



**HAL**  
open science

## An integrated toolbox to profile macrophage immunometabolism

Sanne G.S. Verberk, Kyra de Goede, Friederike Gorki, Xanthe A.M.H. van Dierendonck, Rafael J Argüello, Jan van den Bossche

► **To cite this version:**

Sanne G.S. Verberk, Kyra de Goede, Friederike Gorki, Xanthe A.M.H. van Dierendonck, Rafael J Argüello, et al. An integrated toolbox to profile macrophage immunometabolism. *Cell Reports Methods*, 2022, 2 (4), pp.100192. 10.1016/j.crmeth.2022.100192 . hal-03867132

**HAL Id: hal-03867132**

**<https://hal-cnrs.archives-ouvertes.fr/hal-03867132>**

Submitted on 23 Nov 2022

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# 1 **An integrated toolbox to profile (macrophage) immunometabolism**

2 Sanne G. S. Verberk<sup>1,4</sup>, Kyra E. de Goede<sup>1,4</sup>, Friederike S. Gorki<sup>1,2</sup>, Xanthe A.M.H. van  
3 Dierendonck<sup>1</sup>, Rafael J. Argüello<sup>3</sup>, Jan Van den Bossche<sup>1,\*</sup>

4 <sup>1</sup> Department of Molecular Cell Biology and Immunology, Amsterdam Cardiovascular  
5 Sciences, Amsterdam Gastroenterology Endocrinology Metabolism, Amsterdam Institute for  
6 Infection and Immunity, Cancer Centre Amsterdam, Amsterdam UMC, Vrije Universiteit  
7 Amsterdam, Amsterdam, Netherlands

8 <sup>2</sup> Institute of Innate Immunity, University Hospital Bonn, University of Bonn, Bonn 53127,  
9 Germany

10 <sup>3</sup> Aix Marseille Univ, CNRS, INSERM, CIML, Centre d'Immunologie de Marseille-Luminy,  
11 Marseille, France

12 <sup>4</sup> These authors contributed equally

13 \*Lead contact: [j.vandenbossche@amsterdamumc.nl](mailto:j.vandenbossche@amsterdamumc.nl)

14

## 15 **Summary**

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

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

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

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

## 32 **Motivation**

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

### 43 **Introduction**

44 Macrophages are innate immune cells that reside in tissues or differentiate from circulating  
45 monocytes and regulate acute inflammatory responses and tissue homeostasis (Wynn et al.,  
46 2013). While they can adopt a broad spectrum of activation states, *in vitro* macrophage  
47 research primarily focusses on lipopolysaccharide (LPS)-, LPS plus interferon (IFN)  $\gamma$ -, and  
48 interleukin (IL)-4-induced activation states. LPS $\pm$ IFN $\gamma$ -induced macrophages (classically  
49 activated macrophages, M[LPS $\pm$ IFN $\gamma$ ]) produce high levels of pro-inflammatory cytokines and  
50 show increased surface expression of (co)stimulatory immune activation markers. Conversely,  
51 IL-4-induced macrophages (alternatively activated macrophages, M[IL-4]) upregulate a  
52 different set of surface markers and enzymes involved in repair and homeostasis (Martinez et  
53 al., 2013; Murray et al., 2014).

54 Metabolic rewiring resides at the core of phenotypic polarization and has been shown to direct  
55 immune responses (Jha et al., 2015). Upon stimulation with LPS, mouse macrophages  
56 increase metabolic flux through glycolysis and the pentose phosphate pathway (PPP) which  
57 in turn fuels reactive oxygen species (ROS) production and nitric oxide (NO) synthesis  
58 (Baardman et al., 2018; Bailey et al., 2019; Van den Bossche et al., 2017). M[LPS] mouse  
59 macrophages also display a disruption of the tricarboxylic acid (TCA) cycle at isocitrate  
60 dehydrogenase (IDH) and succinate dehydrogenase (SDH) (Jha *et al.*, 2015). The LPS-  
61 induced downregulation of IDH results in the shunting of (iso)citrate towards synthesis of  
62 aconitate and subsequent accumulation of anti-microbial itaconate, mediated by the  
63 *immunoresponse gene 1 (Irg1)*-encoded enzyme ACOD1 (Lampropoulou et al., 2016). As a  
64 result of blunted SDH activity, succinate accumulates and functions as an immunoregulatory  
65 metabolite in macrophages that directs the immune response via hypoxia-inducible factor 1  
66 alpha (HIF1 $\alpha$ ) and other mechanisms (Harber et al., 2020; Mills et al., 2016). In sharp contrast  
67 with M[LPS $\pm$ IFN $\gamma$ ], IL-4-stimulated mouse macrophages are characterized by an intact TCA

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

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

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

93 A combination of several -omics techniques can explain delicate changes in cellular  
94 metabolism. As such, metabolomics measures the abundance of metabolites and can reveal  
95 metabolic changes dictated by increased production or decreased substrate usage (Jha *et al.*,  
96 2015; Rattigan *et al.*, 2018). Also, (single-cell) RNA-sequencing (transcriptomics) shows  
97 regulation on gene expression level, and aids in evaluating the transcriptional regulation of all  
98 metabolic pathways (Artyomov and Van den Bossche, 2020; Jha *et al.*, 2015). However, RNA-  
99 sequencing neither allows the assessment of post-translational modifications that may dictate  
100 the function of metabolic enzymes, nor of metabolic enzyme activity. While new methods are  
101 arising that permit the measurement of metabolic enzyme abundance and activation by (flow)  
102 cytometry (Ahl *et al.*, 2020; Hartmann *et al.*, 2021; Levine *et al.*, 2021), these are relatively

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

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

## 122 **Results**

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

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

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

### 134 Metabolic pre-screening gives first indications about altered macrophage metabolism

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

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

### 149 XF analysis validates metabolic rewiring in LPS±IFN $\gamma$ and IL-4-activated macrophages

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

155 upregulated mitochondrial respiration after IL-4 treatment (Figure 3C, Supplementary Figure  
156 2C), and decreased OCR following LPS+IFN $\gamma$  activation (Figure 3C, E) (Jha *et al.*, 2015;  
157 Koenis *et al.*, 2018; Van den Bossche *et al.*, 2016). The calculated mitochondrial and glycolytic  
158 contribution to ATP production followed a similar pattern as OCR and ECAR parameters in the  
159 different conditions (Figure 3F). In HMDMs, LPS+IFN $\gamma$  significantly increased glycolysis  
160 parameters, while none of the stimuli significantly affected OCR-derived readouts (Figure 3B,  
161 D, G, Supplementary Figure 2B, D). Akin to mouse macrophages, the mitochondrial  
162 contribution to ATP production was significantly decreased by LPS $\pm$ IFN $\gamma$  and increased by IL-  
163 4 (Figure 3H).

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

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

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

183 High puromycin incorporation (displayed as median fluorescence intensity, MFI) was detected  
184 in control samples, indicating a high level of protein synthesis in naïve BMDMs, and this was  
185 further increased after stimulation with LPS or IL-4 which correlated significantly with XF-  
186 derived total mitochondrial plus glycolytic ATP production (Figure 4A, Supplementary Figure  
187 3A). This was not the case for HMDMs (Figure 4B, Supplementary Figure 3B). Addition of

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

190 Measurement of puromycin MFI after addition of inhibitors allowed for the calculation of distinct  
191 metabolic parameters as visualized in Figure 4C:

192 - Glucose and mitochondrial dependency were calculated as the proportion of protein  
193 synthesis dependent on glucose oxidation and OXPHOS, respectively (Figure 4A-C).

194 - Glycolytic capacity and fatty acid and/or amino acid oxidation (FAO/AAO) capacity indicate  
195 the maximum potential of cells to sustain protein synthesis when OXPHOS and glucose  
196 oxidation are inhibited, respectively (Arguello *et al.*, 2020).

197 DG almost completely abolished the puromycin signal in all macrophage stimulations,  
198 indicating overall high glucose dependency (Figure 4A-E). While M[LPS±IFN $\gamma$ ] depended  
199 almost exclusively on glucose, naïve and IL-4-treated BMDMs and HMDMs had the capacity  
200 to use FAO/AAO as fuel (Figure 4D, E). Accordingly, inhibiting mitochondrial ATP production  
201 with oligomycin (O) had the smallest effect in M[LPS+IFN $\gamma$ ] (Figure 4A, B, D, E). Together, the  
202 SCENITH-based calculations revealed reduced mitochondrial dependency and high glycolysis  
203 in M[LPS+IFN $\gamma$ ] (Figure 4D, E, F, H), which aligns with data obtained by XF analysis (Figure  
204 4G, I).

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

219 Measuring incorporation of fluorescent nutrient analogs yields complementary information to  
220 XF analysis and SCENITH

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

227 2NB-DG is a fluorescent glucose analog that is used to assess glucose uptake by cells during  
228 the 30-minute incubation in the presence of this dye. Flow cytometry revealed an elevated  
229 uptake of 2NB-DG in LPS-stimulated BMDMs, which correlated with the increased glycolysis  
230 as determined by XF analysis (Figure 5C). In comparison to naïve macrophages, LPS+IFN $\gamma$ -  
231 activated BMDMs showed decreased 2NB-DG uptake. Yet, they consumed more glucose over  
232 a 24-hour time period (Figure 2D), probably indicating that they were saturated and were no  
233 longer capable of taking up more 2NB-DG. Although HMDMs did not show differences in 2NB-  
234 DG uptake (Figure 5E), it is a single-cell approach to assess glucose uptake that significantly  
235 correlates with bulk glycolysis measurements during XF analysis (Figure 5G).

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

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

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

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

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

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

273 This technique is based on the reduction of a redox dye by NAD(P)H or FADH $_2$  production as  
274 a result of mitochondrial respiration (Figure 1). Intact cells added to carbon-substrate-coated  
275 plates (PM-M01 plates) reveal insight in their utilization of sugars. Alternatively, permeabilized  
276 cells added to plates coated with mitochondrial respiration-related carbon sources (MitoPlate  
277 S-1) reveal increased insight into mitochondrial function.

278 Using carbon-substrate-coated plates, we observed that intact BMDMs highly upregulated D-  
279 glucose utilization after both LPS- and IL-4-activation (Supplementary Figure 5A). The  
280 maximum rate of D-glucose oxidation correlated well with glycolysis as determined by XF  
281 analysis and with 2NB-DG uptake (Supplementary Figure 5B). In parallel, we used  
282 mitochondrial-substrate-coated plates to obtain information about TCA cycle and mitochondrial  
283 function in distinct macrophage subsets (Supplementary Figure 5C, D). In agreement with the  
284 described breaks in the TCA cycle at IDH and SDH in inflammatory macrophages (Jha *et al.*,  
285 2015), we observed decreased oxidation of isocitrate and succinate in LPS-activated BMDMs  
286 but not HMDMs (Supplementary Figure 6C, D). As such, the use of substrate-coated plates  
287 can be a useful follow-up method to study enzyme activity across a variety of metabolic  
288 pathways. Yet, it should be noted that this approach also yielded unexpected results that

289 appear in contrast with published results and data obtained by more established techniques  
290 such as XF analysis. For example, upregulation of mitochondrial respiration in M[IL-4] (Figure  
291 3C, Supplementary Figure 2C) could not be reproduced in either plate type and alterations in  
292 FAO as observed with SCENITH (Figure 4D, E) could not be confirmed (Supplementary Figure  
293 6E, F).

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

## 297 Discussion

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

### 309 Profiling immunometabolism highlights distinct metabolic rewiring in mouse and human 310 macrophage activation states

311 As an example, we applied our immunometabolic profiling platform to LPS±IFN $\gamma$ - and IL-4-  
312 stimulated BMDMs for which metabolic reprogramming is well-described (Lauterbach *et al.*,  
313 2019; Van den Bossche *et al.*, 2015; Vijayan *et al.*, 2019), and also applied the same stimuli  
314 to HMDMs for which metabolic processes are less well understood. While focused on  
315 macrophages, after optimization of certain factors (detailed in Supplementary Table 1) the  
316 approach can also be extended to other cells since individual techniques have previously been  
317 applied to, for example T cells and dendritic cells (Arguello *et al.*, 2020; Buck *et al.*, 2016;  
318 Everts *et al.*, 2012; Little *et al.*, 2020; Lopes *et al.*, 2021; Scharping *et al.*, 2016; Thomas and  
319 Mattila, 2014).

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

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

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

336 (iii) Since HMDMs do not produce NO *in vitro* (Figure 2C) (Gross et al., 2014), this could explain  
337 why they don't show the drop in mitochondrial respiration that is observed in their LPS±IFN $\gamma$ -  
338 induced NO-producing mouse counterparts (Van den Bossche et al., 2016; Vijayan et al.,  
339 2019).

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

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

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

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

362 Interrogation of distinct metabolic pathways by complementary techniques

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

#### 366 *Readouts of glycolysis*

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

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

#### 384 *Mitochondrial function*

385 To measure mitochondrial parameters, we here showed the use of XF-derived OCR,  
386 SCENITH, MitoTracker Green, TMRM, and mitochondrial-substrate-coated plates. As  
387 expected, OCR measurements showed decreased mitochondrial respiration in inflammatory  
388 BMDMs but not HMDMs due to species differences as discussed above. Still, mitochondrial  
389 contribution to total ATP production as calculated when taking along CO<sub>2</sub>-based acidification  
390 in XF analysis, and mitochondrial dependence as assessed by SCENITH was decreased in  
391 both mouse and human inflammatory macrophages. Mitochondrial-substrate-coated plates  
392 confirmed this reduction in LPS-activated mouse (but not human) inflammatory macrophages.  
393 However, the substrate utilization assays appear less sensitive than XF and SCENITH since  
394 the metabolism of the individual mitochondrial substrates was not indicative of the well-  
395 established elevated mitochondrial respiration in IL-4-activated BMDMs when analyzing intact  
396 cells with carbon-substrate-coated plates nor permeabilized cells with mitochondrial-substrate-

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

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

#### 413 *Fatty acid metabolism*

414 Readouts for fatty acid metabolism demonstrated here include SCENITH, BODIPY C16 uptake  
415 and fatty acid utilization in mitochondrial-substrate-coated plates. SCENITH-derived FAO/AAO  
416 capacity followed the expected pattern for both BMDMs and HMDMs, although SCENITH  
417 cannot formally distinguish between FAO and AAO capacities. SCENITH-derived FAO/AAO  
418 capacity was paralleled by BODIPY C16 signal for BMDMs but not for HMDMs, indicating that  
419 uptake of fatty acids is not a direct measure of FAO. Rather, fatty acids can be stored in lipid  
420 droplets instead of being used to fuel metabolic processes (Feingold *et al.*, 2012; Huang *et al.*,  
421 2014b). mitochondrial-substrate-coated plates did not pick up differences in fatty acid  
422 metabolism between conditions. Therefore, SCENITH and BODIPY C16 uptake can give first  
423 indications about fatty acid metabolism and could be extended by a BODIPY dye staining lipid  
424 droplets (Qiu and Simon, 2016) or an adapted XF protocol allowing to probe fuel preferences  
425 (Voss *et al.*, 2021).

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

430 Practical considerations

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

433 *Pre-screening of metabolic alterations*

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

446 *XF analysis profiles glycolysis and mitochondrial function in parallel*

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

452 However, it also comes with certain disadvantages limiting its applications. Whereas extremely  
453 useful for homogeneous samples of which many cells are present (such as *in vitro* stimulated  
454 cells), only the core metabolic pathways that directly result in H<sup>+</sup> production or oxygen  
455 consumption are represented. Additionally, analysis of complex *in vivo* samples such as tumor  
456 tissue is much more challenging. Such samples include many distinct cell populations which  
457 cannot be separately assessed without prior cell sorting. Both cell sorting and cell culturing  
458 may affect the metabolic state of cells (Binek *et al.*, 2019; Llufrio *et al.*, 2018; Voss *et al.*, 2021).  
459 Generally, XF analysis requires large cell numbers in the 96-well format (typically 5,000-  
460 200,000 cells in 3-8 replicates).

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

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

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

#### 471 *SCENITH allows metabolic phenotyping of distinct cell subsets*

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

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

#### 486 *Fluorescent metabolic probes enable assessing metabolic features at single-cell resolution*

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

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

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

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

513 Limitations and potential follow-up beyond the toolbox

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

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

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

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

## 560 Conclusion and practical guidance

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

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

#### 568 **Author contributions**

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

#### 574 **Acknowledgements**

575 We thank Sam de Decker, Enrico Tatti and Barry Bochner for supplying all materials to perform  
576 the Biolog mitochondrial-substrate-coated and carbon-substrate-coated assays, their help with  
577 setting up the assays, with interpreting the results and their helpful comments on the  
578 manuscript. We further thank Amber Eggert for optimizing the Hoechst cell counting protocol  
579 on the Cytation 5 and Karl Harber for proofreading the manuscript. We thank ANR and Inserm  
580 Transfert for the ANR-20-CE-CE14-0028-01 and CoPoC MAT-PI-17493-A-04 grants (to RJA).  
581 JVdB received a Netherlands Heart Foundation senior fellowship (2017T048). Additionally,  
582 JVdB received an Enw-Klein-1 grant from NWO (OCENW.KLEIN.268) and a consortia grant  
583 from European Research Area network on Cardiovascular Diseases (ERA-CVD 2019T108).  
584 Lastly, we acknowledge support from Cancer Center Amsterdam.

#### 585 **Declarations of interest**

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

## 588 Figure Legends

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

601  
602 **Figure 2: Metabolic pre-screening of BMDMs and HMDMs indicate metabolic differences after varying**  
603 **macrophage activation.** (A) BMDMs and HMDMs were left untreated (Naive), or stimulated with either LPS,  
604 LPS+IFN $\gamma$  or IL-4 for 24 h. (B, C) Levels of NO in supernatants from BMDMs (B) or HMDMs (both  $1 \times 10^5$  cells per  
605 well) (C) and arginase activity in BMDMs ( $5 \times 10^4$  cells per well) (B) or HMDMs ( $1 \times 10^5$  cells per well) (C) following  
606 stimulation. (D, E) Levels of glucose consumption and lactate production in supernatants of BMDMs (D) or HMDMs  
607 (E) (all  $1 \times 10^5$  cells per well). Data are shown as mean  $\pm$  SEM. For BMDMs, N=12 mice with 3 technical replicates  
608 in 4 independent experiments were included for NO, glucose and lactate assays, and N=6 with 3 technical replicates  
609 in 2 independent experiments for arginase activity assay. For HMDMs, N=5 human donors with 3 technical  
610 replicates in 2 independent experiments were included for all assays. \*\* P<0.01, \*\*\* P<0.001 by one-way ANOVA  
611 with Dunnett's post-hoc test for multiple comparisons.

612  
613 **Figure 3: XF analyses of BMDMs and HMDMs yield insight into metabolic profiles of macrophages after**  
614 **LPS $\pm$ IFN $\gamma$  and IL-4-activation.** (A, B) Normalized (to relative Hoechst<sup>+</sup> objects) ECAR, with injections of glucose,  
615 oligomycin, FCCP and Antimycin A/Rotenone/Hoechst for BMDMs (A) and HMDMs (B). (C, D) Normalized (to  
616 relative Hoechst<sup>+</sup> objects) OCR with same injections as for ECAR for BMDMs (C) and HMDMs (D). (E, G) Metabolic  
617 profiles outlining basal respiration and glycolysis for BMDMs (E) and HMDMs (G). (F, H) Mitochondrial and glycolytic  
618 contribution to overall ATP production in BMDMs (F) and HMDMs (H). N=6 mice or N=6 donors were included with  
619 4-5 technical replicates each. Values shown as mean  $\pm$  SEM calculated from the average of technical replicates  
620 per mouse/donor. \* P<0.05, \*\* P<0.01, \*\*\* P<0.001 by one-way ANOVA with Dunnett's post-hoc test for multiple  
621 comparisons. For F and H, significance on top of bar graphs indicates changes in total ATP production rate,  
622 significance within bars indicates significant differences between either the glycolytic or mitochondrial contribution  
623 to ATP production rate compared with N.

624  
625 **Figure 4: Metabolic analysis of BMDMs and HMDMs with SCENITH reveals expected macrophage activation**  
626 **by LPS $\pm$ IFN $\gamma$  and IL-4.** (A, B) MFI of puromycin across samples treated with different inhibitors for BMDMs (A) and  
627 HMDMs (B). DG, O and DGO indicate Deoxyglucose- (DG), Oligomycin- (O) or Deoxyglucose+Oligomycin-treated  
628 (DGO) samples. (C) Calculations of metabolic SCENITH parameters based on puromycin MFI. (D, E, F, H)  
629 SCENITH parameters as calculated for mouse (D, F) and human (E, H) macrophages. (G, I) Correlation of glycolytic  
630 capacity as measured with XF analysis with glycolytic capacity as measured with SCENITH for BMDMs (G) and  
631 HMDMs (I). (J, K) tSNE dimensionality reduction of naive, LPS $\pm$ IFN $\gamma$ - and IL-4-treated BMDMs (J) and HMDMs (K)  
632 and clustered heatmaps showing the expression of activation markers and puromycin per stimulus. Data are shown  
633 as mean  $\pm$  SEM. Each dot marks a separate mouse (N=6) or human donor (N=6). \* P<0.05, \*\* P<0.01, \*\*\* P<0.001  
634 by two-way (A, B) or ordinary one-way ANOVA (D, E, F, H) with Dunnett's post-hoc test for multiple comparisons.  
635 Correlations were fitted using a simple linear regression model (G, I).

636  
637 **Figure 5: Uptake of fluorescent probes provides additional insight into macrophage metabolism.** (A, B)  
638 Representative images of BMDM (A) and HMDM (B) staining by fluorescent dyes and uptake of fluorescent nutrient  
639 analogs as assessed by multi-mode reader. Scale bar represents 200  $\mu$ m. (C-F) Fluorescent intensity of 2NB-DG  
640 (C, E) and BODIPY C16 (D, F) uptake by BMDMs (C, D) and HMDMs (E, F) as examined by flow cytometry,  
641 correlated with relevant parameters of XF analysis. (G-J) Fluorescent intensity of MitoTracker Green (G, I) and  
642 TMRM (H, J) analysis as examined by flow cytometry and correlations with relevant parameters of XF analysis in  
643 BMDMs (G, H) and HMDMs (I, J). Data are shown as mean  $\pm$  SEM. For graphs of fluorescent probes, each dot  
644 marks a separate mouse (N=9) or donor (N=8).  $\Delta$ MFI was calculated as MFI (median fluorescent intensity) of  
645 sample – MFI of unstained control. \* P<0.05, \*\* P<0.01, \*\*\* P<0.001 by one-way ANOVA with Dunnett's post-hoc  
646 test for multiple comparisons. Correlations were fitted using a simple linear regression model.

647  
648 **Figure 6: An actionable workflow to guide researchers from simple screening towards complex**  
649 **measurement of immunometabolism.** Immunometabolic alterations can be pre-screened by quick and easy  
650 assays such as NO production and arginase activity in mouse macrophages and glucose consumption and lactate  
651 production in both species (yellow). Cytokine and viability measurements (green) can be performed in parallel to  
652 connect cellular metabolic changes and function. These assays can be followed up by metabolic characterization  
653 (purple) with a bulk (XF analysis) or single-cell approaches (SCENITH and fluorescent metabolic dyes).

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

**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		

662 **STAR Methods**

663 **RESOURCE AVAILABILITY**

664 **Lead contact**

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

667 **Materials availability**

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

672 **Data and code availability**

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

676 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

677 **Bone marrow isolation and BMDM culture**

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

692 **Monocyte isolation and HMDM culture**

693 Buffy coats (50 ml) were purchased from Sanquin blood Bank (Amsterdam, Netherlands).  
694 Information regarding sex and age of donors was not supplied by the blood bank. PBMCs were  
695 isolated with a Ficoll/Lymphoprep gradient (Greiner Biosciences) and careful centrifugation at  
696 800g for 30 minutes. Next, monocytes were isolated by applying  $120\text{-}150 \times 10^6$  cells on top of a  
697 46% Percoll™ (Cytiva) solution followed by careful centrifugation at 2000 rpm for 20 minutes.  
698 Monocytes were counted in the same manner as BMDMs and plated at a density of  $2 \times 10^6$   
699 cells/ml in the appropriate 96-well plates for each experiment in 100  $\mu$ l IMDM medium  
700 containing HEPES (Gibco) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100  
701  $\mu$ g/ml streptomycin (full IMDM medium), and containing 1% FCS. After settling for one hour,  
702 medium was replaced with full IMDM medium with 10% FCS and 50 ng/ml M-CSF (Miltenyi)  
703 for 6-day differentiation, resulting in >95% macrophage purity (Supplementary Figure 1C). On  
704 day 3, medium was replaced with fresh medium supplemented with M-CSF (Miltenyi). On day  
705 6, cells (near 100% macrophage purity (Supplementary Figure 1A, C)) were left untreated or  
706 stimulated in fresh medium without M-CSF with 100 ng/ml LPS (Sigma Aldrich), 10 ng/ml LPS  
707 + 20 ng/ml IFN $\gamma$  (Peprotech), or 20 ng/ml IL-4 (Peprotech) for 24 hours.

## 708 **METHOD DETAILS**

### 709 **NO production and Arginase activity assay**

710 100  $\mu$ l supernatant was collected from 96-well plates with  $1 \times 10^5$  cells (BMDMs/HMDMs) per  
711 well. Subsequently, NO production was measured by adding 50  $\mu$ l Griess reagent (2.5% H $_3$ PO $_4$   
712 (Merck), 1% sulfanilamide (Sigma Aldrich), and 0.1% naphthylene diamide dihydrochloride  
713 (Sigma Aldrich) in H $_2$ O) to 50  $\mu$ l cell supernatants (1:1) and optical density was measured at  
714 540 nm.

715 Arginase activity was determined on cell lysates of  $5 \times 10^4$  cells (BMDMs) or  $1 \times 10^5$  cells  
716 (HMDMs). Cells were washed with PBS and lysed by incubating for 30 minutes with 100  $\mu$ l  
717 0.1% Triton X-100 (Sigma Aldrich), 25 mM Tris-HCl (pH 7.5, Roche) supplemented with 1x  
718 protease inhibitor cocktail (Roche). Arginase was activated by adding 3.5  $\mu$ l of 10 mM MnCl $_2$   
719 (Sigma Aldrich) to 10  $\mu$ l sample and incubated at 56°C for 10 minutes. Next, samples were  
720 incubated with 10  $\mu$ l 0.5 M L-arginine (pH 9.7, Sigma Aldrich) for 60 minutes at 37°C. The  
721 reaction was stopped by adding 90  $\mu$ l stop solution (96% H $_2$ SO $_4$ /85% H $_3$ PO $_4$ /H $_2$ O 1:3:7, Merck)  
722 and incubated with 4  $\mu$ l  $\alpha$ -isonitrosopropiophenone (9%, Sigma Aldrich) for 30 minutes at 95°C.  
723 Samples were left in the dark to cool down to room temperature until measurement of optical  
724 density at 540 nm. Enzymatic activity was calculated by [Urea]\*(total volume\* $10^6$ )/(tested  
725 volume\*Time(incubated at 37°C)\*1000).

### 726 **Glucose consumption assay**

727 100  $\mu$ l supernatant was collected from 96-well plates with  $1 \times 10^5$  cells (BMDMs/HMDMs) per  
728 well. To determine glucose levels in supernatant, samples and standard (5  $\mu$ l per well) were  
729 pipetted into a 96-well plate. 250  $\mu$ l glucose reagent (BIOLABO) was added to standard and  
730 samples, mixed by pipetting and incubated for 30 minutes in the dark. Absorbance was  
731 measured at 490 nm. Glucose consumption was calculated as the difference between glucose  
732 levels of medium without cells and glucose levels in cell supernatants.

### 733 Lactate production assay

734 100  $\mu$ l supernatant was collected from 96-well plates with  $1 \times 10^5$  cells (BMDMs/HMDMs) per  
735 well. Lactate levels in cellular supernatants were determined by conversion of lactate into  
736 NADH by lactate dehydrogenase (LDH). First, samples were incubated for 15 minutes at 4°C  
737 with 3% metaphosphoric acid (Sigma Aldrich), centrifuged at 20.000 g for 10 minutes, and  
738 supernatants were used further. 5  $\mu$ l of thus deproteinized samples were transferred into a 96-  
739 wells plate and 150  $\mu$ l Master Mix consisting of 0.5 M Glycine - 0.4 M hydrazine buffer (pH =  
740 9.0, Sigma Aldrich) with 27 mM NAD (Cayman Chemicals) was added per well. NADH  
741 fluorescence was measured using a Mithras LB 940 with  $\lambda_{ex}/\lambda_{em}$  = 340-10 /450-10 nm every 2  
742 minutes for 5 cycles as background measurement. Next, 50  $\mu$ l of start solution consisting of  
743 0.5 M Glycine - 0.4 M hydrazine buffer (pH = 9.0) and 5 mg/ml LDH was added to each well.  
744 Fluorescence was measured every 2 minutes with shaking until a stable read was achieved.

### 745 Extracellular flux analysis

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

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

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

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

$$767\ mitoPER = CCF * (OCR_{basal} - OCR_{Rot/AA}),$$

768 where CCF (CO<sub>2</sub> contribution factor) was determined as 0.61 for XFe96 plates by the  
769 manufacturer for a range of cell types. Total proton efflux rate (PER) can be calculated as:

$$770\ total\ PER = ECAR * BF * Vol_{measurement\ chamber} * K_{vol},$$

771 where the buffer factor (BF) of the medium refers to the amount of H<sup>+</sup> necessary to change the  
772 pH of the medium by one unit, and was measured according to manufacturer instructions in  
773 the Buffer Factor Protocol Quick Reference Guide (Agilent). Vol<sub>measurement chamber</sub> and K<sub>vol</sub> are  
774 scaling factors to determine the effective volume of the well and were determined as 2.28 µl  
775 and 1.60, respectively, by the manufacturer for XFe96 plates. From this, glycolytic proton efflux  
776 rate and therefore glycolytic ATP production rate can be determined:

$$777\ glycoATP = glycoPER = total\ PER - mitoPER.$$

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

$$779\ mitoATP = (OCR_{basal} - OCR_{Rot/AA}) * 2 * P/O,$$

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

## 785 **SCENITH and Flow cytometry and analysis**

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

791 added without washing and incubated for another 30 min at 37°C. After incubation, cells were  
792 washed with cold PBS, harvested by incubating with PBS + 5 mM EDTA for 10 min at 4°C and  
793 proceeded with Fc receptor blockade (eBioScience for mouse, BD Biosciences for human  
794 cells) and fixable viability dye staining for 15 min at 4°C in the dark. Subsequently, cells were  
795 washed and stained with surface antibody mix in PBS/0.5% BSA/0.02% sodium azide (PBA)  
796 for 30 min at 4°C in the dark. Cells were then washed, fixed and permeabilized using the  
797 FOXP3 fixation and permeabilization kit (eBioScience) according to manufacturer's  
798 instructions. For intracellular staining of iNOS, Arg1 and puromycin, cells were incubated for  
799 1h at 4°C in antibody staining solution in permeabilization buffer. Samples were then  
800 transferred to a plate-reader compatible 96-well U-bottom plate.

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

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

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

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

### 819 **Fluorescent metabolic dyes**

820 Mitochondrial mass was measured using MitoTracker Green (Invitrogen). Mitochondrial  
821 membrane potential was measured using Tetramethylrhodamine methyl ester (TMRM,  
822 Thermo Fisher) and fatty acid and glucose uptake was measured using BODIPY<sup>TM</sup> FL C16  
823 (BODIPY, Thermo Fisher) or 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose  
824 (2NB-DG, Invitrogen) fluorescent probes. Cells were plated in 96-well black culture plates at a  
825 density of  $8 \times 10^4$  cells/well. Cells were starved for 2 hours in basal RPMI-medium with (for  
826 BODIPY C16) or without glucose (for 2NB-DG) and subsequently stained by incubation for 30

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

834 For FACS analysis, cells were harvested using ice-cold PBS+5 mM EDTA, transferred to a  
835 plate-reader compatible 96-well U-bottom plate and immediately acquired on an X20 Fortessa  
836 flow cytometer with plate reader (BD Biosciences) and analyzed as described in the section  
837 'Flow cytometry and analysis'. To validate metabolic dye signal, MitoTracker Green and TMRM  
838 signal were inhibited by the uncoupler FCCP (Sigma-Aldrich, 5  $\mu$ M final concentration), 2NB-  
839 DG by phloretin, an inhibitor of glucose transport (Sigma-Aldrich, 150  $\mu$ M final concentration),  
840 and BODIPY C16 by lipofermata, an inhibitor of fatty acid transporter 2 (Cayman Chemicals,  
841 10  $\mu$ M final concentration) (Supplementary Figure 4).

#### 842 **Mitochondrial functional substrate assay**

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

854 Additionally, intact BMDMs were plated as  $4 \times 10^4$  cells/well in 50  $\mu$ l MC-0 Assay Medium into  
855 carbon-substrate-coated plates (Biolog) to further assess whole-cell substrate usage as  
856 opposed to only mitochondrial substrate use. Cells and substrates were pre-incubated for 24  
857 hours at 37°C and 5% CO<sub>2</sub>, and metabolism was assessed after adding 20  $\mu$ l 6X Biolog Redox  
858 Dye MB.

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

## 862 **QUANTIFICATION AND STATISTICAL ANALYSIS**

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

- 875 Ahl, P.J., Hopkins, R.A., Xiang, W.W., Au, B., Kaliaperumal, N., Fairhurst, A.M., and  
876 Connolly, J.E. (2020). Met-Flow, a strategy for single-cell metabolic analysis highlights  
877 dynamic changes in immune subpopulations. *Commun Biol* 3, 305. 10.1038/s42003-020-  
878 1027-9.
- 879 Arguello, R.J., Combes, A.J., Char, R., Gigan, J.P., Baaziz, A.I., Bousiquot, E., Camosseto,  
880 V., Samad, B., Tsui, J., Yan, P., et al. (2020). SCENITH: A Flow Cytometry-Based Method to  
881 Functionally Profile Energy Metabolism with Single-Cell Resolution. *Cell Metab* 32, 1063-  
882 1075 e1067. 10.1016/j.cmet.2020.11.007.
- 883 Artyomov, M.N., and Van den Bossche, J. (2020). Immunometabolism in the Single-Cell Era.  
884 *Cell Metab* 32, 710-725. 10.1016/j.cmet.2020.09.013.
- 885 Baardman, J., Verberk, S.G.S., Prange, K.H.M., van Weeghel, M., van der Velden, S., Ryan,  
886 D.G., Wust, R.C.I., Neele, A.E., Speijer, D., Denis, S.W., et al. (2018). A Defective Pentose  
887 Phosphate Pathway Reduces Inflammatory Macrophage Responses during  
888 Hypercholesterolemia. *Cell Rep* 25, 2044-2052 e2045. 10.1016/j.celrep.2018.10.092.
- 889 Bailey, J.D., Diotallevi, M., Nicol, T., McNeill, E., Shaw, A., Chuaiphichai, S., Hale, A., Starr,  
890 A., Nandi, M., Stylianou, E., et al. (2019). Nitric Oxide Modulates Metabolic Remodeling in  
891 Inflammatory Macrophages through TCA Cycle Regulation and Itaconate Accumulation. *Cell*  
892 *Rep* 28, 218-230 e217. 10.1016/j.celrep.2019.06.018.
- 893 Bambouskova, M., Potuckova, L., Paulenda, T., Kerndl, M., Mogilenko, D.A., Lizotte, K.,  
894 Swain, A., Hayes, S., Sheldon, R.D., Kim, H., et al. (2021). Itaconate confers tolerance to  
895 late NLRP3 inflammasome activation. *Cell Rep* 34, 108756. 10.1016/j.celrep.2021.108756.
- 896 Bauerfeld, C.P., Rastogi, R., Pirockinaite, G., Lee, I., Huttemann, M., Monks, B., Birnbaum,  
897 M.J., Franchi, L., Nunez, G., and Samavati, L. (2012). TLR4-mediated AKT activation is  
898 MyD88/TRIF dependent and critical for induction of oxidative phosphorylation and  
899 mitochondrial transcription factor A in murine macrophages. *J Immunol* 188, 2847-2857.  
900 10.4049/jimmunol.1102157.
- 901 Binek, A., Rojo, D., Godzien, J., Ruperez, F.J., Nunez, V., Jorge, I., Ricote, M., Vazquez, J.,  
902 and Barbas, C. (2019). Flow Cytometry Has a Significant Impact on the Cellular Metabolome.  
903 *J Proteome Res* 18, 169-181. 10.1021/acs.jproteome.8b00472.
- 904 Buck, M.D., O'Sullivan, D., Klein Geltink, R.I., Curtis, J.D., Chang, C.H., Sanin, D.E., Qiu, J.,  
905 Kretz, O., Braas, D., van der Windt, G.J., et al. (2016). Mitochondrial Dynamics Controls T  
906 Cell Fate through Metabolic Programming. *Cell* 166, 63-76. 10.1016/j.cell.2016.05.035.
- 907 D'Souza, L.J., Wright, S.H., and Bhattacharya, D. (2021). 10.1101/2021.12.13.472409.
- 908 Everts, B., Amiel, E., van der Windt, G.J., Freitas, T.C., Chott, R., Yarasheski, K.E., Pearce,  
909 E.L., and Pearce, E.J. (2012). Commitment to glycolysis sustains survival of NO-producing  
910 inflammatory dendritic cells. *Blood* 120, 1422-1431. 10.1182/blood-2012-03-419747.
- 911 Farren, M.R., Sayegh, L., Ware, M.B., Chen, H.R., Gong, J., Liang, Y., Krasinskas, A.,  
912 Maithel, S.K., Zaidi, M., Sarmiento, J.M., et al. (2020). Immunologic alterations in the  
913 pancreatic cancer microenvironment of patients treated with neoadjuvant chemotherapy and  
914 radiotherapy. *JCI Insight* 5. 10.1172/jci.insight.130362.
- 915 Feingold, K.R., Shigenaga, J.K., Kazemi, M.R., McDonald, C.M., Patzek, S.M., Cross, A.S.,  
916 Moser, A., and Grunfeld, C. (2012). Mechanisms of triglyceride accumulation in activated  
917 macrophages. *J Leukoc Biol* 92, 829-839. 10.1189/jlb.1111537.
- 918 Freerman, A.J., Johnson, A.R., Sacks, G.N., Milner, J.J., Kirk, E.L., Troester, M.A.,  
919 Macintyre, A.N., Goraksha-Hicks, P., Rathmell, J.C., and Makowski, L. (2014). Metabolic  
920 reprogramming of macrophages: glucose transporter 1 (GLUT1)-mediated glucose  
921 metabolism drives a proinflammatory phenotype. *J Biol Chem* 289, 7884-7896.  
922 10.1074/jbc.M113.522037.
- 923 Galli, G., and Saleh, M. (2020). Immunometabolism of Macrophages in Bacterial Infections.  
924 *Front Cell Infect Microbiol* 10, 607650. 10.3389/fcimb.2020.607650.
- 925 Gross, T.J., Kremens, K., Powers, L.S., Brink, B., Knutson, T., Domann, F.E., Philibert, R.A.,  
926 Milhem, M.M., and Monick, M.M. (2014). Epigenetic silencing of the human NOS2 gene:

927 rethinking the role of nitric oxide in human macrophage inflammatory responses. *J Immunol*  
928 *192*, 2326-2338. 10.4049/jimmunol.1301758.

929 Harber, K.J., de Goede, K.E., Verberk, S.G.S., Meinster, E., de Vries, H.E., van Weeghel,  
930 M., de Winther, M.P.J., and Van den Bossche, J. (2020). Succinate Is an Inflammation-  
931 Induced Immunoregulatory Metabolite in Macrophages. *Metabolites* *10*.  
932 10.3390/metabo10090372.

933 Hartmann, F.J., and Bendall, S.C. (2020). Immune monitoring using mass cytometry and  
934 related high-dimensional imaging approaches. *Nat Rev Rheumatol* *16*, 87-99.  
935 10.1038/s41584-019-0338-z.

936 Hartmann, F.J., Mrdjen, D., McCaffrey, E., Glass, D.R., Greenwald, N.F., Bharadwaj, A.,  
937 Khair, Z., Verberk, S.G.S., Baranski, A., Baskar, R., et al. (2021). Single-cell metabolic  
938 profiling of human cytotoxic T cells. *Nat Biotechnol* *39*, 186-197. 10.1038/s41587-020-0651-  
939 8.

940 Huang, S.C., Everts, B., Ivanova, Y., O'Sullivan, D., Nascimento, M., Smith, A.M., Beatty, W.,  
941 Love-Gregory, L., Lam, W.Y., O'Neill, C.M., et al. (2014a). Cell-intrinsic lysosomal lipolysis is  
942 essential for alternative activation of macrophages. *Nat Immunol* *15*, 846-855.  
943 10.1038/ni.2956.

944 Huang, Y.L., Morales-Rosado, J., Ray, J., Myers, T.G., Kho, T., Lu, M., and Munford, R.S.  
945 (2014b). Toll-like receptor agonists promote prolonged triglyceride storage in macrophages.  
946 *J Biol Chem* *289*, 3001-3012. 10.1074/jbc.M113.524587.

947 Jaguin, M., Houlbert, N., Fardel, O., and Lecreur, V. (2013). Polarization profiles of human  
948 M-CSF-generated macrophages and comparison of M1-markers in classically activated  
949 macrophages from GM-CSF and M-CSF origin. *Cell Immunol* *281*, 51-61.  
950 10.1016/j.cellimm.2013.01.010.

951 Jha, A.K., Huang, S.C., Sergushichev, A., Lampropoulou, V., Ivanova, Y., Loginicheva, E.,  
952 Chmielewski, K., Stewart, K.M., Ashall, J., Everts, B., et al. (2015). Network integration of  
953 parallel metabolic and transcriptional data reveals metabolic modules that regulate  
954 macrophage polarization. *Immunity* *42*, 419-430. 10.1016/j.immuni.2015.02.005.

955 Kalvala, A., Wallet, P., Yang, L., Wang, C., Li, H., Nam, A., Nathan, A., Mambetsariev, I.,  
956 Poroyko, V., Gao, H., et al. (2019). Phenotypic Switching of Naive T Cells to Immune-  
957 Suppressive Treg-Like Cells by Mutant KRAS. *J Clin Med* *8*. 10.3390/jcm8101726.

958 Kam, Y., Rogers, G.W., Jastromb, N., Dranka, B.P. (2018). Methods and strategies for  
959 normalizing XF metabolic data to cellular parameters.  
960 [https://www.agilent.com/cs/library/technicaloverviews/public/Methods\\_and\\_Strategies\\_for\\_N](https://www.agilent.com/cs/library/technicaloverviews/public/Methods_and_Strategies_for_Normalizing_Tech_Overview_022118.pdf)  
961 [ormalizing\\_Tech\\_Overview\\_022118.pdf](https://www.agilent.com/cs/library/technicaloverviews/public/Methods_and_Strategies_for_Normalizing_Tech_Overview_022118.pdf).

962 Keren, L., Bosse, M., Thompson, S., Risom, T., Vijayaragavan, K., McCaffrey, E., Marquez,  
963 D., Angoshtari, R., Greenwald, N.F., Fienberg, H., et al. (2019). MIBI-TOF: A multiplexed  
964 imaging platform relates cellular phenotypes and tissue structure. *Sci Adv* *5*, eaax5851.  
965 10.1126/sciadv.aax5851.

966 Koenis, D.S., Medzikovic, L., van Loenen, P.B., van Weeghel, M., Huveneers, S., Vos, M.,  
967 Evers-van Gogh, I.J., Van den Bossche, J., Speijer, D., Kim, Y., et al. (2018). Nuclear  
968 Receptor Nur77 Limits the Macrophage Inflammatory Response through Transcriptional  
969 Reprogramming of Mitochondrial Metabolism. *Cell Rep* *24*, 2127-2140 e2127.  
970 10.1016/j.celrep.2018.07.065.

971 Lacey, D.C., Achuthan, A., Fleetwood, A.J., Dinh, H., Roiniotis, J., Scholz, G.M., Chang,  
972 M.W., Beckman, S.K., Cook, A.D., and Hamilton, J.A. (2012). Defining GM-CSF- and  
973 macrophage-CSF-dependent macrophage responses by in vitro models. *J Immunol* *188*,  
974 5752-5765. 10.4049/jimmunol.1103426.

975 Lachmandas, E., Boutens, L., Ratter, J.M., Hijmans, A., Hooiveld, G.J., Joosten, L.A.,  
976 Rodenburg, R.J., Fransen, J.A., Houtkooper, R.H., van Crevel, R., et al. (2016). Microbial  
977 stimulation of different Toll-like receptor signalling pathways induces diverse metabolic  
978 programmes in human monocytes. *Nat Microbiol* *2*, 16246. 10.1038/nmicrobiol.2016.246.

979 Lampropoulou, V., Sergushichev, A., Bambouskova, M., Nair, S., Vincent, E.E., Loginicheva,  
980 E., Cervantes-Barragan, L., Ma, X., Huang, S.C., Griss, T., et al. (2016). Itaconate Links

981 Inhibition of Succinate Dehydrogenase with Macrophage Metabolic Remodeling and  
982 Regulation of Inflammation. *Cell Metab* 24, 158-166. 10.1016/j.cmet.2016.06.004.  
983 Lauterbach, M.A., Hanke, J.E., Serefidou, M., Mangan, M.S.J., Kolbe, C.C., Hess, T., Rothe,  
984 M., Kaiser, R., Hoss, F., Gehlen, J., et al. (2019). Toll-like Receptor Signaling Rewires  
985 Macrophage Metabolism and Promotes Histone Acetylation via ATP-Citrate Lyase. *Immunity*  
986 51, 997-1011 e1017. 10.1016/j.immuni.2019.11.009.  
987 Levine, L.S., Hiam-Galvez, K.J., Marquez, D.M., TenVooren, I., Madden, M.Z., Contreras,  
988 D.C., Dahunsi, D.O., Irish, J.M., Oluwole, O.O., Rathmell, J.C., and Spitzer, M.H. (2021).  
989 Single-cell analysis by mass cytometry reveals metabolic states of early-activated CD8(+) T  
990 cells during the primary immune response. *Immunity* 54, 829-844 e825.  
991 10.1016/j.immuni.2021.02.018.  
992 Lim, A.R., Rathmell, W.K., and Rathmell, J.C. (2020). The tumor microenvironment as a  
993 metabolic barrier to effector T cells and immunotherapy. *Elife* 9, e55185.  
994 Little, A.C., Kovalenko, I., Goo, L.E., Hong, H.S., Kerk, S.A., Yates, J.A., Purohit, V.,  
995 Lombard, D.B., Merajver, S.D., and Lyssiotis, C.A. (2020). High-content fluorescence  
996 imaging with the metabolic flux assay reveals insights into mitochondrial properties and  
997 functions. *Commun Biol* 3, 271. 10.1038/s42003-020-0988-z.  
998 Lufrio, E.M., Wang, L., Naser, F.J., and Patti, G.J. (2018). Sorting cells alters their redox  
999 state and cellular metabolome. *Redox Biol* 16, 381-387. 10.1016/j.redox.2018.03.004.  
1000 Lopes, N., McIntyre, C., Martin, S., Raverdeau, M., Sumaria, N., Kohlgruber, A.C., Fiala,  
1001 G.J., Agudelo, L.Z., Dyck, L., Kane, H., et al. (2021). Distinct metabolic programs established  
1002 in the thymus control effector functions of gammadelta T cell subsets in tumor  
1003 microenvironments. *Nat Immunol* 22, 179-192. 10.1038/s41590-020-00848-3.  
1004 Makowski, L., Chaib, M., and Rathmell, J.C. (2020). Immunometabolism: From basic  
1005 mechanisms to translation. *Immunological reviews* 295, 5-14.  
1006 Martinez, F.O., Helming, L., Milde, R., Varin, A., Melgert, B.N., Draijer, C., Thomas, B.,  
1007 Fabbri, M., Crawshaw, A., Ho, L.P., et al. (2013). Genetic programs expressed in resting and  
1008 IL-4 alternatively activated mouse and human macrophages: similarities and differences.  
1009 *Blood* 121, e57-69. 10.1182/blood-2012-06-436212.  
1010 Miller, A., Nagy, C., Knapp, B., Laengle, J., Ponweiser, E., Groeger, M., Starkl, P.,  
1011 Bergmann, M., Wagner, O., and Haschemi, A. (2017). Exploring Metabolic Configurations of  
1012 Single Cells within Complex Tissue Microenvironments. *Cell Metab* 26, 788-800 e786.  
1013 10.1016/j.cmet.2017.08.014.  
1014 Mills, E.L., Kelly, B., Logan, A., Costa, A.S.H., Varma, M., Bryant, C.E., Tourlomousis, P.,  
1015 Dabritz, J.H.M., Gottlieb, E., Latorre, I., et al. (2016). Succinate Dehydrogenase Supports  
1016 Metabolic Repurposing of Mitochondria to Drive Inflammatory Macrophages. *Cell* 167, 457-  
1017 470 e413. 10.1016/j.cell.2016.08.064.  
1018 Mills, E.L., and O'Neill, L.A. (2016). Reprogramming mitochondrial metabolism in  
1019 macrophages as an anti-inflammatory signal. *Eur J Immunol* 46, 13-21.  
1020 10.1002/eji.201445427.  
1021 Munder, M., Eichmann, K., and Modolell, M. (1998). Alternative metabolic states in murine  
1022 macrophages reflected by the nitric oxide synthase/arginase balance: competitive regulation  
1023 by CD4+ T cells correlates with Th1/Th2 phenotype. *J Immunol* 160, 5347-5354.  
1024 Murphy, M.P., and O'Neill, L.A.J. (2020). How should we talk about metabolism? *Nat*  
1025 *Immunol* 21, 713-715. 10.1038/s41590-020-0691-8.  
1026 Murray, P.J., Allen, J.E., Biswas, S.K., Fisher, E.A., Gilroy, D.W., Goerdts, S., Gordon, S.,  
1027 Hamilton, J.A., Ivashkiv, L.B., Lawrence, T., et al. (2014). Macrophage activation and  
1028 polarization: nomenclature and experimental guidelines. *Immunity* 41, 14-20.  
1029 10.1016/j.immuni.2014.06.008.  
1030 Namgaladze, D., and Brune, B. (2014). Fatty acid oxidation is dispensable for human  
1031 macrophage IL-4-induced polarization. *Biochim Biophys Acta* 1841, 1329-1335.  
1032 10.1016/j.bbali.2014.06.007.  
1033 Palmer, A., Phapale, P., Chernyavsky, I., Lavigne, R., Fay, D., Tarasov, A., Kovalev, V.,  
1034 Fuchser, J., Nikolenko, S., Pineau, C., et al. (2017). FDR-controlled metabolite annotation for  
1035 high-resolution imaging mass spectrometry. *Nat Methods* 14, 57-60. 10.1038/nmeth.4072.

1036 Qiu, B., and Simon, M.C. (2016). BODIPY 493/503 Staining of Neutral Lipid Droplets for  
1037 Microscopy and Quantification by Flow Cytometry. *Bio Protoc* 6. 10.21769/BioProtoc.1912.  
1038 Rattigan, K.M., Pountain, A.W., Regnault, C., Achcar, F., Vincent, I.M., Goodyear, C.S., and  
1039 Barrett, M.P. (2018). Metabolomic profiling of macrophages determines the discrete  
1040 metabolomic signature and metabolomic interactome triggered by polarising immune stimuli.  
1041 *PLoS One* 13, e0194126. 10.1371/journal.pone.0194126.  
1042 Romero, N., Swain, P., Neilson A., and Dranka, B.P. (2017). Improving Quantification of  
1043 Cellular Glycolytic Rate Using Agilent Seahorse XF Technology  
1044 [https://seahorseinfo.agilent.com/acton/fs/blocks/showLandingPage/a/10967/p/p-](https://seahorseinfo.agilent.com/acton/fs/blocks/showLandingPage/a/10967/p/p-00ca/t/page/fm/1)  
1045 [00ca/t/page/fm/1](https://seahorseinfo.agilent.com/acton/fs/blocks/showLandingPage/a/10967/p/p-00ca/t/page/fm/1).  
1046 Salabei, J.K., Gibb, A.A., and Hill, B.G. (2014). Comprehensive measurement of respiratory  
1047 activity in permeabilized cells using extracellular flux analysis. *Nat Protoc* 9, 421-438.  
1048 10.1038/nprot.2014.018.  
1049 Scharping, N.E., Menk, A.V., Moreci, R.S., Whetstone, R.D., Dadey, R.E., Watkins, S.C.,  
1050 Ferris, R.L., and Delgoffe, G.M. (2016). The Tumor Microenvironment Represses T Cell  
1051 Mitochondrial Biogenesis to Drive Intratumoral T Cell Metabolic Insufficiency and  
1052 Dysfunction. *Immunity* 45, 701-703. 10.1016/j.immuni.2016.08.009.  
1053 Sinclair, L.V., Barthelemy, C., and Cantrell, D.A. (2020). Single Cell Glucose Uptake Assays:  
1054 A Cautionary Tale. *Immunometabolism* 2, e200029. 10.20900/immunometab20200029.  
1055 Tavakoli, S., Zamora, D., Ullevig, S., and Asmis, R. (2013). Bioenergetic profiles diverge  
1056 during macrophage polarization: implications for the interpretation of 18F-FDG PET imaging  
1057 of atherosclerosis. *J Nucl Med* 54, 1661-1667. 10.2967/jnumed.112.119099.  
1058 Thomas, A.C., and Mattila, J.T. (2014). "Of mice and men": arginine metabolism in  
1059 macrophages. *Front Immunol* 5, 479. 10.3389/fimmu.2014.00479.  
1060 Thwe, P.M., Pelgrom, L.R., Cooper, R., Beauchamp, S., Reisz, J.A., D'Alessandro, A.,  
1061 Everts, B., and Amiel, E. (2019). Cell-Intrinsic Glycogen Metabolism Supports Early  
1062 Glycolytic Reprogramming Required for Dendritic Cell Immune Responses. *Cell Metab* 30,  
1063 225. 10.1016/j.cmet.2019.05.017.  
1064 Van den Bossche, J., Baardman, J., and de Winther, M.P. (2015). Metabolic characterization  
1065 of polarized M1 and M2 bone marrow-derived macrophages using real-time extracellular flux  
1066 analysis. *JoVE (Journal of Visualized Experiments)*, e53424.  
1067 Van den Bossche, J., Baardman, J., Otto, N.A., van der Velden, S., Neele, A.E., van den  
1068 Berg, S.M., Luque-Martin, R., Chen, H.J., Boshuizen, M.C., Ahmed, M., et al. (2016).  
1069 Mitochondrial Dysfunction Prevents Repolarization of Inflammatory Macrophages. *Cell Rep*  
1070 17, 684-696. 10.1016/j.celrep.2016.09.008.  
1071 Van den Bossche, J., O'Neill, L.A., and Menon, D. (2017). Macrophage Immunometabolism:  
1072 Where Are We (Going)? *Trends Immunol* 38, 395-406. 10.1016/j.it.2017.03.001.  
1073 van der Windt, G.J., Everts, B., Chang, C.H., Curtis, J.D., Freitas, T.C., Amiel, E., Pearce,  
1074 E.J., and Pearce, E.L. (2012). Mitochondrial respiratory capacity is a critical regulator of  
1075 CD8+ T cell memory development. *Immunity* 36, 68-78. 10.1016/j.immuni.2011.12.007.  
1076 Vats, D., Mukundan, L., Odegaard, J.I., Zhang, L., Smith, K.L., Morel, C.R., Wagner, R.A.,  
1077 Greaves, D.R., Murray, P.J., and Chawla, A. (2006). Oxidative metabolism and PGC-1beta  
1078 attenuate macrophage-mediated inflammation. *Cell Metab* 4, 13-24.  
1079 10.1016/j.cmet.2006.05.011.  
1080 Vijayan, V., Pradhan, P., Braud, L., Fuchs, H.R., Gueler, F., Motterlini, R., Foresti, R., and  
1081 Immenschuh, S. (2019). Human and murine macrophages exhibit differential metabolic  
1082 responses to lipopolysaccharide - A divergent role for glycolysis. *Redox Biol* 22, 101147.  
1083 10.1016/j.redox.2019.101147.  
1084 Voss, K., Hong, H.S., Bader, J.E., Sugiura, A., Lyssiotis, C.A., and Rathmell, J.C. (2021). A  
1085 guide to interrogating immunometabolism. *Nat Rev Immunol*. 10.1038/s41577-021-00529-8.  
1086 Wynn, T.A., Chawla, A., and Pollard, J.W. (2013). Macrophage biology in development,  
1087 homeostasis and disease. *Nature* 496, 445-455. 10.1038/nature12034.  
1088 Yu, W., Wang, X., Zhao, J., Liu, R., Liu, J., Wang, Z., Peng, J., Wu, H., Zhang, X., Long, Z.,  
1089 et al. (2020). Stat2-Drp1 mediated mitochondrial mass increase is necessary for pro-

1090 inflammatory differentiation of macrophages. *Redox Biol* 37, 101761.  
1091 10.1016/j.redox.2020.101761.  
1092 Zhang, I.W., Curto, A., Lopez-Vicario, C., Casulleras, M., Duran-Guell, M., Flores-Costa, R.,  
1093 Colsch, B., Aguilar, F., Aransay, A.M., Lozano, J.J., et al. (2021). Mitochondrial dysfunction  
1094 governs immunometabolism in leukocytes of patients with acute-on-chronic liver failure. *J*  
1095 *Hepatol.* 10.1016/j.jhep.2021.08.009.  
1096 Zorova, L.D., Popkov, V.A., Plotnikov, E.Y., Silachev, D.N., Pevzner, I.B., Jankauskas, S.S.,  
1097 Babenko, V.A., Zorov, S.D., Balakireva, A.V., Juhaszova, M., et al. (2018). Mitochondrial  
1098 membrane potential. *Anal Biochem* 552, 50-59. 10.1016/j.ab.2017.07.009.

1099