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Intermediates of Metabolism: From Bystanders to Signalling Molecules.

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TRENDS BOX

- Metabolic intermediates of biochemical pathways are able to act as intra- and extracellular signalling molecules affecting immune cell responses.

- The signalling effects of metabolites are concentration and localization dependent.

- Their functions go beyond self-regulatory mechanisms and include cell to cell communication as well as sensing of micro-environmental conditions to elicit stress responses and cellular adaptation.

1   **Intermediates of metabolism: from bystanders to signalling molecules**

2

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12

14 **Summary**

15

16   The integration of biochemistry into immune cell biology has immensely contributed to our

17 understanding of immune cell function and the associated pathologies. So far, most studies have

18 focused on the regulation of metabolic pathways during an immune response and their contribution

19 to its success. More recently, novel signalling functions of metabolic intermediates are being

20 discovered that might play important roles in the regulation of immunity. Here, we describe the

21 three long-known small metabolites lactate, acetyl-CoA and succinate in the context of immuno-

22 metabolic signalling. Functions of these ubiquitous molecules are largely dependent on their intra-

23 and extra-cellular concentrations as well as their sub-compartmental localization. Importantly, the

24 signalling functions of these metabolic intermediates extend beyond self-regulatory roles and

25 include cell to cell communication and sensing of micro-environmental conditions to elicit stress

26 responses and cellular adaptation.

27

Metabolite signaling in immunity

The metabolic regulation of immune cells during health and disease has gained a lot of attention as the active reconfiguration of immune cell metabolism enables these cells to sustain certain effector functions. The focus so far has been on the necessity of the main catabolic pathways glycolysis, fatty acid oxidation, the anaplerotic tricarboxylic acid (TCA) cycle and oxidative phosphorylation as well as amino acid metabolism (Figure 1) during activation, proliferation, differentiation and function as a response to extracellular signals.

It is now becoming increasingly evident that small molecule intermediates of these metabolic pathways, besides their anabolic and catabolic function, can act as intra- and extracellular signals that influence the outcome of an immune response. The roles of metabolite signalling stretch from regulation of cytokine production via indirect effects on the cellular redox state [1] or direct interaction with transcription factors binding the specific cytokine promoter elements [2] and modulating the activity of transmembrane ion channels [3], to interference with cell migration and differentiation. Interestingly, a few G-protein coupled receptors that are activated by intermediates of metabolism have recently been identified supporting a role for metabolites as extracellular signals [4, 5]. In this review, we discuss the three well known metabolites lactate, succinate and acetyl-CoA in more detail; identify their differences and similarities in signal transduction and effect on immunity and inflammation that defines them as novel signalling molecules in physiology and pathology.

Lactate is a signalling molecule

51   Lactate is a ubiquitous molecule, whose presence in the mammalian body was first observed

52 in muscle tissue at the beginning of the 19th century [6]. Since its discovery, lactate has been

53 intensely studied and it has been shown to have numerous metabolic functions (Figure 2, Key

54 Figure), including as a central metabolite in the Cori cycle (also known as the lactic acid cycle), which

55 mediates metabolic cross talk between the liver and the muscle. In the Cori cycle, muscle tissue

56 metabolizes liver-derived glucose to lactate, which in turn is shuttled back to the liver and acts as a

57 fuel source for hepatic gluconeogenesis [7]. By contrast, in the brain lactate acts as a metabolic

58 signal and fuel for oxidative metabolism, which is the basis of the neuron-astrocyte lactate shuttle

59 [8]. Briefly, the neurotransmitter glutamate induces high glycolytic activity in astrocytes, which

60 secrete lactate into the synaptic cleft. The increased availability of extracellular lactate enables

61 neurons to import it and use it as an alternative fuel source.

62   Although lactate has been known to biochemists for over two hundred years, it has been

63 long neglected, seen as a by-product or a bio-marker at best rather than a bio-active molecule. As a

64 consequence its potential functional effects have been under appreciated.

65   Recently, lactate is being rediscovered as an active signalling metabolite in multiple fields of

66 biology and medicine that has two main ways of signal transduction – transporter and receptor

67 mediated. Its direct regulation of global gene transcription [9, 10], endothelial and cancer cell

68 migration [11, 12], cancer progression [13] and functional polarization of immune cells are being

69 described [14-16].

70   Lactate production occurs mainly in the cytoplasm during hypoxia or aerobic glycolysis in

71 proliferative cells and is then secreted through the plasma membrane. This transport is dependent

72 on six so far described solute carrier transporters that perform proton – lactate symport (Mct1-4) or

73 a sodium-dependent symport (Slc5a8, Slc5a12) [17, 18]. The MCT family harbours 14 members that

74 all share conserved sequence motifs, yet differ in their substrate specificity, transport rate and

75 expression pattern [17]. Indeed, only Mct1 (Slc16a1, Km 4.5) and Mct4 (Slc16a3, Km 28) have been

76 shown to have a high specificity for lactate in concord with broad tissue expression [17, 19].

77 Similarly, sodium-coupled lactate transport is carried out by the ubiquitously expressed high affinity

78 transporter Slc5a8 or the low affinity transporter Slc5a12 [18]. The transport direction of both

79 systems depends on the intra- and extracellular concentration of lactate, favouring lactate import

80 (even through low affinity transporters) only in the presence of high extracellular lactate

81 concentrations.

82 The physiological lactate concentration is about 1.5mM – 3mM [18] in blood and healthy

83 tissues, but can rise up to 10mM in inflammatory pathologies such as atherosclerotic plaques or

84 rheumatic synovial fluid and even up to 20-30mM in cancerous tissue [14, 15, 20]. Far from being

85 inert, accumulating lactate – a feature of most inflammatory sites – has tremendous effects on

86 tissue resident or infiltrating immune cells as well as stromal cells. In the tumour microenvironment,

87 lactate produced by tumour cells is taken up by macrophages where it promotes polarization

88 towards Arginase 2 (Arg2) expressing M2-like phenotype via Hypoxia inducible factor 1α (Hif-1α)

89 stabilization and the resulting increased production of vascular endothelial growth factor (VEGF).

90 These effects further enhance tumour growth in a vicious loop [14]. The authors applied unbiased

91 high-throughput platforms to look for hypothetical protein factors perpetuating such vicious loop,

92 but surprisingly found lactate as the orchestrating factor. Similarly, an independent study recently

93 found lactate to be the driving force behind tumour associated macrophage (TAM) development

94 during epithelial to mesenchymal transition [16].

95 The involvement of Hif-1α in the response to lactate is currently under scrutiny. On the one

96 hand, it has been demonstrated that targeting the lactate transporter Mct1 in endothelial cells or

97 cervix squamous carcinoma cells rescues lactate-mediated Hif-1α activation and inhibits the

98 consequential angiogenesis [21, 22]. On the other hand, these authors could show a lactate

99 mediated Hif-1α independent induction of angiogenesis. Here, reactive oxygen species (ROS)

100 induced the NF-κB pathway that led to IL-8 expression, a known chemotactic molecule that resulted

101 in increased cell migration and tumour metastasis [23]. Additionally, a recent study demonstrates

102 the Hif-1α independent, direct binding of lactate to NDRG3 during hypoxia. Upon lactate binding,

103 NDRG3 is stabilized and executes a Raf-ERK1/2 mediated signalling cascade promoting angiogenesis

104 and cell growth [24]. Surprisingly, not only migration-promoting but also inhibiting functions of

105 lactate have been described.

106   Activated T cells that infiltrate inflammatory sites are exposed to the increased lactate

107 concentration that is commonly found in these sites (e.g., 10-12mM in arthritic synovium) [15]. Due

108 to the high extracellular concentration, lactate internalization through the CD8$^+$ T cell specific

109 transporter Mct1 and CD4$^+$ T cell specific transporter Slc5a12 is favoured. This inhibits glycolysis via

110 inhibition of Pfk or downregulation of Hk1 [15, 25], causing T cells to lose their responsiveness to

111 chemokines and effectively trapping them in the inflamed site. Of note, these effects are not only

112 observed in *in vitro* assays; in an animal model of peritonitis, lactate levels and T cell numbers in the

113 peritoneum are indeed increased 5 days after intra peritoneal (i.p.) injection with zymosan, a glucan

114 commonly used to induce sterile inflammation. Of note, inhibition of lactate transporters re-

115 establishes T cell migration not only *in vitro* but also in the peritonitis model [15]. These findings

116 describe a mechanism explaining at least in part the well-known clinical observation that T cells are

117 entrapped in inflamed tissue [15] (Figure 2).

118   In addition, lactate also triggers the production of the pro-inflammatory cytokine IL-17 in the

119 CD4$^+$ subset and inhibits the cytolytic function of cytotoxic CD8$^+$ T cells (CTL) (Figure 2). The observed

120 inhibition of CTL function was also reported in an earlier publication, showing that both proliferation

121 and cytokine production in human CTLs is severely impaired in the presence of lactic acid [26].

122   The observed effects of T cell entrapment, CTL inhibition and increased production of pro-

123 inflammatory cytokines are common features of many chronic inflammatory diseases that might be

124 in part explained by lactate signalling. The detailed molecular mechanisms for lactate mediated

125 inhibition of T cell migration and change in function, however, are yet to be elucidated.

126

127   Interestingly, the above effects of lactate/lactic acid on macrophages, endothelial cells and T

128 cells are independent of the pH change that is caused by the acidic form of lactate [15, 16], yet

129 protons are still required for the translocation of lactate into the cytoplasm [15].

130   Additional evidence establishing lactate as a signalling molecule comes from the

131 identification of the lactate receptor Gpr81, first cloned in 2001 [27]. Seven years later, lactate was

132 identified as the primary ligand for Gpr81, being involved in lactate-mediated reduction of lipolysis

133 in adipocytes, the primary Gpr81-expressing cell type [4]. It is now known that Gpr81 is a G-protein

134 coupled receptor that inhibits adenylyl cyclase via the Gi signalling pathway [28] and mediates the

135 insulin-induced reduction of lipolysis [29, 30]. Interestingly, several reports identify lactate receptor

136 activation as a critical survival signal for cancer cells [31, 32], and define it as a therapeutic target in

137 ischemic brain injury [33] (Figure 2).

138   In contrast to the roles of lactate on cell signalling, its effect on regulating other metabolic

139 pathways is better understood. Once in the cytoplasm, lactate is readily oxidized to pyruvate by

140 lactate dehydrogenase (LDH). This reaction proceeds with a concomitant proton transfer from

141 lactate to nicotinamide dinucleotide (NAD$^+$) thereby generating NADH and affecting the redox state

142 of the cell (see Glossary). Although LDH is mainly considered a cytoplasmic enzyme, after years of

143 controversy the existence of a mitochondrial LDH has finally been proven [34]. Thus, given the

144 presence of Mct1 in the mitochondrial membrane [35], lactate metabolism is now being considered

145 as an active part of mitochondrial metabolism. Recently, the LDH subunit B (LDHB) has also been

146 shown to localize to peroxisomes in fibroblasts and HeLa cells [36]. This might be a hint towards the

147 possible involvement of lactate in fatty acid oxidation or lipid metabolism in general as discussed in

148 the following excellent review [37].

149   Contrarily, the effects on glycolysis remain questionable. It was shown that 10mM

150 extracellular lactate inhibits glycolytic activity in T cells, which could be facilitated by downregulation

151 of hexokinase 1 or direct inhibition of phosphofructokinase. By contrast, in heart tissue lactate

152 causes increased Glut1 and Glut4 expression on the plasma membrane, which is in general an

153 indicator of increased glucose uptake and flux [38].

154   Taken together, lactate-induced signalling is an important pathway in health and disease. As

155 discussed here, lactate has two primary means of relaying signals into the cell (receptor- and

156 transporter-mediated), and lactate signalling has several possible outcomes that depend on the cell

157 type. Moreover, the concentration of extracellular lactate that lie between 1.5mM in physiological

158 and 10-30mM in pathological settings has immense impact on cell function as it will lead to the

159 activation of different signalling pathways.

160

161 Effects beyond metabolism of TCA cycle intermediates: focus on succinate

162

163   It is now becoming clear that the reaction intermediates of the TCA cycle (Figure 1; Box 1)

164 can act as proper signalling molecules once they are withdrawn from the cycle and redirected

165 towards different functions. This occurs both in physiological and pathological conditions; for

166 instance, citrate accumulates in the cytosol of Lipopolysaccharide (LPS) -treated macrophages where

167 it is necessary for the synthesis of NO, ROS and prostaglandins [39]. Fumarate has been recently

168 defined as a proto-oncometabolite [40, 41] because its accumulation, due to loss of function

169 mutations in the fumarate hydratase (FH) (see Glossary) [42, 43], results in stabilization of Hif-1α via

170 two different mechanisms: the inhibition of PHD2 (prolyl hydroxylase domain-containing protein 2),

171 which targets HIF factors for degradation [44], and the amplification of ROS signalling through the

172 consumption of reduced glutathione and NADPH [41]. Another TCA cycle metabolite that can

173 regulate non-metabolic activities is NAD^+, an important cofactor for sirtuins, a family of deacetylases

174 that targets important transcription factors of the inflammatory response, such as NF-kB [45] and

175 AP1 [46], and controls mitochondrial quality and biogenesis [47].

176   For the rest of this section we focus on succinate, as in the past few years it has been

177 reported to be a central metabolite in the biology of immune cells (such as macrophages) as well as

178 in cancer and other pathological contexts, providing a clear example of a new potential therapeutic

179 target.

180 Succinate is synthesized from α-ketoglutarate (first converted to succinyl-CoA and then to

181 succinate) and subsequently utilized as a substrate by succinate dehydrogenase (SDH) to produce

182 fumarate (Figure 3). Inhibition of SDH results in accumulation of succinate, stabilization of Hif-1α,

183 induction of Hif-1α transcriptional activity and oncogenic events. Stabilization of Hif-1α is due to the

184 ability of succinate to inhibit PHD enzymes (prolyl hydroxylases). Hydroxylation of HIF by PHD

185 enzymes is necessary for its binding to pVHL, part of an E3 ubiquitin ligase targeting HIF for

186 degradation [48]. Thus, reduction of this hydroxylation results in HIF stabilization leading to the

187 transcription of genes involved in proliferation, angiogenesis and metastasis [49] (Figure 3).

188 Stabilization of Hif-1α by succinate was also observed in LPS-treated macrophages and was

189 associated with enhanced production of IL-1β [2]. The authors observed an accumulation of

190 succinate in macrophages after Toll-like receptor 4 (TLR4) engagement by LPS, which was

191 responsible for the stabilization of Hif-1α. The stabilization of Hif-1α in activated macrophages was

192 directly linked to the increased transcription and production of IL-1β; indeed, the authors

193 demonstrated that Hif-1α can bind the promoter of IL-1β, activating its transcription. LPS-treated

194 macrophages undergo a switch from oxidative phosphorylation to glycolysis, lowering the activity of

195 the TCA cycle, which raises questions as to the source of succinate accumulation. Indeed, the main

196 source of succinate after LPS treatment appears to be glutamine, mainly via anaplerosis of α-

197 ketoglutarate feeding into the TCA cycle and replenishing succinate, and, to a lesser extent, via the

198 GABA (γ-aminobutyric acid) shunt (Figure 3). These findings suggest succinate is an inflammatory

199 signal that is necessary to activate macrophages and stabilize a fundamental player of immune

200 response, Hif-1α, leading to the production of IL-1β [2] (Figure 3).

201 A new depth of understanding of the metabolic rewiring of intracellular metabolism was

202 obtained by a study that used metabolomics and transcriptomics to characterize, in detail, the

203 changes that occur in macrophages during polarization towards M1 or M2 phenotypes [50]. With

204 regard to M1 polarization, they identified two TCA cycle break-points: the first at the conversion

205 point of citrate to α-ketoglutarate, and the second after succinate synthesis at the conversion point

206 of succinate to fumarate. The first break-point, due to the downregulation of isocitrate

207 dehydrogenase, results in citrate accumulation, which is then redirected towards production of

208 itaconic acid, an anti-microbial metabolite [51]. The authors also described a second break-point, at

209 the TCA step of converting succinate into fumarate; succinate accumulation increased, as previously

210 reported [2]. Despite the low efficiency of succinate to fumarate conversion, an accumulation of

211 malate (produced from fumarate) was also observed. This was due to the upregulation of the

212 arginosuccinate shunt, a series of reactions feeding first into fumarate and then malate. This shunt is

213 important not only to replenish malate and subsequently citrate (to complete the cycle) but also to

214 produce NO and IL-6, both necessary for appropriate activation of macrophages [50].

215 Ischaemia reperfusion (IR) also causes the specific accumulation of succinate and

216 subsequent production of mitochondrial ROS. Production of ROS during ischaemia reperfusion has

217 always been thought to be a nonspecific response due to reperfusion; however, a recent study

218 demonstrates that succinate accumulation during reperfusion of ischaemic tissues is a selective

219 response that drives the generation of ROS responsible for tissue damage [52]. Using an *in vivo*

220 model of ischaemia in combination with unsupervised metabolomics analysis, succinate was found

221 to specifically accumulate during ischaemia in different tissues (liver, kidney, heart and brain) and it

222 was rapidly re-oxidized during reperfusion. To assess the source of succinate, they performed stable

223 isotope tracing experiments and found that succinate derived mainly from the malate/aspartate

224 shuttle (MAS) and the purine nucleotide cycle (PNC) (Figure 3). These two pathways led to the

225 accumulation of fumarate which was then converted to succinate by the reversal of SDH. During

226 reperfusion the accumulated succinate was rapidly re-oxidized to fumarate by SDH, leading to a

227 massive production of mROS, mainly superoxide (Figure 3), due to the reverse electron transport

228 (RET) through mitochondrial complex I (see Box 1) [52]. This observation describes succinate as a

229 damage signal during reperfusion, making it an intriguing target for therapy development.

230   In addition to these intracellular non-metabolic effects of succinate, it also binds to a specific

231 receptor localized onto the cytoplasmic membrane, which suggests it can signal as an extracellular

232 molecule (Figure 3). The succinate receptor, GPR91 (also known as SUCNR), is a G protein-coupled

233 receptor whose activation triggers intracellular calcium release and inhibits cAMP production. In

234 mouse, it is expressed mainly in the kidney, liver, spleen and small intestine [5]. GPR91 is expressed

235 on human and mouse dendritic cells (DC), where it enhances their immune-stimulatory capacity

236 [53]. Specifically, succinate stimulation of GPR91 promotes migration of DC in a dose-dependent

237 manner, and it cooperates with TLR ligands to induce cytokines via Erk1/2 phosphorylation.

238 Furthermore, succinate sustains and empowers DC-mediated T cell activation. These effects were

239 shown to be dependent on succinate stimulation of GPR91, as they were abrogated in Sucnr-/- mice

240 [53].

241

Immune regulation by fatty acid oxidation: in search of a signalling role for lipid intermediates

243

244   Unlike glycolysis and the TCA cycle, the intermediates of fatty acid metabolism have not yet

245 been shown to regulate T cell fate and functional specification (Figure 1 and Box 1). However, both

246 the induction of fatty acid synthesis (FAS) and its inverse metabolic pathway, fatty acid oxidation

247 (FAO), have been linked to T cell function. Specifically, while the induction FAS is known to be an

248 integral part of the T cell activation program that is associated with increased glucose metabolism,

249 which is essential for the differentiation of naïve T cells into their T effector subsets, FAO has been

250 shown to be critical for the development of CD8$^+$ memory T cells and the induction of CD4$^+$

251 regulatory T cells [54, 55] (Figure 4).

252   Mice with a T cell-specific deletion of tumour necrosis factor (TNF) receptor-associated

253 factor 6 (TRAF6) displayed a profound defect in CD8$^+$ memory T cell generation, and were unable to

254 upregulate FAO after growth factors withdrawal during contraction phase of immune response.

255 TRAF6-deficient CD8$^+$ T cells exhibited defective AMP-activated kinase (AMPK) activation, whereas

256 activation of AMPK with metformin was able to rescue both FAO and the generation of CD8^{+}

257 memory T cells, suggesting TRAF6 regulates a metabolic switch towards FAO important for

258 generation of long-lived CD8^{+} memory T cells [54]. Regulatory CD4^{+} T cells (Tregs) have also been

259 shown to rely primarily on FAO during their development [55, 56]. Naturally occurring Tregs display

260 low levels of Glut1, and thus low rates of glycolysis, while having increased activation of AMPK.

261 Treatment with etomoxir, an inhibitor of carnitine palmitoyl transferase (CPT1), the rate limiting

262 enzyme in FAO, was sufficient to abrogate Treg development, suggesting that FAO is essential for

263 Treg development [55].

264 Interestingly, it has recently been demonstrated that the increase in FAO in memory CD8^{+} T is

265 surprisingly the direct result of *de novo* FAS, rather than uptake of fatty acids from the extracellular

266 environment [57]. After observing no increase in extracellular fatty acid uptake, the authors showed

267 that extracellular glucose fuels mitochondrial fatty acid oxidation and oxidative phosphorylation

268 (OXPHOS) indicating that fatty acids are synthesised for subsequent oxidation. Upon treatment with

269 a fatty acid synthase inhibitor, memory T cell death increased suggesting fatty acid synthesis is

270 necessary for their survival. Memory T cells lack typical fatty acid storage droplets; instead,

271 lysosomal acid lipase (LAL) activity plays a role in non-classical fatty acid storage. LAL is required for

272 lipolysis of stored fatty acids to generate available fatty acids for oxidation and necessary for

273 memory T cell survival. These data support the phenomenon known as fatty acid "futile cycling"

274 whereby intracellular fatty acids are catabolized rather than acquired from extracellular sources, for

275 use in the mitochondria for fatty acid oxidation. With no net gain of ATP, this cycling of fatty acids is

276 bio-energetically redundant. However, it has been suggested that fatty acid cycling in memory T cells

277 may provide a mechanism to maintain their survival and, sustaining their glycolytic and

278 mitochondrial metabolism, may enable rapid recall responses after antigen recognition.

279   Extracellular signalling by IL-7 and IL-15 has been shown to impact on T cell development by

280 affecting FAO [58, 59] (Figure 4). The cytokine IL-15, which is critical for the development and

281 maintenance of CD8^{+} memory cells, enhanced the expression of the rate-limiting FAO enzyme CPT1,

282 and thus FAO. Importantly, blocking CPT1 with etomoxir impaired the mitochondrial spare

283 respiratory capacity (see Glossary) and survival of CD8^{+} memory T cells. Conversely, over-expression

284 of CPT1 increased the formation of CD8^{+} memory T cells following infection [58]. IL-7 is known to

285 control CD8^{+} memory T cell longevity and homeostasis [60, 61]. Recent findings demonstrate a new

286 pathway in which IL-7 promotes survival via glycerol import, triglyceride synthesis and storage.

287 Triglycerides are synthetized combining glycerol-3-phosphate and Acyl-CoAs (free fatty acids

288 activated with a Coenzyme-A moiety), thus the amount of glycerol affects triglyceride synthesis. In

289 this study IL-7 was shown to induce expression of the glycerol channel aquaporin 9 (AQP9), which

290 was required for long term survival of CD8^{+} memory cells. AQP9 deficiency resulted in impaired

291 glycerol import, and thus esterification of fatty acids and reduced triglyceride synthesis and storage.

292 These defects were rescued by ectopic expression of triglyceride synthases, which restored lipid

293 stores and CD8^{+} memory T cells [59].

294   Acyl-CoA is the main intermediate metabolite of lipid metabolism within the cell. FAO

295 involves the sequential removal of 2-carbon units from a fatty acyl-CoA molecule to yield acetyl-CoA,

296 which can be directly shuttled into the TCA cycle. While intermediate products of glycolysis and the

297 TCA cycle have been shown to have active signalling roles, little research has been done into

298 whether FAO metabolites may have direct effects on the cell fate decision of T cells. However, a

299 recent paper has shown that long chain acyl-CoAs, which are the activated form of free fatty acids

300 and represent the pre-step reaction for β-oxidation, can act as positive modulators of ion channels

301 and exchangers [3]. Specifically, long chain acyl-CoAs were shown to be potent activators of TRPV1

302 cation channels independently of $Ca2^+$, and increasing the level of long chain acyl-CoAs in intact

303 Jurkat T cells leads to a significant increase in agonist-induced $Ca2^+$ levels. This novel mechanism

304 indicates that long chain acyl-CoAs could play an active role in T cell functions under both

305 physiological and pathophysiological conditions that alter fatty acid transport and metabolism.

306   Acetyl-CoA, the end product of FAO, has also been implicated to have roles beyond the TCA

307 cycle, as it can also act as a substrate for post-translational modifications such as acetylation [62].

308 One potential mechanism is through histone acetylation, which is known to be important for

309 promoting gene transcription. In the context of immune cells, CD8^{+} T cells histone acetylation occurs

310 at specific loci and may be involved in determining the decision between memory and short-lived

311 effector cell fate [63]. Similarly, different histone acetylation patterns have been shown in CD4^{+}

312 effector T cells compared to CD4^{+} Tregs. Specifically, in Tregs there is increased acetylation of the

313 Foxp3 locus [64], whereas in T effector cells the IL-13, IL-15 and IL-4 loci have all been shown to have

314 increased acetylation induced by IL-4 [65].

315

Concluding remarks

317

318 The discovery of extra-metabolic functions of metabolic enzymes (referred to as moon-

319 lighting) has been the first evidence that metabolism and its players have a role in the regulation of

320 signalling pathways inside cells. Here, we have described how a similar concept applies to

321 metabolites, which for decades were considered to be only the building blocks of biomasses. The

322 observation that intermediates and end products of the main metabolic pathways can desert their

323 metabolic roles to function, in certain circumstances, as transduction signals shows how these

324 molecules can play an active and crucial role in regulating some of the most important biological

325 processes. We currently have evidence that some metabolites play roles in the immune and

326 inflammatory responses, and influence cytokine production, proliferation and angiogenesis, both in

327 physiology and pathology. It remains to be established if other metabolites might display similar

328 functions.

329 In this new scenario, the accumulation or the depletion of specific metabolites does not

330 represent just a metabolic adaptation or a choice merely dictated by energy demand; it is rather an

331 elected form of signalling-mediated regulation of biological processes. The loss of this intrinsic

332 regulation is associated with several pathological conditions, and this might explain the current

333 efforts to target metabolic pathways for therapeutic purpose. In doing so, we need to bear in mind

334 that some processes, such as ROS production, are necessary but must be tightly regulated to

335 maintain homeostasis; hence in some cases mild intervention could be safer and even more

336 beneficial than blunt blockade.

337 **ACKNOWLEDGEMENTS**

338

345

346 **BOX1 - Basics of TCA cycle, FAO and ETC/RET**

347

348 **TCA cycle**

349   The tricarboxylic acid (TCA) cycle, also known as citric acid cycle or Krebs Cycle, is a series of

350 biochemical reactions occurring into the mitochondrion that provide energy and reducing

351 equivalents through the complete oxidation of acetate, in form of acetyl-CoA, produced from the

352 breakdown of sugars, lipids and amino-acids (Figure 1).

353

354 **FAO**

355   Fatty acids are an alternative source of fuel for cells yielding large amounts of ATP during

356 their oxidation. This catabolic process occurs in the mitochondria to generate acetyl-CoA, which

357 directly enters into the TCA cycle (Figure 1).

358

359 **ETC**

360 The mitochondrial electron transport chain (ETC) is a series of protein complexes embedded into the

361 inner mitochondrial membrane responsible for the transfer of electrons from donors to acceptors

362 via redox reactions. The transfer of electrons through the chain is coupled to the pumping of protons

363 into the intermembrane space. This proton gradient provides the proton-motive force necessary for

364 the generation of energy in the form of ATP. In eukaryotes, ETC is composed of 4 complexes:

365 complex I or NADH dehydrogenase, which oxidizes NADH and transfers electrons to ubiquinone Q

366 (thus turned to ubiquinol QH2) while pumping four protons into the intermembrane space; complex

367 II or succinate dehydrogenase, which oxidizes succinate to fumarate and transfers electrons to

368 ubiquinone; complex III or cytochrome c oxidoreductase, which re-oxidizes ubiquinol to ubiquinone

369 while reducing cytochrome c and pumping two protons across the membrane; and complex IV or

370 cytochrome c oxidase, which oxidizes cytochrome c passing electrons to the final acceptor of the

371 chain, molecular oxygen O_2, generating water and pumping four protons across the membrane.

372   Finally, ATP synthase utilizes the energy stored into the protons gradient to phosphorylate ADP to

373   ATP. Complex I and II represent two independent entry points to the ETC.

374

375   **RET**

376   Reverse electron transfer (RET) is the flow of electrons from ubiquinone to NAD^+ catalysed by

377   complex I in the presence of a reduced pool of QH2 and a high proton gradient forcing electrons

378   backward from QH2 into complex I, which leads to the production of superoxide [66, 67].

379

380

381 **GLOSSARY**

382 **REDOX STATE**

383 The cellular redox state is often described as the balance of **red**uced and **ox**idized glutathione

384 (GSH/GSSG), Nicotinamidedinuclotide (NAD$^+$/NADH) and Nicotinamidedinuclotide-phosphate

385 (NADP$^+$/NADPH). These redox couples are central mediators for catabolic and anabolic reactions

386 acting as cofactors and regulators for enzymes, scavengers for reactive oxygen species or substrates

387 for the mitochondrial electron transport chain.

388 **FUMARATE HYDRATASE (FH):**

389 The TCA cycle enzyme responsible for the conversion of fumarate to malate.

390 **SPARE RESPIRATORY CAPACITY:**

391 The extra capacity available in cell to produce energy via mitochondrial respiration.

392

393

394   **FIGURE LEGENDS**

395

396   **Figure 1 – Metabolic pathways and regulatory intermediates of metabolism**. The main cellular

397   catabolic pathways (glycolysis: blue; tricarboxylic acid (TCA) cycle: red; fatty acid oxidation (FAO):

398   green) not only produce ATP, but also metabolic intermediates, such as lactate, acetyl-CoA and

399   succinate, highlighted in colour. These are substrates for anabolic processes including lipid and

400   nucleotide synthesis, but can also act as regulatory signalling molecules.

401

402   **Figure 2, Key Figure – Lactate-mediated signalling pathways and their biological outcomes.**

403   Extracellular lactate (left side of the figure) has staggering functional effects on several cell types,

404   including production of pro- and anti-inflammatory mediators by T cells and macrophages (Mφ), or

405   migratory changes and metabolic adaptation in T cells, endothelial cells (EC) and neurons.

406   Intracellularly (right side of the figure), lactate can directly bind to proteins (i.e. NDRG3), influence

407   the redox state via the lactate dehydrogenase (LDH) reaction, stabilize Hif-1α, induce reactive

408   oxygen species (ROS) and act as an inhibitor of glucose breakdown. The occurrence of these effects

409   might depend on the investigated cell type.

410

411   **Figure 3 – Succinate signalling and its biological effects.** Succinate exerts several biological

412   responses. By stabilizing Hif-1α, it promotes proliferation, angiogenesis and cytokine production.

413   Succinate can also regulate proteins activity via succinylation. During ischaemia, fumarate

414   accumulates through the malate-aspartate shuttle (MAS) and the purine nucleotide cycle (PNC) and

415   is converted into succinate by succinate dehydrogenase reversal (SDH); during reperfusion, the rapid

416   re-oxidation of succinate to fumarate by SDH leads to the production of mitochondrial reactive

417   oxygen species (mROS) responsible for tissue damage. Furthermore, succinate in the extracellular

418   microenvironment can signal through its receptor (GPR91), sustaining cytokine production and

419   migration of dendritic cells (DC). TCA, tricarboxylic acid cycle; AMP, adenosine monophosphate; IMP,

420 inosine monophosphate; PHD, prolyl hydroxylases; GABA, γ-aminobutyric acid; IR, ischeamia-

421 reperfusion.

422

423 **Figure 4 – Fatty acids oxidation and its role in signalling.** FAO has been shown to be necessary for

424 CD8+ memory T cells survival and CD4+ regulatory T cells induction. Moreover, upregulation of

425 glycerol transporter AQP9 by cytokines is important to increase triglycerides synthesis and thus FAO.

426 The futile cycling of lipolysis and subsequent FAO seems to be important for memory T cell survival.

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573

OUTSTANDING QUESTIONS BOX

- Lactate has two primary means of relaying signals into the cell, receptor- and transporter mediated, and depending on the cell type has several possible outcomes. The downstream pathways still need to be worked out and might illuminate new knowledge in the near future.

- Besides succinate, other metabolites of the TCA cycle might be directly regulating immune cell functions but this is yet to be fully investigated.

- Unlike glycolysis and the TCA cycle, whose intermediates have been shown to play important roles in determining specific T cell fates and functional specifications, the same has yet to be defined for the cellular metabolism of fatty acids.

Glycerol import via AQP9

TAG synthesis

FA synthesis

Lipolysis

Free FA

TCA cycle

Glucose

FA oxidation

ATP generation

CD8+ memory T cell Survival