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Summary

Macrophages are plastic immune cells that can adopt several activation states. Fundamental to these functional activation states is the regulation of cellular metabolic processes. Especially in mice, the metabolic alterations underlying pro-inflammatory or homeostatic phenotypes have been assessed thoroughly using various techniques. However, researchers new to the field may encounter ambiguity in choosing which combination of techniques is best suited to profile immunometabolism.

To guide readers, we provide a toolbox to assess cellular metabolism in a semi-high-throughput 96-well-plate-based format. We applied the approach to activated mouse and human macrophages by metabolically pre-screening cells, followed by measuring extracellular fluxes, mitochondrial mass and membrane potential, glucose and lipid uptake, along with the application of SCENITH.

Hereby, we not only validated established activation-induced metabolic rewiring in mouse macrophages, but also uncovered new insights in human macrophage immunometabolism. By thoroughly discussing each techniques, we guide readers with a practical workflow to interrogate immunometabolism.

Keywords: immunometabolism, macrophages, toolbox, semi-high-throughput, metabolism

Motivation

In the last decade, it has become increasingly clear that cellular metabolism regulates immune cell function. This has sparked an expansion of immunometabolism research. However, the different techniques available may seem daunting to researchers new to the field and can be very time-consuming and costly. We therefore established a 96-well-plate-based immunometabolic toolbox and illustrate different techniques on mouse and human macrophages. Next to established methods such as extracellular flux analysis, NO, arginase, lactate and glucose measurements, the toolbox includes the recently published method SCENITH which has not been previously applied to macrophages *in vitro*. We further discuss advantages and limitations of each technique to guide readers to effectively use the toolbox in their own research setting.

Introduction

Macrophages are innate immune cells that reside in tissues or differentiate from circulating monocytes and regulate acute inflammatory responses and tissue homeostasis (Wynn et al., 2013). While they can adopt a broad spectrum of activation states, *in vitro* macrophage research primarily focusses on lipopolysaccharide (LPS)-, LPS plus interferon (IFN) γ-, and interleukin (IL)-4-induced activation states. LPS±IFNγ-induced macrophages (classically activated macrophages, M[LPS±IFNγ]) produce high levels of pro-inflammatory cytokines and show increased surface expression of (co)stimulatory immune activation markers. Conversely, IL-4-induced macrophages (alternatively activated macrophages, M[IL-4]) upregulate a different set of surface markers and enzymes involved in repair and homeostasis (Martinez et al., 2013; Murray et al., 2014).

Metabolic rewiring resides at the core of phenotypic polarization and has been shown to direct immune responses (Jha et al., 2015). Upon stimulation with LPS, mouse macrophages increase metabolic flux through glycolysis and the pentose phosphate pathway (PPP) which in turn fuels reactive oxygen species (ROS) production and nitric oxide (NO) synthesis (Baardman et al., 2018; Bailey et al., 2019; Van den Bossche et al., 2017). M[LPS] mouse macrophages also display a disruption of the tricarboxylic acid (TCA) cycle at isocitrate dehydrogenase (IDH) and succinate dehydrogenase (SDH) (Jha *et al.*, 2015). The LPS-induced downregulation of IDH results in the shunting of (iso)citrate towards synthesis of aconitate and subsequent accumulation of anti-microbial itaconate, mediated by the *immunoresponsive gene 1* (*Irg1*)-encoded enzyme ACOD1 (Lampropoulou et al., 2016). As a result of blunted SDH activity, succinate accumulates and functions as an immunoregulatory metabolite in macrophages that directs the immune response via hypoxia-inducible factor 1 alpha (HIF1α) and other mechanisms (Harber et al., 2020; Mills et al., 2016). In sharp contrast with M[LPS±IFNγ], IL-4-stimulated mouse macrophages are characterized by an intact TCA

cycle, increased fatty acid oxidation (FAO) (Van den Bossche *et al.*, 2017; Vats et al., 2006) and increased arginase-1 (*Arg1*) activity that aids in the metabolic conversion of arginine to proline for the resolution of inflammation (Mills and O'Neill, 2016).

Although human macrophages have been less well described, they also undergo metabolic reprogramming in response to inflammatory stimuli which slightly differs from that in mouse macrophages. Firstly, opposed to mouse macrophages, human macrophages do not produce NO upon stimulation with LPS±IFNγ (Gross et al., 2014). Additionally, LPS-induced glycolysis is less pronounced or sometimes absent in human monocytes and macrophages (Lachmandas et al., 2016; Vijayan et al., 2019). Lastly, the disrupted TCA cycle and mitochondrial dysfunction as seen in mouse macrophages has not yet been shown in human macrophages (Vijayan *et al.*, 2019).

As metabolism dictates functional responses in macrophages and other immune cells, there is substantial potential in generating targeted therapeutics that combat chronic inflammatory disorders, infectious diseases and cancer (Galli and Saleh, 2020; Lim et al., 2020; Makowski et al., 2020). In the study of new therapeutic metabolic interventions lies the importance of measuring cellular metabolism in a time- and cost-effective manner. Currently, a commonly used method is extracellular flux (XF, also known as Seahorse) analysis, which measures extracellular acidification rates (ECAR) and oxygen consumption rates (OCR) as markers for glycolysis and mitochondrial oxidative phosphorylation (OXPHOS), respectively (Van den Bossche et al., 2015). This technique contributed to the key concept that classically activated mouse macrophages are more glycolytic, whereas mouse M[IL-4] macrophages show higher OXPHOS and FAO (Huang et al., 2014a; Tavakoli et al., 2013; Vats *et al.*, 2006). While the method gives a proper overview of core metabolic pathways, it does not reveal the regulation and activation of more specific cellular metabolic pathways or the direct uptake of nutrients from the environment.

A combination of several -omics techniques can explain delicate changes in cellular metabolism. As such, metabolomics measures the abundance of metabolites and can reveal metabolic changes dictated by increased production or decreased substrate usage (Jha *et al.*, 2015; Rattigan et al., 2018). Also, (single-cell) RNA-sequencing (transcriptomics) shows regulation on gene expression level, and aids in evaluating the transcriptional regulation of all metabolic pathways (Artyomov and Van den Bossche, 2020; Jha *et al.*, 2015). However, RNA-sequencing neither allows the assessment of post-translational modifications that may dictate the function of metabolic enzymes, nor of metabolic enzyme activity. While new methods are arising that permit the measurement of metabolic enzyme abundance and activation by (flow) cytometry (Ahl et al., 2020; Hartmann et al., 2021; Levine et al., 2021), these are relatively

expensive in terms of time and costs, resulting in the inclusion of only a limited number of samples or experimental conditions. Therefore, we evaluate and integrate methods allowing for an effective screening of metabolic pathway activity to facilitate drug and inhibitor screens.

Here, we demonstrate an integrated approach in which cells and their supernatants can be used in parallel in different metabolic and phenotypic or functional readouts. While the individual methods have been published in separate papers, combining these distinct readouts facilitates a fast and cost-effective way to profile macrophage immunometabolism in a 96-well-plate-based format. First, we perform NO production and arginase activity measurements to quickly show metabolic alterations as a preliminary readout tool for mouse macrophages. Measurements of glucose consumption and lactate production serve as a first indication of altered glycolytic flux in both mouse and human macrophages. Next, we compare XF analysis with the recently developed method SCENITH for measuring metabolism in cell subsets (Arguello et al., 2020). Additionally, we apply fluorescent dyes to assess glucose and fatty acid uptake, mitochondrial mass, and mitochondrial membrane potential. Lastly, we provide a comprehensive protocol to measure specific substrate usage in intact and permeabilized cells as an optional follow-up method. The methods are applied to LPS±IFNγ- and IL-4-induced mouse and human macrophages, providing the tools for semi-high-throughput immunometabolic research that can be applied in macrophages and may be extended to different immune cells.

Results

Integration of 96-well-plate metabolic readouts into an immunometabolic profiling toolbox

We evaluated and combined existing techniques and integrated them in a toolbox to interrogate immunometabolism in a semi-high-throughput manner using 96-well-plates both in parallel and consecutively.

The approach presented here includes an easy and quick pre-screening for metabolic alterations (Figure 1) that can be combined with phenotypic/functional analyses (ELISA, flow cytometry, viability, etc.) and followed up by more dedicated readouts including XF analysis, SCENITH-based analysis of cell subsets (Arguello *et al.*, 2020) and by measuring uptake of fluorescent probes by flow cytometry or fluorescent imaging. Together, this integrated approach allows for fast (1 assay-day after 1-week culture) screening of macrophage metabolism and function.

Metabolic pre-screening gives first indications about altered macrophage metabolism

Mouse bone marrow cells and human monocytes were differentiated into macrophages (Supplementary Figure 1A-D) and subsequently left untreated or stimulated with either LPS±IFNγ or IL-4 (Figure 2A). Consistent with earlier studies, bone marrow-derived macrophages (BMDMs) displayed an LPS±IFNγ-mediated increase of NO production by inducible nitric oxide synthase (iNOS) and increased IL-4-induced arginase activity (Figure 2B) (Van den Bossche et al., 2016). Confirming literature, human monocyte-derived macrophages (HMDMs) did not show detectable NO production and arginase activity (Figure 2C).

While NO/arginase measurements are not applicable to human macrophages, measuring extracellular glucose and lactate levels in macrophage supernatants are fast approaches to interrogate the LPS±IFNγ-induced glycolytic switch in both mouse and human macrophages (Figure 2D, E). Increased glycolytic flux, as evidenced by increased glucose consumption and lactate secretion, is generally associated with increased inflammatory signaling and immune effector functions in macrophages and other immune cells (Freemerman et al., 2014; Voss et al., 2021).

XF analysis validates metabolic rewiring in LPS±IFNγ and IL-4-activated macrophages

More insight into alterations in glycolysis can be obtained by measuring ECAR via XF analysis. This method confirmed the increase in glycolysis in both mouse and human M[LPS±IFNγ] and additionally revealed increased glycolysis in IL-4-stimulated BMDMs (Figure 3A, E, Supplementary Figure 2A). An added value of XF analysis is that it simultaneously measures OCR as a proxy of mitochondrial function. Mouse macrophages displayed the expected

upregulated mitochondrial respiration after IL-4 treatment (Figure 3C, Supplementary Figure 2C), and decreased OCR following LPS+IFNγ activation (Figure 3C, E) (Jha *et al.*, 2015; Koenis et al., 2018; Van den Bossche *et al.*, 2016). The calculated mitochondrial and glycolytic contribution to ATP production followed a similar pattern as OCR and ECAR parameters in the different conditions (Figure 3F). In HMDMs, LPS+IFNγ significantly increased glycolysis parameters, while none of the stimuli significantly affected OCR-derived readouts (Figure 3B, D, G, Supplementary Figure 2B, D). Akin to mouse macrophages, the mitochondrial contribution to ATP production was significantly decreased by LPS±IFNγ and increased by IL-4 (Figure 3H).

Since metabolic readouts like XF analysis depend on cell count, data need to be normalized. Normalization methods are further discussed in the 'Practical considerations' section of the discussion. To normalize ECAR and OCR for cell counts, cell-permeable Hoechst dye was injected in the last step of XF analysis, followed by fluorescent imaging and counting as detailed previously (Little et al., 2020) (Supplementary Figure 2E). Normalization of XF data with relative Hoechst$^+$ object counts (counts per well / average counts in all wells (See methods for more detail)) reduced the variation between wells as indicated by reduced standard deviation of both ECAR and OCR-based parameters (Supplementary Figure 2F-H).

Flow cytometry-based SCENITH method allows metabolic profiling of cell subsets and correlates well with XF analysis

While XF analysis provides valuable insight into the glycolytic and mitochondrial function of cells, this bulk analysis requires relatively large cell numbers in the 96-well format. Moreover, it requires an XF analyzer that is not available in all laboratories. We therefore examined the SCENITH technology which can profile metabolism of distinct and small cell subsets (Arguello *et al.*, 2020). This technique is based on the fact that protein synthesis and ATP levels are often tightly connected and determines the effect of metabolic inhibitors on puromycin incorporation during protein translation to estimate glycolytic and mitochondrial dependency via flow cytometry. We assessed mouse and human macrophages with SCENITH and compared it with XF analysis, as done previously for T cells (Arguello *et al.*, 2020).

High puromycin incorporation (displayed as median fluorescence intensity, MFI) was detected in control samples, indicating a high level of protein synthesis in naïve BMDMs, and this was further increased after stimulation with LPS or IL-4 which correlated significantly with XF-derived total mitochondrial plus glycolytic ATP production (Figure 4A, Supplementary Figure 3A). This was not the case for HMDMs (Figure 4B, Supplementary Figure 3B). Addition of

Deoxy-D-Glucose (DG) for 30 minutes affected viability in some conditions, highlighting the need of including a viability dye (Supplementary Figure 3C, D).

Measurement of puromycin MFI after addition of inhibitors allowed for the calculation of distinct metabolic parameters as visualized in Figure 4C:
- Glucose and mitochondrial dependency were calculated as the proportion of protein synthesis dependent on glucose oxidation and OXPHOS, respectively (Figure 4A-C).
- Glycolytic capacity and fatty acid and/or amino acid oxidation (FAO/AAO) capacity indicate the maximum potential of cells to sustain protein synthesis when OXPHOS and glucose oxidation are inhibited, respectively (Arguello *et al.*, 2020).

DG almost completely abolished the puromycin signal in all macrophage stimulations, indicating overall high glucose dependency (Figure 4A-E). While M[LPS±IFNγ] depended almost exclusively on glucose, naïve and IL-4-treated BMDMs and HMDMs had the capacity to use FAO/AAO as fuel (Figure 4D, E). Accordingly, inhibiting mitochondrial ATP production with oligomycin (O) had the smallest effect in M[LPS+IFNγ] (Figure 4A, B, D, E). Together, the SCENITH-based calculations revealed reduced mitochondrial dependency and high glycolysis in M[LPS+IFNγ] (Figure 4D, E, F, H), which aligns with data obtained by XF analysis (Figure 4G, I).

An important advantage of SCENITH is that it can be combined with larger flow cytometry panels including for example M[LPS±IFNγ]- and M[IL-4]-associated activation markers. To test the capacity of SCENITH to directly link macrophage metabolism (as measured by puromycin inhibition) to phenotype (surface markers), we performed tSNE dimensionality reduction on oligomycin-treated BMDMs and HMDMs (Figure 4J, K). In tSNE graphs, differentially activated macrophages cluster separately for both mouse and human, with substantial overlap between the LPS and LPS+IFNγ populations (Figure 4J, K). Puromycin levels of oligoymcin-treated cells were highest, and thus the least affected by oligomycin, in ($iNOS^{hi}$) $CD80^{hi}$ $CD40^{hi}$ M[LPS+IFNγ] (Figure 4J, K, Supplementary Figure 3E, F), which in other analyses also showed to be the least dependent on mitochondria for ATP production. While oligomycin stimulation in the SCENITH experiment can be analyzed at single-cell resolution as shown in Figure 4J, K, cells need to be split into distinct wells and conditions to calculate all metabolic dependency and capacity parameters shown in the rest of Figure 4. As such, SCENITH should be regarded as cell subset analysis and not truly single-cell analysis.

<u>Measuring incorporation of fluorescent nutrient analogs yields complementary information to XF analysis and SCENITH</u>

To advance from SCENITH-based subset analysis to single-cell resolution, the uptake of fluorescent dyes that provide distinct insights into cellular metabolism can be measured by flow cytometry or imaging. We first titrated the dyes, followed by qualitative assessment via fluorescent imaging (Figure 5A, B), and validation that obtained signal could be inhibited by specific inhibitors to ensure that the selected concentration was not too high (Supplementary Figure 4).

2NB-DG is a fluorescent glucose analog that is used to assess glucose uptake by cells during the 30-minutes incubation in the presence of this dye. Flow cytometry revealed an elevated uptake of 2NB-DG in LPS-stimulated BMDMs, which correlated with the increased glycolysis as determined by XF analysis (Figure 5C). In comparison to naïve macrophages, LPS+IFNγ-activated BMDMs showed decreased 2NB-DG uptake. Yet, they consumed more glucose over a 24-hour time period (Figure 2D), probably indicating that they were saturated and were no longer capable of taking up more 2NB-DG. Although HMDMs did not show differences in 2NB-DG uptake (Figure 5E), it is a single-cell approach to assess glucose uptake that significantly correlates with bulk glycolysis measurements during XF analysis (Figure 5G).

In parallel to 2NB-DG, the fluorescent BODIPY C16 can be applied as a marker to estimate fatty acid uptake by cells via flow cytometry. In mouse macrophages, fatty acid uptake was increased by IL-4 and decreased by LPS±IFNγ (Figure 5D). This aligns with the observed FAO/AAO capacity by SCENITH and the fact that M[IL-4] macrophages show enhanced mitochondrial OXPHOS and FAO, whilst their LPS±IFNγ-induced inflammatory counterparts do not (Figures 3C, 4D). In HMDMs, fatty acid uptake was mostly increased by macrophage activation with both LPS+IFNγ and IL-4. Whereas fatty acid uptake cannot always be directly correlated to mitochondrial oxygen consumption (Figure 5F), BODIPY C16 provides an easy cytometry-based approach to assess fatty acid uptake in single cells.

MitoTracker Green is a fluorescent mitochondrial stain that is commonly used to estimate mitochondrial mass of cells by flow cytometry. Its signal aligns well with other mitochondrial mass measurements including mitochondrial DNA:genomic DNA ratio and mitochondrial complex immunoblotting and in certain conditions it relates to the spare respiratory capacity of cells (Baardman *et al.*, 2018; van der Windt et al., 2012). Here, MitoTracker Green signal was unchanged in all BMDMs and was decreased by the different stimuli in HMDMs (Figure 5G, I). In this setting, no correlation was found with XF analysis-derived mitochondrial readouts. In BMDMs, MitoTrackerGreen signal was unaffected by FCCP treatment, whereas a decrease was visible for HMDMs, indicating that MitoTracker Green staining may not be independent of mitochondrial membrane potential for all species or cell types (Supplementary Figure 4A-D).

TMRM is a red fluorescent probe that is designed to quantify changes in mitochondrial membrane potential. Such changes can relate to metabolic stress, proton leak, reverse electron transport and ROS production (Zorova et al., 2018), and as such TMRM signals often do not directly correlate with XF-derived mitochondrial parameters. Here, we observed a drop in TMRM signal in LPS+IFNγ- and IL-4-stimulated BMDMs and HMDMs, which did not correlate with OCR-derived mitochondrial readouts (Figure 5H, J).

Overall, the fluorescent probes provide an accessible option to estimate metabolic changes at single-cell resolution using equipment that is present in most laboratories. For good biological interpretation and in-depth insight, these readouts should be combined with other metabolic analyses presented above or followed up by more advanced readouts described below.

Potential follow-up beyond the 96-well plate-based metabolic profiling approach

When researchers want to obtain more in-depth knowledge about the metabolic changes observed in the readouts described above, we refer to a detailed overview of the distinct possibilities (Voss *et al.*, 2021), as well as to novel approaches providing single-cell and/or spatial resolution that are especially applicable in patient material (Artyomov and Van den Bossche, 2020). One potential follow-up option we further tested here is the use of substrate-coated plates as this is a less established technique. However, it might provide additional insight into macrophage immunometabolism.

This technique is based on the reduction of a redox dye by NAD(P)H or $FADH_2$ production as a result of mitochondrial respiration (Figure 1). Intact cells added to carbon-substrate-coated plates (PM-M01 plates) reveal insight in their utilization of sugars. Alternatively, permeabilized cells added to plates coated with mitochondrial respiration-related carbon sources (MitoPlate S-1) reveal increased insight into mitochondrial function.

Using carbon-substrate-coated plates, we observed that intact BMDMs highly upregulated D-glucose utilization after both LPS- and IL-4-activation (Supplementary Figure 5A). The maximum rate of D-glucose oxidation correlated well with glycolysis as determined by XF analysis and with 2NB-DG uptake (Supplementary Figure 5B). In parallel, we used mitochondrial-substrate-coated plates to obtain information about TCA cycle and mitochondrial function in distinct macrophage subsets (Supplementary Figure 5C, D). In agreement with the described breaks in the TCA cycle at IDH and SDH in inflammatory macrophages (Jha *et al.*, 2015), we observed decreased oxidation of isocitrate and succinate in LPS-activated BMDMs but not HMDMs (Supplementary Figure 6C, D). As such, the use of substrate-coated plates can be a useful follow-up method to study enzyme activity across a variety of metabolic pathways. Yet, it should be noted that this approach also yielded unexpected results that

appear in contrast with published results and data obtained by more established techniques such as XF analysis. For example, upregulation of mitochondrial respiration in M[IL-4] (Figure 3C, Supplementary Figure 2C) could not be reproduced in either plate type and alterations in FAO as observed with SCENITH (Figure 4D, E) could not be confirmed (Supplementary Figure 6E, F).

Together, the techniques assessed here investigate metabolism from different angles and all contribute to a deeper understanding of metabolic phenotypes. In the next sections, results will be discussed followed by a consideration of strengths and limitations of each technique.

Discussion

The recently increased appreciation that metabolic reprogramming is essential during innate immune responses now requires immunologists to select between different techniques and interpret data without formal training in metabolism. To help researchers entering the immunometabolism field, we laid out an approach that integrates distinct 96-well-plate-based metabolic assays and can be combined with common immune readouts (e.g. ELISA, cytometry) (Figure 1). This allows for easy, fast and cost-efficient pre-screening of the effect of candidate drugs (or other interventions) on immune cell metabolism along with function, before moving towards more advanced metabolic assays. In this discussion, we describe the strengths, weaknesses, pitfalls and caveats associated with the available assays, and provide a practical workflow that guides readers through the different possibilities, ranging from basic to more advanced.

Profiling immunometabolism highlights distinct metabolic rewiring in mouse and human macrophage activation states

As an example, we applied our immunometabolic profiling platform to LPS±IFNγ- and IL-4-stimulated BMDMs for which metabolic reprogramming is well-described (Lauterbach et al., 2019; Van den Bossche *et al.*, 2015; Vijayan *et al.*, 2019), and also applied the same stimuli to HMDMs for which metabolic processes are less well understood. While focused on macrophages, after optimization of certain factors (detailed in Supplementary Table 1) the approach can also be extended to other cells since individual techniques have previously been applied to, for example T cells and dendritic cells (Arguello *et al.*, 2020; Buck et al., 2016; Everts et al., 2012; Little *et al.*, 2020; Lopes et al., 2021; Scharping et al., 2016; Thomas and Mattila, 2014).

Mouse macrophages from different (sub)strains and mouse and human macrophages cannot be directly compared due to differences in metabolism between mice (sub)strains, different cell sources and diverse differentiation methods. However, it is worth mentioning some exposed similarities and differences between both species before proceeding with a technical discussion and practical guidance:

(i) Both mouse and human macrophages induced glycolysis upon inflammatory activation, as measured consistently with multiple methods. Since inflammatory signaling and glycolysis are often strongly connected, the distinct readouts that estimate glycolytic function can be applied as a first and easy way to assess whether functional changes induced by pharmacological and/or genetic interventions are paralleled by metabolic rewiring.

(ii) While measuring L-arginine metabolism via iNOS or arginase-1 was one of the first metabolic ways to discriminate between classical (LPS±IFNγ) and alternative (IL-4) macrophage activation in mice (Munder et al., 1998), we here confirm it is not a valid approach to monitor *in vitro* responses in human macrophages. This is due to *NOS2* being epigenetically silenced in HMDMs and arginase usually not being regulated in human monocytes/macrophages *in vitro* (Gross *et al.*, 2014; Thomas and Mattila, 2014).

(iii) Since HMDMs do not produce NO *in vitro* (Figure 2C) (Gross *et al.*, 2014), this could explain why they don't show the drop in mitochondrial respiration that is observed in their LPS±IFNγ-induced NO-producing mouse counterparts (Van den Bossche *et al.*, 2016; Vijayan *et al.*, 2019).

(iv) Notwithstanding the lack of OCR reduction in human inflammatory macrophages, they depend less on mitochondria for ATP production and as such this can be regarded as a commonality between both species.

(v) Fatty acids fuel mitochondrial respiration in reparative macrophages and this supposedly anti-inflammatory metabolic feature was most apparent in IL-4-activated BMDMs in XF analysis and HMDMs during SCENITH analysis.

(vi) It should be noted that variation between human donors is higher than the variation between mice as it is also the case for other (immunological) readouts. Further increasing the number of replicates (+6) could help to reach significance in some human conditions.

Importantly, the observed metabolic rewiring was sometimes context- and method-dependent, as highlighted in the technical discussion. Lastly, it is worth mentioning that we used M-CSF to differentiate human monocytes to macrophages while they can also be differentiated with GM-CSF. The latter induces a more inflammatory state when compared to M-CSF-differentiated macrophages (Jaguin et al., 2013; Lacey et al., 2012) and the limited research that compared both growth factors revealed that they can yield metabolic differences (Namgaladze and Brune, 2014). Dissecting the metabolic rewiring induced by different differentiation and activation factors is an avenue for future research. To address these and other questions, our integrated immunometabolic profiling approach will be valuable to efficiently investigate many conditions simultaneously. The practical workflow presented here (summarized in Figure 1) allows starting with a fast metabolic pre-screening of a broad range of conditions before narrowing down to a selective set of conditions for comprehensive metabolic characterization with specialized high-end techniques.

Interrogation of distinct metabolic pathways by complementary techniques

In this section, we elucidate how the different methods can give complementary insights into cellular metabolism. Due to the nature and sensitivity of the readouts, some differences in results may occur as detailed below.

Readouts of glycolysis

Extracellular glucose and lactate measurements, ECAR-derived parameters in XF analysis, SCENITH, 2NB-DG uptake and analyzing glucose utilization with carbon-substrate-coated plates allow inferences about glycolysis.

The uptake of 2NB-DG, together with ECAR-derived glycolysis, and glucose usage as analyzed with carbon-substrate-coated plates all correlated significantly for BMDMs. For both BMDMs and HMDMs, glycolytic capacity determined by SCENITH significantly correlated with ECAR-derived glycolytic capacity. Therefore, these techniques give similar results and the choice of method can thus depend on practical considerations such as sample type or desired resolution (i.e., bulk or single cell) as discussed in the workflow below. On the other hand, glucose and lactate measurements in the supernatant can differ from ECAR-derived glycolysis or 2NB-DG uptake due to the nature of the readouts (i.e., cumulative over 24 h or a snapshot at the 24 h timepoint). This is illustrated in LPS+IFNγ-activated BMDMs, which showed increased glucose consumption and lactate production over the course of 24 h but did not show increased ECAR or 2NB-DG uptake after this period. Therefore, these are complementary techniques that examine cumulative changes in extracellular glucose and lactate during the stimulation versus effects on glycolysis after this period, and results may not always be identical.

Mitochondrial function

To measure mitochondrial parameters, we here showed the use of XF-derived OCR, SCENITH, MitoTracker Green, TMRM, and mitochondrial-substrate-coated plates. As expected, OCR measurements showed decreased mitochondrial respiration in inflammatory BMDMs but not HMDMs due to species differences as discussed above. Still, mitochondrial contribution to total ATP production as calculated when taking along CO_2-based acidification in XF analysis, and mitochondrial dependence as assessed by SCENITH was decreased in both mouse and human inflammatory macrophages. Mitochondrial-substrate-coated plates confirmed this reduction in LPS-activated mouse (but not human) inflammatory macrophages. However, the substrate utilization assays appear less sensitive than XF and SCENITH since the metabolism of the individual mitochondrial substrates was not indicative of the well-established elevated mitochondrial respiration in IL-4-activated BMDMs when analyzing intact cells with carbon-substrate-coated plates nor permeabilized cells with mitochondrial-substrate-

coated plates. Since XF and SCENITH yield similar results, they can be selected based on other criteria.

Other parameters related to mitochondrial function include mitochondrial mass and membrane potential, measured by MitoTracker Green and TMRM, respectively. While MitoTracker Green is a reliable estimate of mitochondrial mass in BMDMs, caution and additional validation are warranted for HMDMs since staining may not be independent of mitochondrial membrane potential. Additionally, while reduced mitochondrial mass can in certain cases (such as in foam cells) explain a decrease in maximal respiration (Baardman *et al.*, 2018), changes in respiration as seen in activated macrophages are not per definition accompanied by a change in mitochondrial mass. Similarly, changes in mitochondrial membrane potential can reflect, among others, metabolic stress and reverse electron transport (Zorova *et al.*, 2018) and are difficult to directly relate to mitochondrial parameters. Furthermore, LPS-treatment has shown both increases (Mills *et al.*, 2016) and decreases (Yu et al., 2020) of mitochondrial membrane potential (TMRM), and is time-dependent (Bauerfeld et al., 2012). Therefore, while these readouts may correlate under certain circumstances, interpretation of results can be difficult and are therefore not a first go-to assay.

Fatty acid metabolism

Readouts for fatty acid metabolism demonstrated here include SCENITH, BODIPY C16 uptake and fatty acid utilization in mitochondrial-substrate-coated plates. SCENITH-derived FAO/AAO capacity followed the expected pattern for both BMDMs and HMDMs, although SCENITH cannot formally distinguish between FAO and AAO capacities. SCENITH-derived FAO/AAO capacity was paralleled by BODIPY C16 signal for BMDMs but not for HMDMs, indicating that uptake of fatty acids is not a direct measure of FAO. Rather, fatty acids can be stored in lipid droplets instead of being used to fuel metabolic processes (Feingold et al., 2012; Huang et al., 2014b). mitochondrial-substrate-coated plates did not pick up differences in fatty acid metabolism between conditions. Therefore, SCENITH and BODIPY C16 uptake can give first indications about fatty acid metabolism and could be extended by a BODIPY dye staining lipid droplets (Qiu and Simon, 2016) or an adapted XF protocol allowing to probe fuel preferences (Voss *et al.*, 2021).

While we now described the readouts per metabolic pathway, many techniques assess parameters of several pathways in parallel. The following section addresses advantages and disadvantages for each technique, in combination with a potential practical workflow to navigate through the distinct readouts.

Practical considerations

Here, we offer practical considerations and assess strengths and limitations of each approach to provide a practical guide on using the distinct techniques (Figure 6, Table 1).

Pre-screening of metabolic alterations

Generally, the metabolic pre-screening assays are quick, cost-efficient and easy to perform and are combinable with functional assays (e.g. cytokine ELISAs and viability assays). Therefore, the pre-screening is well-suited to screen many conditions simultaneously. NO and arginase assays are particularly useful to profile altered L-arginine metabolism in mouse macrophages, and can also be used for neutrophils, dendritic cells and NK cells (Thomas and Mattila, 2014). In case NO production is altered, assessing mitochondrial OCR with XF analysis is recommended since NO is known to affect mitochondrial function (Everts *et al.*, 2012; Van den Bossche *et al.*, 2016). Given the basic nature of these measurements which does not always reflect the intricate regulation of the metabolic pathways, researchers should be aware that no changes in glucose, lactate, NO or arginase levels do not automatically imply no metabolic changes. Therefore, if other indications exist to study metabolism (e.g., results of RNA-seq), more advanced metabolic readouts are a recommended next step.

XF analysis profiles glycolysis and mitochondrial function in parallel

XF analysis currently is a commonly used tool to study glycolysis and mitochondrial respiration. Besides standard use as described in the present study, injected inhibitors can be customized to assess dependence on main fuels or activity of electron transport chain components (Salabei et al., 2014; Voss *et al.*, 2021). Therefore, it is often a standard tool in an immunometabolism researcher's repertoire.

However, it also comes with certain disadvantages limiting its applications. Whereas extremely useful for homogeneous samples of which many cells are present (such as *in vitro* stimulated cells), only the core metabolic pathways that directly result in H^+ production or oxygen consumption are represented. Additionally, analysis of complex *in vivo* samples such as tumor tissue is much more challenging. Such samples include many distinct cell populations which cannot be separately assessed without prior cell sorting. Both cell sorting and cell culturing may affect the metabolic state of cells (Binek et al., 2019; Llufrio et al., 2018; Voss *et al.*, 2021). Generally, XF analysis requires large cell numbers in the 96-well format (typically 5,000-200,000 cells in 3-8 replicates).

Furthermore, since changes in viability, adherence, proliferation and plating may affect XF readouts, these data need to be carefully normalized. This can be done by measuring protein

content (e.g. BCA protein assay), DNA content (e.g. CyQUANT), biomass (e.g. Crystal Violet) on standard (fluorescent) plate readers or by cell counts using a 96-well plate imager. We refer to a comparison for details about the specific advantages and disadvantages of distinct normalization methods for XF analysis (Kam, 2018).

Additionally, for further assessing viability, we recommend assays based on cell membrane integrity (such as adding a live/dead marker in flow cytometry) instead of assays based on mitochondrial activity (such as the MTT assay) since mitochondrial activity may be affected without decreasing viability.

SCENITH allows metabolic phenotyping of distinct cell subsets

When the sample to assess consists of distinct cell subsets and when isolation of rare populations yields cell numbers too low for XF analysis, SCENITH can be used to evaluate metabolism using just one fluorescent channel in flow cytometry. It can provide additional phenotypic (and metabolic) insights when combined with antibodies against immune activation markers (and key metabolic mediators) in larger cytometry panels. Similar to XF analysis, the inhibitors used can also be extended or customized to assess different metabolic dependencies.

Although a range of cell types was tested in the original publication, situations may exist for which protein synthesis is not directly related to ATP synthesis, e.g. in quiescent stem cells (Arguello *et al.*, 2020). Furthermore, as the metabolic parameters are calculated from samples treated with inhibitors in parallel rather than from one single sample, metabolic parameters cannot be calculated for each single cell but rather for cell subsets. Additionally, combining SCENITH with large panels can become costly, since each sample needs to be split, stained, and measured in parallel in order to accommodate the different inhibitors.

Fluorescent metabolic probes enable assessing metabolic features at single-cell resolution

Fluorescent metabolic probes can be measured by flow cytometry or microscopy to provide single-cell resolution, and are practical when no XF analyzer is available. Flow cytometry allows for easier quantification of data but lacks the option for visual information. While metabolic dyes can in principle be combined into larger flow cytometry panels, this is practically limited due to protocol incompatibilities (e.g. staining temperature and duration, and not all dyes are fixable). Furthermore, care needs to be taken when assessing metabolic properties solely by fluorescent labeling as it was previously shown that 2NB-DG staining yielded substantially different results compared to more established glucose transport assays (D'Souza et al., 2021; Sinclair et al., 2020). This illustrates the importance of proper controls to ensure that dyes are taken up specifically. Dyes should also be titrated to prevent unspecific

staining of cellular compartments. Additionally, MitoTracker Green signal should be checked for independence of mitochondrial membrane potential to reliably estimate mitochondrial mass, since this may be species- or cell-type-dependent. Lastly, interpretation of results may be difficult due lack of robustness and timing- and context-dependent effects on mitochondrial function, particularly for MitoTracker Green and TMRM.

Next to the fluorescent probes measured here, a range of other fluorescent dyes and analyses exist to analyze metabolic alterations in response to specific treatments. Examples of other probes are mitoSOX for the measurement of mitochondrial ROS or propidium iodide for the analysis of cell cycle distribution. Additionally, a confocal microscope or multi-mode reader with high resolution can be used to reveal visual differences in subcellular localization or mitochondrial fragmentation (Little *et al.*, 2020).

The toolbox as discussed here gives an overview of metabolic alterations occurring after compound treatment and can help to narrow down stimuli of interest. These can be investigated in more detail with more complex techniques which will be discussed briefly in the following section. For more details we refer the reader to recent reviews (Artyomov and Van den Bossche, 2020; Voss *et al.*, 2021).

Limitations and potential follow-up beyond the toolbox

It should be noted that the readouts within the toolbox provide a good estimation of the immunometabolic state of macrophages, but that more dedicated high-end follow-up studies can be required to obtain more in-depth insight. One potential follow-up technique that we here illustrate is substrate utilization analysis for intact and permeabilized cells that facilitates the screening of fuel preference in homogeneous *in vitro* cultured cells. Due to the range of different substrates, a greater variety of metabolic pathways can be assessed in extensive detail.

Carbon-substrate-coated plates can measure substrate utilization in intact cells, but the assessment of mitochondria-specific substrate utilization requires cell permeabilization. This results in a rather artificial system due to the lack of feedback and inhibition by other metabolites, the inability to regulate cellular substrate uptake and because of ad libitum access to substrates by mitochondria. For these reasons, the assessed substrate oxidation reflects theoretical enzyme activity, but does not reflect the physiological metabolic pathway. While this technique has been applied in other immune cells (Kalvala et al., 2019; Thwe et al., 2019; Zhang et al., 2021), it has not previously been used in cultured macrophages *in vitro*. In combination with the limited overlap with the other techniques tested here, it makes this technique a potential follow-up tool when additional validation is included.

Transcriptomics and metabolomics measurements can provide additional insight into metabolic changes. The acquired data may be complementary and can be integrated (Jha *et al.*, 2015), but may seem contradictory when enzyme regulation takes place on different levels. For example, post-transcriptional or post-translational modifications are not included in transcriptomics data, and differences in gene expression do not always correlate to active translation into protein and enzyme activity. We refer the reader to recent reviews (Artyomov and Van den Bossche, 2020; Voss *et al.*, 2021) for a further discussion of (single-cell) transcriptomics, metabolomics and their integration and other single-cell techniques to study cellular metabolism such as cytometry-based metabolic panels (Ahl *et al.*, 2020; Hartmann *et al.*, 2021; Levine *et al.*, 2021). These methods will greatly enhance insight into metabolic alterations upon treatment with compounds of interest, but they do require more specialized machines, and can complicate data analysis.

In the metabolic analysis of complex *in vivo* samples, spatial and temporal knowledge of metabolic processes are of major additional value (Artyomov and Van den Bossche, 2020; Murphy and O'Neill, 2020). Therefore, imaging-based techniques (Miller et al., 2017; Palmer et al., 2017) such as GeoMX (Farren et al., 2020) or MIBI-TOF (Hartmann and Bendall, 2020; Keren et al., 2019) have been developed that combine transcriptomics, metabolic and functional readouts with spatial information. Also, approaches that assess subcellular metabolism are important since metabolites accumulate to a different extent in diverse subcellular locations and may affect (signaling) targets differently. This was for example indicated for acyl intermediates and their ability to modify proteins (Bambouskova et al., 2021; Murphy and O'Neill, 2020). Additionally, to prove whether a metabolic change is cause or consequence of phenotypic alterations, it is desirable to assess temporal aspects. This can be implemented e.g. by repeated ^{13}C-metabolomic flux measurements in combination with phenotypic assessment or the analysis of pseudo-time in single-cell RNA-sequencing. By including spatiotemporal approaches in immunometabolic research, discrepancies in the literature may be resolved. Lastly, causality needs to be demonstrated by targeting metabolic enzymes with genetic or pharmacological tools as described previously in more detail (Voss *et al.*, 2021).

Conclusion and practical guidance

We here present an integrated 96-well-plate-based approach to screen metabolic alterations. We recommend to start with quick and easy pre-screening methods such as NO, arginase, glucose and lactate assays before continuing with XF analysis for bulk samples, SCENITH for subset analysis of complex samples or fluorescent dyes for single-cell resolution (Figure 6). This approach serves to efficiently analyze metabolic alterations and narrow down conditions

of interest before applying complex and costly follow up techniques such as (single-cell) transcriptomics or (spatial) metabolomics.

Author contributions

SV and KdG contributed equally to this work, order of authorship was decided by flipping a coin. Conceptualization: JVdB, SV, KdG. Methodology: SV, KdG, FG, RA. Formal analysis: SV, KdG. Investigation: SV, KdG, FG, XvD. Visualization: SV, KdG. Writing-review&editing: SV, KdG, FG, XvD, RA, JVdB, Funding acquisition: JVdB. All authors read and approved the final manuscript.

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Declarations of interest

The authors declare no competing interests. There are restrictions to the commercial use of SCENITH due to a pending patent application (PCT/EP2020/060486).

Figure Legends

Figure 1: Overview of semi-high-throughput techniques encompassed in metabolic pre-screening and toolbox. (i) Metabolic pre-screening, consisting of arginase activity assay in cell lysates and NO, lactate and glucose levels in cellular supernatant (yellow). (ii) The core metabolic characterization, consisting of extracellular flux (XF) analysis, SCENITH and uptake of fluorescent metabolic dyes (purple), and (iii) substrate oxidation analysis as a potential follow-up on the toolbox (green). XF analysis measures extracellular acidification and oxygen consumption in XF96-well plates in response to metabolic inhibitors to estimate glycolysis and OXPHOS, respectively. The flow cytometry-based metabolic profiling technique SCENITH (Arguello *et al.*, 2020) measures changes in the level of translation in response to inhibitors as a measure for cellular metabolism. This can be assessed in plate-reader compatible FACS 96-well plates. Fluorescent measurement of the uptake of several metabolic dyes can be measured by an imaging multi-mode plate reader in black 96-well plates and by flow cytometry. (iii) These readouts can be followed up by more extensive metabolic profiling using substrate oxidation, metabolomics, transcriptomics or various types of single-cell profiling.

Figure 2: Metabolic pre-screening of BMDMs and HMDMs indicate metabolic differences after varying macrophage activation. (A) BMDMs and HMDMs were left untreated (Naive), or stimulated with either LPS, LPS+IFNγ or IL-4 for 24 h. (B, C) Levels of NO in supernatants from BMDMs (B) or HMDMs (both $1*10^5$ cells per well) (C) and arginase activity in BMDMs ($5*10^4$ cells per well) (B) or HMDMs ($1*10^5$ cells per well) (C) following stimulation. (D, E) Levels of glucose consumption and lactate production in supernatants of BMDMs (D) or HMDMs (E) (all $1*10^5$ cells per well). Data are shown as mean ± SEM. For BMDMs, N=12 mice with 3 technical replicates in 4 independent experiments were included for NO, glucose and lactate assays, and N=6 with 3 technical replicates in 2 independent experiments for arginase activity assay. For HMDMs, N=5 human donors with 3 technical replicates in 2 independent experiments were included for all assays. ** P<0.01, *** P<0.001 by one-way ANOVA with Dunnett's post-hoc test for multiple comparisons.

Figure 3: XF analyses of BMDMs and HMDMs yield insight into metabolic profiles of macrophages after LPS±IFNγ and IL-4-activation. (A, B) Normalized (to relative Hoechst$^+$ objects) ECAR, with injections of glucose, oligomycin, FCCP and Antimycin A/Rotenone/Hoechst for BMDMs (A) and HMDMs (B). (C, D) Normalized (to relative Hoechst$^+$ objects) OCR with same injections as for ECAR for BMDMs (C) and HMDMs (D). (E, G) Metabolic profiles outlining basal respiration and glycolysis for BMDMs (E) and HMDMs (G). (F, H) Mitochondrial and glycolytic contribution to overall ATP production in BMDMs (F) and HMDMs (H).. N=6 mice or N=6 donors were included with 4-5 technical replicates each. Values shown as mean ± SEM calculated from the average of technical replicates per mouse/donor. * P<0.05, ** P<0.01, *** P<0.001 by one-way ANOVA with Dunnett's post-hoc test for multiple comparisons. For F and H, significance on top of bar graphs indicates changes in total ATP production rate, significance within bars indicates significant differences between either the glycolytic or mitochondrial contribution to ATP production rate compared with N.

Figure 4: Metabolic analysis of BMDMs and HMDMs with SCENITH reveals expected macrophage activation by LPS±IFNγ and IL-4. (A, B) MFI of puromycin across samples treated with different inhibitors for BMDMs (A) and HMDMs (B). DG, O and DGO indicate Deoxyglucose- (DG), Oligomycin- (O) or Deoxyglucose+Oligomycin-treated (DGO) samples. (C) Calculations of metabolic SCENITH parameters based on puromycin MFI. (D, E, F, H) SCENITH parameters as calculated for mouse (D, F) and human (E, H) macrophages. (G, I) Correlation of glycolytic capacity as measured with XF analysis with glycolytic capacity as measured with SCENITH for BMDMs (G) and HMDMs (I). (J, K) tSNE dimensionality reduction of naïve, LPS±IFNγ- and IL-4-treated BMDMs (J) and HMDMs (K) and clustered heatmaps showing the expression of activation markers and puromycin per stimulus. Data are shown as mean ± SEM. Each dot marks a separate mouse (N=6) or human donor (N=6). * P<0.05, ** P<0.01, *** P<0.001 by two-way (A, B) or ordinary one-way ANOVA (D, E, F, H) with Dunnett's post-hoc test for multiple comparisons. Correlations were fitted using a simple linear regression model (G, I).

Figure 5: Uptake of fluorescent probes provides additional insight into macrophage metabolism. (A, B) Representative images of BMDM (A) and HMDM (B) staining by fluorescent dyes and uptake of fluorescent nutrient analogs as assessed by multi-mode reader. Scale bar represents 200 µm. (C-F) Fluorescent intensity of 2NB-DG (C, E) and BODIPY C16 (D, F) uptake by BMDMs (C, D) and HMDMs (E, F) as examined by flow cytometry, correlated with relevant parameters of XF analysis. (G-J) Fluorescent intensity of MitoTracker Green (G, I) and TMRM (H, J) analysis as examined by flow cytometry and correlations with relevant parameters of XF analysis in BMDMs (G, H) and HMDMs (I, J). Data are shown as mean ± SEM. For graphs of fluorescent probes, each dot marks a separate mouse (N=9) or donor (N=8). ΔMFI was calculated as MFI (median fluorescent intensity) of sample – MFI of unstained control. * P<0.05, ** P<0.01, *** P<0.001 by one-way ANOVA with Dunnett's post-hoc test for multiple comparisons. Correlations were fitted using a simple linear regression model.

Figure 6: An actionable workflow to guide researchers from simple screening towards complex measurement of immunometabolism. Immunometabolic alterations can be pre-screened by quick and easy assays such as NO production and arginase activity in mouse macrophages and glucose consumption and lactate production in both species (yellow). Cytokine and viability measurements (green) can be performed in parallel to connect cellular metabolic changes and function. These assays can be followed up by metabolic characterization (purple) with a bulk (XF analysis) or single-cell approaches (SCENITH and fluorescent metabolic dyes).

Normalization should be performed in parallel to XF analysis, and optional phenotyping can be done by adding activation and/or lineage markers to SCENITH and fluorescent metbolic dyes. Experiments can be further extended using more complex techniques (green), such as substrate utilization, metabolomics/fluxomics, RNA-seq for bulk analysis of homogeneous samples or metabolic profiling using cytometry, single-cell RNA-seq, spatial metabolomics or immunohistochemistry for complex samples where single-cell or spatial resolution is required. Solid lines indicate preferred workflow, dotted lines indicate optional readouts.

660 **Table 1. Tools for 96-well-plate-based immunometabolic profiling**

Methods	Assay principle	Advantages/disadvantages	Equipment
Extracellular glucose / lactate assay	Estimate of glycolysis based on cumulative glucose consumption / lactate secretion	+ Simple, fast, cheap - Limited insight - Bulk analysis	Absorbance (glucose) or fluorescent (lactate) plate reader
NO / arginase assay	Estimates L-arginine metabolism via iNOS / arginase	+ Simple, fast, cheap - Not all species/cell types - Bulk analysis	Absorbance plate reader
Extracellular flux analysis	Measures ECAR and OCR as proxies of glycolysis and mitochondrial OXPHOS, respectively	+ Parallel readouts of glycolysis and OXPHOS + Adaptable injections and protocol - Dedicated (costly) instrument/consumables - Normalization needed - Bulk analysis, require cell purification/sorting	Seahorse XF analyzer
SCENITH	Estimates metabolic capacities and dependencies by measuring changes in the level of protein synthesis	+ Suitable for rare cells and complex samples + Compatible with complex immune phenotyping - Assay principle requires active protein synthesis - Subset analysis rather than single-cell for calculated parameters	Flow cytometer
2-NBDG uptake	Fluorescent glucose analogue to estimate glucose uptake	+ Simple, fast, single-cell - Need to be validated with complementary readouts and appropriate controls to ensure correct interpretation	Flow cytometer / multi-mode fluorescence imager / microscope
BODIPY C16	Fluorescent fatty acid to estimate fatty acid uptake		
MitoTracker Green	Fluorescent mitochondrial mass indicator		
TMRM (TMRE)	Fluorescent dye to measure mitochondrial membrane potential		

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STAR Methods

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jan Van den Bossche (j.vandenbossche@amsterdamumc.nl).

Materials availability

This study did not generate new unique reagents. As described previously, adapted SCENITH protocols and all the reagents including the panel of inhibitors, puromycin and the monoclonal antibody clone R4743L-E8, conjugated with Alexa Fluor 647 or Alexa Fluor 488 (SCENITH kit) are available upon application at http://www.scenith.com/.

Data and code availability

All data reported in this paper will be shared by the lead contact upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Bone marrow isolation and BMDM culture

Mouse experiments were approved by the Committee for Animal Welfare of the VU University Amsterdam. 8-16 week old male and female C57Bl/6J mice were purchased from Charles River and housed in groups of four in SPF conditions at 21°C until sacrifice. Bone marrow cells were isolated from femurs and tibias by flushing with PBS. Bone marrow-derived macrophages (BMDMs) were generated by culturing in 145x20 mm petri dishes (greiner bio-one) in 20 ml complete RPMI-1640 (Gibco) containing 2 mM L-glutamine, 10% FCS (Gibco), 100 U/ml penicillin, 100 µg/ml streptomycin (all Gibco), and 15% L929-conditioned medium (LCM) for 6 days, which resulted in >90% macrophage purity (Supplementary Figure 1B). 10 ml fresh medium was added on day 3. On day 6, cells were harvested with cold PBS and gentle scraping and counted using a Bürker cell counting chamber with 0.0025 mm^2 grid (Optik Labor). Cells were subsequently plated at a density of $1*10^6$ cells/ml in fresh medium with 5% LCM in 96-well plates for the experiments. On day 7, medium was refreshed and cells were either left untreated or stimulated with 100 ng/ml LPS (Sigma Aldrich), 10 ng/ml LPS+100 U IFNγ (Peptrotech), or 20 ng/ml IL-4 (Peprotech) for 24 hours in the presence of 5% LCM.

Monocyte isolation and HMDM culture

Buffy coats (50 ml) were purchased from Sanquin blood Bank (Amsterdam, Netherlands). Information regarding sex and age of donors was not supplied by the blood bank. PBMCs were isolated with a Ficoll/Lymphoprep gradient (Greiner Biosciences) and careful centrifugation at 800g for 30 minutes. Next, monocytes were isolated by applying 120-150*10^6 cells on top of a 46% Percoll™ (Cytiva) solution followed by careful centrifugation at 2000 rpm for 20 minutes. Monocytes were counted in the same manner as BMDMs and plated at a density of 2*10^6 cells/ml in the appropriate 96-well plates for each experiment in 100 µl IMDM medium containing HEPES (Gibco) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (full IMDM medium), and containing 1% FCS. After settling for one hour, medium was replaced with full IMDM medium with 10% FCS and 50 ng/ml M-CSF (Miltenyi) for 6-day differentiation, resulting in >95% macrophage purity (Supplementary Figure 1C). On day 3, medium was replaced with fresh medium supplemented with M-CSF (Miltenyi). On day 6, cells (near 100% macrophage purity (Supplementary Figure 1A, C)) were left untreated or stimulated in fresh medium without M-CSF with 100 ng/ml LPS (Sigma Aldrich), 10 ng/ml LPS + 20 ng/ml IFNγ (Peprotech), or 20 ng/ml IL-4 (Peprotech) for 24 hours.

METHOD DETAILS

NO production and Arginase activity assay

100 µl supernatant was collected from 96-well plates with 1*10^5 cells (BMDMs/HMDMs) per well. Subsequently, NO production was measured by adding 50 µl Griess reagent (2.5% H_3PO_4 (Merck), 1% sulfanilamide (Sigma Aldrich), and 0.1% naphtylene diamide dihydrochloride (Sigma Aldrich) in H_2O) to 50 µl cell supernatants (1:1) and optical density was measured at 540 nm.

Arginase activity was determined on cell lysates of 5*10^4 cells (BMDMs) or 1*10^5 cells (HMDMs). Cells were washed with PBS and lysed by incubating for 30 minutes with 100 µl 0.1% Triton X-100 (Sigma Aldrich), 25 mM Tris-HCl (pH 7.5, Roche) supplemented with 1x protease inhibitor cocktail (Roche). Arginase was activated by adding 3.5 µl of 10 mM $MnCl_2$ (Sigma Aldrich) to 10 µl sample and incubated at 56ºC for 10 minutes. Next, samples were incubated with 10 µl 0.5 M L-arginine (pH 9.7, Sigma Aldrich) for 60 minutes at 37ºC. The reaction was stopped by adding 90 µl stop solution (96%H_2SO_4/85% H_3PO_4/H_2O 1:3:7, Merck) and incubated with 4 µl α-isonitrosopropiophenone (9%, Sigma Aldrich) for 30 minutes at 95ºC. Samples were left in the dark to cool down to room temperature until measurement of optical density at 540 nm. Enzymatic activity was calculated by [Urea]*(total volume*10^6)/(tested volume*Time(incubated at 37ºC)*1000).

Glucose consumption assay

100 µl supernatant was collected from 96-well plates with $1*10^5$ cells (BMDMs/HMDMs) per well. To determine glucose levels in supernatant, samples and standard (5 µl per well) were pipetted into a 96-well plate. 250 µl glucose reagent (BIOLABO) was added to standard and samples, mixed by pipetting and incubated for 30 minutes in the dark. Absorbance was measured at 490 nm. Glucose consumption was calculated as the difference between glucose levels of medium without cells and glucose levels in cell supernatants.

Lactate production assay

100 µl supernatant was collected from 96-well plates with $1*10^5$ cells (BMDMs/HMDMs) per well. Lactate levels in cellular supernatants were determined by conversion of lactate into NADH by lactate dehydrogenase (LDH). First, samples were incubated for 15 minutes at 4°C with 3% metaphosphoric acid (Sigma Aldrich), centrifuged at 20.000 g for 10 minutes, and supernatants were used further. 5 µl of thus deproteinized samples were transferred into a 96-wells plate and 150 µl Master Mix consisting of 0.5 M Glycine - 0.4 M hydrazine buffer (pH = 9.0, Sigma Aldrich) with 27 mM NAD (Cayman Chemicals) was added per well. NADH fluorescence was measured using a Mithras LB 940 with $\lambda_{ex}/\lambda_{em}$= 340-10 /450-10 nm every 2 minutes for 5 cycles as background measurement. Next, 50 µl of start solution consisting of 0.5 M Glycine - 0.4 M hydrazine buffer (pH = 9.0) and 5 mg/ml LDH was added to each well. Fluorescence was measured every 2 minutes with shaking until a stable read was achieved.

Extracellular flux analysis

XF analysis was performed using the Seahorse XFe-96 Flux Analyzer (Agilent) to examine oxygen consumption (OCR) and extracellular acidification rates (ECAR) as described previously (Van den Bossche *et al.*, 2015). Briefly, BMDMs and HMDMs were plated at a density of $7.5*10^4$ cells per well in XF-96-cell culture plates (Agilent) and stimulated for 24 hours with LPS or IL-4, or left untreated. 1 hour prior to the assay, cells were washed and medium was replaced by Seahorse base medium (Sigma-Aldrich) without glucose, phenol red, and sodium bicarbonate, supplemented with 5 mM HEPES and 2 mM L-glutamine. The run consisted of 2 minutes mixing, 3 minutes measuring and subsequent 4 injections; Glucose (final concentration in well 25 mM), Oligomycin (O, final concentration 1.5 µM), FCCP (final concentration 1.5 µM), and antimycin A (AA, final concentration 2.5 µM) with rotenone (rot, final concentration 1.25 µM) and Hoechst 33342 (Thermo Fisher) (final concentration 5 µg/ml). Directly after the run, Hoechst signal was measured on the Cytation 5 Cell Imaging multi-mode reader (BioTek) with a 4X magnification using a 365 nm LED in combination with an EX377/50 EM 447/60 filter cube and cell counts were analyzed using Gen 5™ software. Subsequently, flux rate data was normalized to cell counts with the following equation:

$$Normalized\ OCR\ or\ ECAR = OCR\ or\ ECAR\ /\ \frac{cell\ count\ in\ center\ of\ well}{average\ of\ plate}.$$

Data were analyzed using Wave software version 2.6.0.31 as described previously (Van den Bossche *et al.*, 2015).

Mitochondrial and glycolytic contributions to total ATP production rate were calculated as described here (Romero, 2017). First, OXPHOS-related acidification (mitochondrial proton efflux rate, mitoPER) of the assay medium was calculated as follows:

$$mitoPER = CCF * (OCR_{basal} - OCR_{Rot/AA})\ ),$$

where CCF (CO_2 contribution factor) was determined as 0.61 for XFe96 plates by the manufacturer for a range of cell types. Total proton efflux rate (PER) can be calculated as:

$$total\ PER = ECAR * BF * Vol_{measurement\ chamber} * K_{vol},$$

where the buffer factor (BF) of the medium refers to the amount of H^+ necessary to change the pH of the medium by one unit, and was measured according to manufacturer instructions in the Buffer Factor Protocol Quick Reference Guide (Agilent). $Vol_{measurement\ chamber}$ and K_{vol} are scaling factors to determine the effective volume of the well and were determined as 2.28 µl and 1.60, respectively, by the manufacturer for XFe96 plates. From this, glycolytic proton efflux rate and therefore glycolytic ATP production rate can be determined:

$$glycoATP = glycoPER = total\ PER - mitoPER.$$

Next, mitochondrial ATP production rate can be calculated as follows:

$$mitoATP = (OCR_{basal} - OCR_{Rot/AA}) * 2 * P/O,$$

where multiplication by 2 is a stoichiometric correction for oxygen atoms consumed, and P/O is the number of ADP molecules phosphorylated to ATP per atom of oxygen which was determined by the manufacturer as 2.75 for a range of cell types. Lastly, total ATP production rate is the sum of glycoATP and mitoATP, as described by the manufacturer in the Real-time ATP Rate assay Kit (Agilent).

SCENITH and Flow cytometry and analysis

SCENITH protocol was performed as described previously (Arguello *et al.*, 2020). Briefly, control or metabolic inhibitors Deoxy-D-glucose (DG, final concentration 100 mM), oligomycin (O, final concentration 1 µM), combination of DG and O (DGO), or Harringtonine (H, final concentration 2 mg/ml) as negative control were added to fully differentiated cells and incubated for 15 min at 37°C. Subsequently, puromycin (final concentration 10 µg/ml) was

added without washing and incubated for another 30 min at 37°C. After incubation, cells were washed with cold PBS, harvested by incubating with PBS + 5 mM EDTA for 10 min at 4°C and proceeded with Fc receptor blockade (eBioScience for mouse, BD Biosciences for human cells) and fixable viability dye staining for 15 min at 4°C in the dark. Subsequently, cells were washed and stained with surface antibody mix in PBS/0.5% BSA/0.02% sodium azide (PBA) for 30 min at 4°C in the dark. Cells were then washed, fixed and permeabilized using the FOXP3 fixation and permeabilization kit (eBioScience) according to manufacturer's instructions. For intracellular staining of iNOS, Arg1 and puromycin, cells were incubated for 1h at 4°C in antibody staining solution in permeabilization buffer. Samples were then transferred to a plate-reader compatible 96-well U-bottom plate.

All samples were acquired within 24 hours of the experiment at the O2 Flow Facility at Amsterdam UMC (Netherlands) on an X20 Fortessa flow cytometer (BD Biosciences) with high-throughput sampler. The flow cytometer was calibrated daily using CS&T calibration beads (BD Biosciences).

Data were analyzed using FlowJo (TreeStar, v10) and were compensated using single stains with UltraComp eBeads (ThermoFisher) labeled with the appropriate fluorochrome. Next, cells were gated on FSC-A/SSC-A to gate out debris, then on FSC-A/FSC-H to identify single-cells and lastly on FVD^- viable cells (Supplementary Figure 1A).

For unbiased tSNE analyses, files of oligomycin-treated cells were uploaded to the OMIQ online analysis platform (https://omiq.ai/), scaled and subsampled to include 10.000 cells live single cells per file. Next, the tSNE-CUDA tool set to 1500 iterations, a perplexity of 30 and a theta of 0.5 was used to create tSNE dimensionality reduction. Cells were overlaid on tSNE dimensionality reduction according to stimulus. Stimulus-associated 'clusters' were further assessed using the 'Clustered Heatmap' tool in OMIQ to identify discriminating markers between activation states.

Vehicle control, DG, O, Harringtonine, puromycin and anti-puromycin antibodies were received as SCENITH kit from (http://www.scenith.com) (Arguello *et al.*, 2020). A complete list of antibodies used can be found in Supplementary Table 2.

Fluorescent metabolic dyes

Mitochondrial mass was measured using MitoTracker Green (Invitrogen). Mitochondrial membrane potential was measured using Tetramethylrhodamine methyl ester (TMRM, Thermo Fisher) and fatty acid and glucose uptake was measured using BODIPY^{TM} FL C16 (BODIPY, Thermo Fisher) or 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2NB-DG, Invitrogen) fluorescent probes. Cells were plated in 96-well black culture plates at a density of $8*10^4$ cells/well. Cells were starved for 2 hours in basal RPMI-medium with (for BODIPY C16) or without glucose (for 2NB-DG) and subsequently stained by incubation for 30

minutes in complete RPMI with either 100 nM MitoTracker, 100 nM TMRM, 100 µM 2NB-DG or with 0.75 µM BODIPY. Concentrations were determined by analysis of unspecific staining and effect on cell viability (Supplementary Figure 4). Hoechst (final concentration 5 µg/ml) was added for the last 5 minutes of incubation. Cells were washed with PBS and imaged in 4X magnification on the Cytation 5 at 37°C using a 465 nm LED in combination with an EX 469/35 EM 525/39 filter cube for MitoTracker Green, BODIPY and 2NB-DG and a 523 nm LED in combination with an EX 531/40 EM 593/40 filter cube for TMRM.

For FACS analysis, cells were harvested using ice-cold PBS+5 mM EDTA, transferred to a plate-reader compatible 96-well U-bottom plate and immediately acquired on an X20 Fortessa flow cytometer with plate reader (BD Biosciences) and analyzed as described in the section 'Flow cytometry and analysis'. To validate metabolic dye signal, MitoTracker Green and TMRM signal were inhibited by the uncoupler FCCP (Sigma-Aldrich, 5 µM final concentration), 2NB-DG by phloretin, an inhibitor of glucose transport (Sigma-Aldrich, 150 µM final concentration), and BODIPY C16 by lipofermata, an inhibitor of fatty acid transporter 2 (Cayman Chemicals, 10 µM final concentration) (Supplementary Figure 4).

Mitochondrial functional substrate assay

Mitochondrial functional substrate assays were performed using mitochondrial-substrate-coated (Biolog). First, cells for mitochondrial functional substrate assay were seeded in a 96 well culture plate at a density of $8*10^4$ cells/well and stimulated for 24 hours with LPS or IL-4, or left untreated. 1 hour prior to the assay, 30 µl assay buffer with 1X saponin (Sigma-Aldrich, final concentration 50 µg/ml) and redox dye MC was dispensed into the wells of the mitochondrial-substrate-coated plate and incubated at 37ºC. Then, cells were washed once with 100 µl assay buffer and subsequently incubated with 35 µl assay buffer with 1X saponin at room temperature for permeabilization. After 15 minute incubation time, permeabilized cells were mixed by pipetting up and down and incubated for another 15 minutes at room temperature. Lastly, 30 µl of permeabilized cell suspension in 1X saponin was transferred to the mitochondrial-substrate-coated plate.

Additionally, intact BMDMs were plated as $4*10^4$ cells/well in 50 µl MC-0 Assay Medium into carbon-substrate-coated plates (Biolog) to further assess whole-cell substrate usage as opposed to only mitochondrial substrate use. Cells and substrates were pre-incubated for 24 hours at 37ºC and 5% CO_2, and metabolism was assessed after adding 20 µl 6X Biolog Redox Dye MB.

Color formation in mitochondrial-substrate-coated and carbon-substrate-coated plates was measured by the automated platform OmniLog (Biolog) and maximum rates between 1 and 4 hours of analysis were determined using Biolog Data Analysis software version 1.7.1.58.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data are presented as mean ± standard error of the mean (SEM) unless specified differently. Statistical significance was analyzed using an ordinary one-way or two-way ANOVA where appropriate followed by Sidak's or Dunnett's correction for multiple comparisons, respectively, in GraphPad Prism software (8.2.1) using paired analysis to minimize effects of mouse/donor variation. For substrate utilization experiments, significant substrates were determined with a Student's t-distribution using Biolog Data Analysis Software. P values < 0.05 were considered statistically significant indicated by $*p<0.05$, $**p<0.01$, $***p<0.001$. The number of mice or donors included in each experiment is indicated in the figure legend. Except for the titration of metabolic dyes, all experiments were performed as at least 2 independent experiments. For NO and Arginase assays, XF analyses and fluorescent probe uptake, at least 3 technical replicates were included to calculate a mean value to represent a mouse or human donor.

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