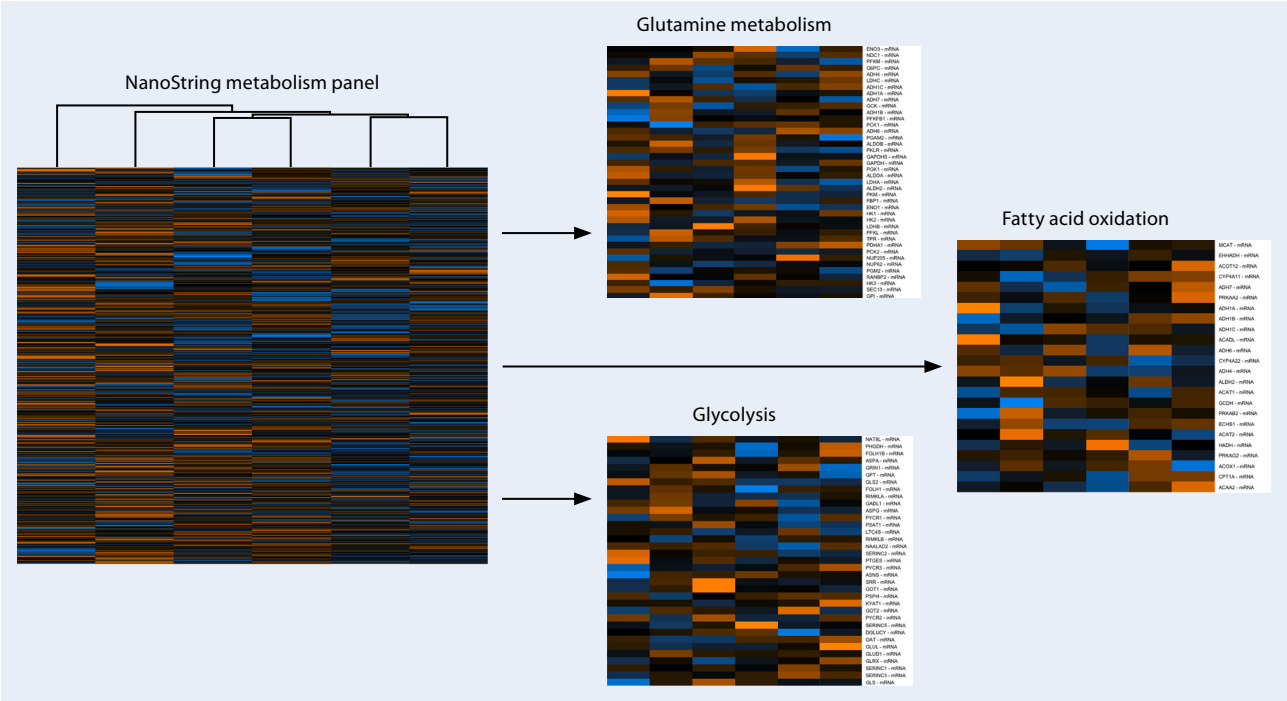

Figure 4: Analysis of monocytes using the NanoString nCounter metabolism panel. A heatmap is produced with an overview of all genes analyzed (up to 770 genes) for each sample (n=6). Individual heatmaps are also produced for genes relating to specific pathways, such as glutamine metabolism, fatty acid oxidation, and glycolysis.

Conclusions

- Monocyte isolation using the autoMACS® Pro Separator enables downstream studies where highly purified cell populations are required such as bioenergetics analysis.
- The immunometabolic profile of monocytes is now known, with a dependence on pyruvate shown and more prevalent genes are revealed.
- A similar workflow can be used to analyze other immune cell populations from T cells to eosinophils.

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