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Title: Interleukin-5 drives glycolysis and reactive oxygen species-dependent citric acid cycling by eosinophils

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35 **Abstract**

36 **Introduction:** Eosinophils have been long implicated in anti-parasite immunity and
37 allergic diseases and, more recently, in regulating adipose tissue homeostasis. The
38 metabolic processes that govern eosinophils, particularly upon activation, are unknown.

39

40 **Methods:** Peripheral blood eosinophils were isolated for analysis of metabolic
41 processes using extracellular flux analysis and individual metabolites by stable isotope
42 tracer analysis coupled to gas chromatography-mass spectrometry following treatment
43 with IL-3, IL-5 or granulocyte-macrophage colony-stimulating factor (GM-CSF).
44 Eosinophil metabolism was elucidated using pharmacological inhibitors.

45

46 **Results:** Human eosinophils engage a largely glycolytic metabolism but also employ
47 mitochondrial metabolism. Cytokine stimulation generates citric acid cycle (TCA)
48 intermediates from both glucose and glutamine revealing this previously unknown role
49 for mitochondria upon eosinophil activation. We further show that the metabolic
50 program driven by IL-5 is dependent on the STAT5/PI3K/Akt signalling axis and that
51 nicotinamide adenine dinucleotide phosphate oxidase (NOX)-dependent ROS
52 production might be a driver of mitochondrial metabolism upon eosinophil activation.

53

54 **Conclusion:** We demonstrate for the first time that eosinophils are capable of metabolic
55 plasticity, evidenced by increased glucose-derived lactate production upon ROS
56 inhibition. Collectively this study reveals a role for both glycolysis and mitochondrial
57 metabolism in cytokine-stimulated eosinophils. Selective targeting of eosinophil
58 metabolism may be of therapeutic benefit in eosinophil-mediated diseases and
59 regulation of tissue homeostasis.

60

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62 **Keywords:** eosinophils, glycolysis, IL-5, metabolism, TCA cycle

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65 **Introduction**

66 Human eosinophils reside primarily in haematopoietic and mucosal tissues. Interest in
67 eosinophil activity stems predominantly from their role in anti-parasite immunity and
68 allergic disease¹⁻⁵ but there is growing interest in their role in tissue homeostasis,
69 especially adipose tissue⁶. Eosinophil-mediated effector function involves
70 degranulation, the release of antimicrobial cytotoxic molecules, and the respiratory
71 burst² yet we know little about the immunometabolic processes that underpin these
72 activities. Activation of other granulocyte populations such as neutrophils and mast
73 cells enhances glycolysis to support biosynthetic intermediate production and rapid
74 ATP generation^{7,8}. Eosinophil metabolism is assumed to be largely homologous to that
75 of neutrophils, where, despite the presence of mitochondria, energy production stems
76 primarily from glycolysis^{5,9,10}.

77

78 Non-metabolic roles for mitochondria within eosinophils have been the focus of several
79 investigations. Mitochondrial DNA can be released in a ‘catapult-like’ fashion from
80 eosinophils and contributes to antibacterial defence, although this remains controversial
81 and has yet to be confirmed^{11,12}. Furthermore, the initiation of apoptosis has been
82 reported as an alternative role to respiration for eosinophil mitochondria¹⁰. As
83 eosinophils produce large amounts of nicotinamide adenine dinucleotide phosphate
84 oxidase (NOX)2-dependent extracellular reactive oxygen species (ROS) upon
85 activation¹³⁻¹⁵, it is commonly thought that oxygen consumption by eosinophils
86 supports ROS production rather than oxidative phosphorylation (OXPHOS). Contrary
87 to this, human eosinophils are sensitive to oligomycin (mitochondrial ATP synthase
88 inhibitor), suggesting that in addition to glycolysis, mitochondria can indeed contribute,
89 at least in part, to ATP production⁵. As such, the role of the mitochondria in eosinophils
90 remains unclear and requires investigation.

91

92 Glycolysis has been reported to be the main source of ATP in numerous cell types¹⁶⁻¹⁸.
93 This is especially true for immune cells such as T cells and mast cells, which undergo
94 a glycolytic switch upon activation to support rapid ATP production^{19,20}. Little is
95 known about the role of glycolysis in eosinophil-mediated immunity, but glycolysis-
96 derived ATP is essential for the removal of schistosomula by human eosinophils²¹ and
97 cytokines such as IL-3, IL-5, granulocyte-macrophage colony-stimulating factor (GM-
98 CSF) and TNF α stimulate glucose uptake in these cells²². The anti-apoptotic cytokines

99 IL-3, IL-5, and GM-CSF, produced primarily by T cell subsets, fibroblasts, and
100 epithelial cells, are critical for eosinophil activation and maturation². Differential
101 effects of IL-3, IL-5, and GM-CSF have been identified, with IL-3 generally being a
102 weaker inducer of eosinophil activation than either IL-5 or GM-CSF; IL-3 induces less
103 glucose uptake, superoxide production and eosinophil-derived neurotoxin (EDN)
104 release than IL-5 or GM-CSF^{22,23}. However, IL-3 can prolong ribosomal protein S6
105 signalling compared to IL-5 and GM-CSF, producing augmented levels of semaphorin-
106 7A and heightened protein translation²⁴. Despite these differences in responses of
107 eosinophils to IL-3, IL-5, and GM-CSF, the impact of these cytokines on human
108 eosinophil metabolic adaptation that underpins these different functional outcomes has
109 not been studied.

110

111 Here, for the first time, we demonstrate that human eosinophils are metabolically plastic
112 cells, up-regulating both glycolytic and TCA cycle intermediates upon activation. We
113 show that IL-3, IL-5 and GM-CSF all increase glycolysis and importantly, that upon
114 activation by these cytokines, eosinophils increase glutaminolysis and subsequent TCA
115 cycling. This is significant as these cells were previously thought to not engage their
116 mitochondria for metabolic purposes. In contrast to earlier studies¹⁰, we report that the
117 IL-5-induced metabolic switch initiates glycolysis and enhances mitochondrial
118 respiration in a mechanism that is dependent on the STAT5/PI3K/Akt axis. Finally, the
119 ability of esoinophils to compensate for inhibition of ROS production and the
120 asscioated reduced levels of TCA cycle intermediates by increased aerobic glycolysis
121 highlights their metabolic plasticity.

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133 **Materials and Methods**

134 **Human eosinophil isolation**

135 Human peripheral blood was collected from both male (n = 9) and female donors (n =
136 25) aged between 18-70 years into heparinised Vacuettes™ (Greiner Bio-one,
137 Frickenhausen, Germany). We recruited both atopic and non-atopic donors with
138 eosinophils comprising between 1-8% of total circulating leukocytes. Specific donor
139 demographics can be found in supplementary table 1. All samples were collected with
140 informed written consent and ethical approval was obtained from Wales Research
141 Ethics Committee 6 (13/WA/0190). Eosinophils were isolated by negative selection
142 using immunomagnetic microbeads (autoMACS; Miltenyi Biotec, Cologne, Germany).
143 Detailed Materials and Methods can be found in the online supplement of this article.

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167 **Results**

168 **Eosinophils increase glycolysis in response to cytokines**

169 In human eosinophils, IL-3, IL-5, and GM-CSF are the predominant cytokines
170 associated with their activation²⁵. Therefore, the effect of IL-3, IL-5 and GM-CSF on
171 eosinophil metabolism was investigated.

172

173 First, we investigated the mode of glucose transport in eosinophils. Gene expression
174 levels of the main glucose transporters (*GLUT1-4; SLC2A1-4*) were determined using
175 qPCR. While there was donor variability, *GLUT1* (*SLC2A1*) and *GLUT3* (*SLC2A3*)
176 were expressed by all donors (Figure 1A). *GLUT4* (*SLC2A4*) expression was not
177 detected, and only some donors expressed detectable *GLUT2* (*SLC2A2*; 3/7) (Figure
178 1A). Uptake of a fluorescent glucose analogue 2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-
179 yl)amino)-2-deoxyglucose (2-NBDG) did not differ between unstimulated and
180 cytokine-stimulated eosinophils (Figure 1B).

181

182 To further investigate the induction of glycolysis upon activation in eosinophils, we
183 performed extracellular flux analysis measuring the extracellular acidification rate
184 (ECAR) upon treatment with IL-3, IL-5, or GM-CSF. Eosinophils were starved of
185 glucose, treated with cytokine followed by the reintroduction of glucose, then addition
186 of the ATP synthase inhibitor oligomycin, with a final addition of 2-deoxy-D-glucose
187 (2-DG) to arrest glycolysis over the timeline shown in Figure 1C. Here we discovered
188 that after a period of glucose starvation, IL-5 or GM-CSF treatment increased ECAR
189 significantly in comparison to either the control or IL-3-treated cells (Figure 1C-D). As
190 there are multiple sources of acidification that may contribute to ECAR in eosinophils²⁶⁻
191 ²⁸, we performed stable isotope tracer analysis (SITA) to determine the fate of glucose-
192 derived carbon atoms upon eosinophil activation. Eosinophils were activated with IL-
193 3, IL-5 or GM-CSF in the presence of ¹³C₆-glucose for 4 h. If eosinophil metabolism is
194 largely homologous to that of neutrophils²⁹, i.e. glycolytic, then it would be expected
195 that the majority of labelled carbon would be present as the m+3 mass isotopologue of
196 lactate produced from the m+3 mass isotopologue of pyruvate (Figure 1E). These data
197 demonstrate that IL-3, IL-5, or GM-CSF treatment promotes the incorporation of ¹³C
198 atoms into pyruvate (Figure 1F-G) and lactate (Figure 1H-I). We also observed ¹³C
199 labelled extracellular lactate within the supernatant (Figure 1J), excess production of
200 lactate upon activation was also confirmed by a standard lactate assay (Figure 1K).

201 Collectively the data demonstrate that eosinophils treated with IL-3, IL-5 or GM-CSF
202 switch to a glycolytic metabolism.

203

204 **Cytokine-stimulated eosinophils consume oxygen for ROS production**

205 It has been reported that eosinophils do not require their mitochondria for ATP
206 production via OXPHOS^{10,30}. However, mitochondria are diverse organelles with
207 multiple metabolic roles. We confirmed the presence of mitochondria using
208 transmission electron microscopy (Figure 2A). Mitochondrial function was assessed by
209 measuring oxygen consumption rate (OCR) in the presence of IL-3, IL-5 or GM-CSF
210 (Figure 2B). Baseline OCR was increased in IL-5 and GM-CSF treated cells post-
211 glucose starvation compared to the control and IL-3-treated cells (Figure 2C). We noted
212 a decrease in OCR upon oligomycin treatment under all conditions, this is indicative of
213 oxygen consumption for ATP generation, which is in agreement with a previous study⁵.
214 Increasing the concentration of IL-3 delivered to the cells caused an increase in both
215 ECAR and OCR (50 and 100 ng/mL; Figure 2D-E) suggestive of a differential kinetic
216 response of eosinophils to IL-3 versus IL-5/GM-CSF.

217

218 In our experiments, eosinophils clearly consume oxygen, especially in response to
219 treatment with IL-5 or GM-CSF. However, oxygen consumption can occur
220 independently of OXPHOS for processes such as respiratory burst, involving the
221 generation of ROS and subsequently hydrogen peroxide via NOX enzymes^{13,14}. To
222 determine whether any of the cytokine-induced oxygen consumption was due to
223 mitochondrial ROS production, we utilised the mitochondrial superoxide indicator
224 MitoSOX and selected a time point to coincide with the extracellular flux assay (15
225 minutes). Regardless of the cytokine used for stimulation, mitochondria did not
226 contribute to oxygen consumption via ROS production at the time point measured;
227 rotenone was used as a positive control (Figure 2F-G).

228

229 To further investigate increased oxygen consumption upon stimulation we investigated
230 whether IL-3, IL-5 and GM-CSF induce total ROS production. We determined total
231 oxidative stress levels using the fluorescent probe CellROX and flow cytometry,
232 phorbol 12-myristate 13-acetate (PMA) was used as a positive control. All three
233 cytokines induced ROS production in comparison to the CellROX control with GM-
234 CSF being the most potent (Figure 2H-I). Using the inhibitor diphenyleneiodonium

235 (DPI), these responses were shown to be NOX-dependent, although this was only
236 significant for GM-CSF and PMA-induced ROS production. These data raise the
237 question of what happens to OCR when ROS production is inhibited, and we have
238 addressed this in relation to IL-5 later in the manuscript, see Figure 5.

239

240 **Cytokine-stimulated eosinophils synthesise TCA cycle intermediates**

241 In addition to the generation of ATP and ROS, mitochondria can act as a biosynthetic
242 hubs, synthesising TCA cycle intermediates and non-essential amino acids, however
243 this has not been previously demonstrated in eosinophils. To test this, eosinophils were
244 activated with IL-3, IL-5, or GM-CSF in the presence of $^{13}\text{C}_6$ -glucose for 4 h. Upon
245 activation, eosinophils incorporated ^{13}C -glucose into TCA cycle intermediates, such as
246 citrate, succinate, malate and fumarate (Figure 3A-E). The metabolite pools analysed
247 were mostly composed of the unlabelled ($m+0$) or $m+2$ mass isotopologue (Figure 3F;
248 Supplementary Figure 1A-C) indicating lack of sustained TCA cycling.

249

250 Next, we determined whether TCA cycle intermediates are used as precursors for the
251 synthesis of non-essential amino acids. Glutamate abundance was increased upon
252 eosinophil stimulation with IL-3, IL-5 or GM-CSF (Figure 3G) and was largely present
253 as the $m+2$ mass isotopologue (Figure 3H). While the eosinophils demonstrated
254 production of glutamine and aspartate at baseline, cytokine stimulation had no further
255 effect on the production of these. However, in comparison to the untreated control,
256 cytokine-stimulated eosinophils had a reduced pool of ^{12}C unlabelled amino acids,
257 indicating consumption of these amino acids (Supplementary Figure 2A-D).

258

259 Fully functional canonical TCA cycling requires two metabolite inputs: acetyl-CoA
260 derived primarily from glucose and α -ketoglutarate derived from glutamine
261 (Supplementary Figure 3A). Having established that eosinophils incorporate ^{13}C -
262 glucose into TCA cycle intermediates, we next wanted to determine whether cytokine-
263 activated eosinophils engage glutaminolysis. To address this, eosinophils were
264 activated with IL-3, IL-5 or GM-CSF in the presence of ^{13}C -glutamine for 4 h.
265 Incorporation of ^{13}C into TCA intermediates was increased in IL-3, IL-5 or GM-CSF
266 treated eosinophils compared to the untreated controls (Supplementary Figure 3B).

267

268 **The STAT5/PI3K/Akt axis governs the immediate metabolic response to IL-5**

269 The development and clinical implementation of IL-5 targeting therapies in the
270 treatment of asthma³¹ prompted us to consider the early signalling mechanisms that
271 govern increased ECAR and OCR in response to IL-5 treatment. STAT5 is activated
272 upon IL-3, IL-5 or GM-CSF ligation^{32,33}, and in certain circumstances can be activated
273 by ROS production via the common β chain³⁴. We initially confirmed STAT5
274 phosphorylation in eosinophils treated with IL-3, IL-5 and GM-CSF. All cytokines
275 induced STAT5 phosphorylation, but this only reached significance above baseline for
276 IL-5 and GM-CSF (Figure 4A). Next, we wanted to determine whether inhibition of
277 STAT5 affected the immediate ECAR and OCR responses of eosinophils treated with
278 IL-5. Pre-treatment with the STAT5 inhibitor N'-((4-oxo-4H-chromen-3-
279 yl)methylene)nicotinohydrazide (STAT5i) completely abrogated the ECAR and OCR
280 response in IL-5-stimulated eosinophils (Figure 4B-C). Calculations of ‘pre-cytokine’
281 and ‘post-cytokine’ data can be found at Supplementary Figure 4.

282

283 In addition to cytokine-mediated STAT5 activation, both IL-5 and ROS can activate
284 the PI3K/Akt axis³⁴, therefore we next investigated the role of PI3K/Akt in human
285 eosinophil metabolism. Treatment with either the PI3K inhibitor LY294002 or the
286 Akt1/2 inhibitor, abrogated IL-5 stimulated induction of ECAR (Figure 4D). The same
287 trend was observed for OCR whereby the PI3K inhibitor reduced the immediate
288 induction of OCR in eosinophils treated with IL-5 (Figure 4E). However, treatment
289 with the Akt1/2 inhibitor did not reduce IL-5 induced OCR (Figure 4E), suggesting
290 other downstream PI3K pathways may be involved. These data show that one of the
291 key immediate effects of IL-5 on eosinophils is up-regulation of glycolysis and this is
292 dependent on the STAT5/PI3K/Akt axis.

293

294 **ROS inhibition reduces TCA cycling of IL-5 stimulated eosinophils**

295 To determine if the observed cytokine-stimulated metabolic changes in eosinophils
296 were promoted by ROS production we next determined whether NOX had a role in
297 increased ECAR and OCR with a focus on IL-5 as before. Bioenergetic analyses were
298 used to show that DPI had no effect on IL-5-stimulated glycolysis (Figure 5A) but
299 significantly reduced peak OCR (Figure 5B). SITA using ¹³C-glucose showed
300 increased incorporation of ¹³C into pyruvate and lactate (indicated as an increased m+3
301 mass isotopologue) in the presence of DPI (Figure 5C-D). This was accompanied by a
302 reduction in the relative abundance of all TCA cycle intermediates (Figure 5E),

303 represented by a decreased abundance of the m+2 mass isotopologue (Figure 5F). DPI
304 treatment negatively impacted on the synthesis of amino acids glutamate and aspartate,
305 from ^{13}C -glucose, by reducing ^{13}C incorporation and the m+2 mass isotopologue
306 (Figure 5G-H). Collectively these data demonstrate that NOX-mediated ROS may have
307 a critical role in driving mitochondrial metabolism.

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337 **Discussion**

338 The study of eosinophil metabolism has been challenging, but recent years have seen
339 the introduction of novel, refined technologies that allow metabolic analyses on low
340 cell numbers with more sensitive readouts. This has been driven by the burgeoning field
341 of immunometabolism and the increasingly recognised role of cellular metabolism in
342 immune cell fate and function. Cellular metabolism through energy production (ATP)
343 and biosynthetic intermediate generation orchestrates numerous effector roles such as
344 cytokine production, migration and proliferation and can have a profound impact on
345 various human pathologies³⁵. Aside from their well-recognised energetic and
346 biosynthetic roles, individual metabolites can have alternative roles. For example, TCA
347 cycle metabolites, succinate and fumarate act as inflammatory signalling molecules. In
348 LPS-stimulated macrophages, succinate stabilizes hypoxia-inducible factor-1α to
349 promote increased glycolysis and IL-1β production^{9,36,37}. Therefore, elucidating the
350 cellular metabolic response of eosinophils not only improves our basic understanding
351 of eosinophil function, especially how it might apply to tissue homeostasis, but has
352 implications for revealing immunopathogenic and therapeutic strategies in eosinophilic
353 disorders.

354

355 The rapid engagement of aerobic glycolysis by eosinophils in response to cytokines
356 demonstrated here was accompanied by accumulation of both intra- and extracellular
357 lactate. Lactate creates an acidic environment in which eosinophils are known to thrive,
358 such as in the lung³⁸. Furthermore, excess lactate retains T cells in pro-inflammatory
359 environments, curtailing their migration³⁹. If the same occurred for eosinophils this
360 would provide a mechanism to retain viable eosinophils in an acidic inflammatory
361 tissue environment. This increased glycolytic rate that supports the accumulation of
362 lactate is presumably due to either GLUT1 or GLUT3 mediated glucose uptake as these
363 transporters were expressed by all donors, or through kinetic effects on the direct
364 phosphorylation of glycolytic enzymes²².

365

366 A key feature of the work presented here is clarity surrounding the role of mitochondria
367 in eosinophil metabolism. It is well established that eosinophils utilise their
368 mitochondria for apoptotic purposes^{10,30}, however definitive metabolic contributions
369 have remained elusive. Here, we confirmed that cytokine-stimulated eosinophils were
370 sensitive to oligomycin treatment through a decrease in OCR. This indicates that

371 mitochondria in eosinophils have an important role in mediating metabolic responses
372 to cytokines which is in agreement with a previous study⁵.

373

374 Whilst the conversion of glucose to lactate seems to be the predominant metabolic
375 pathway in response to cytokine stimulation, we used stable isotope tracing to show
376 that eosinophils use both glucose and glutamine to generate TCA cycle intermediates
377 and support OXPHOS upon activation. To our knowledge we are the first to provide
378 evidence that carbons from glucose and glutamine are incorporated into TCA
379 metabolites upon cytokine stimulation in eosinophils. Collectively, we reveal a novel
380 role for human eosinophil mitochondria that extends beyond apoptosis and antibacterial
381 defence. We demonstrate that eosinophils can utilise their mitochondria for TCA
382 cycling contributions to OXPHOS and biosynthesis of amino acids. In support of a role
383 for mitochondrial metabolism in eosinophils as we described here, a recent study
384 indicated that peripheral blood eosinophils have increased oxidative parameters in
385 comparison to neutrophils⁵. However, this interpretation was based solely on decreased
386 oxygen consumption upon exposure to oligomycin and did not definitively characterise
387 the metabolic fuels consumed by eosinophils.

388

389 The effects of IL-3, IL-5 and GM-CSF on eosinophil metabolism were broadly similar.
390 To better understand the signalling processes that govern cytokine-mediated changes
391 to eosinophil cellular metabolism we chose to focus on a single cytokine. IL-5 was
392 chosen as it is a therapeutic target for treating eosinophilic asthma via monoclonal
393 antibodies to IL-5 itself or IL-5R α ³¹. IL-5 ligation in human eosinophils has been
394 shown to activate the JAK/STAT pathway, specifically STAT5^{40,41}. With use of a
395 specific STAT5 inhibitor we determined that increases in both ECAR and OCR upon
396 IL-5 stimulation were dependent on STAT5 signalling. Because activated eosinophils
397 increased their glucose utilization substantially, our attention turned to the PI3K/Akt
398 axis as it is known to control glycolysis in other immune cell types such as T cells and
399 macrophages^{42,43}. PI3K and Akt inhibitors had a profound effect on the IL-5 mediated
400 metabolic switch, especially glycolysis, showing that the IL-5 induced metabolic
401 switch in human eosinophils is mediated by the STAT5/PI3K/Akt signalling axis. The
402 IL-5 induced OCR was abrogated with PI3K inhibition but not Akt. This suggests that
403 there are alternative downstream PI3K pathways contributing to increased oxygen

404 consumption, such as the PI3K/Rac pathway⁴⁴. Respiratory burst in eosinophils has
405 been closely linked previously with the Rac pathway, thus offering a plausible
406 explanation for our observations¹⁵. Elucidating roles of multiple Akt-independent
407 downstream PI3K targets and their contributions to eosinophil metabolism warrants
408 further investigation.

409

410 Finally, we considered the link between ROS production and metabolic pathway
411 activity in eosinophils again focussing on the effects of IL-5. Treatment of eosinophils
412 with IL-5 has been shown to induce ROS production²³ and here we show that IL-5
413 increases oxygen consumption. As NOX-dependent respiratory burst is a fundamental
414 effector function of eosinophils¹⁴ we sought to investigate the role of ROS in eosinophil
415 metabolism. Inhibiting NOX-dependent ROS production reduced the abundance of
416 TCA cycle intermediates while increasing the accumulation of glucose-derived lactate
417 suggesting that ROS may be a driver of eosinophil mitochondria metabolism in
418 particular. This highlights that different bioactive molecules in the immediate
419 microenvironment of eosinophils shape their metabolic plasticity.

420

421 Our study outlines the metabolic requirements of mitochondria in cytokine-activated
422 eosinophils. We also show that ROS may enable metabolic plasticity. Taken together,
423 this provides further insight into the mechanistic control of eosinophil function. It is
424 likely that terminally differentiated cells such as eosinophils do not require extensive
425 energy production and biosynthesis to support homeostasis or activation. Instead
426 multiple cytokines and important mediators such as eosinophil-derived neurotoxins and
427 peroxidases are contained within pre-formed granules. However, cytokine-mediated
428 activation clearly up-regulates cytoplasmic and mitochondrial metabolic pathways.
429 This raises further questions about the links between eosinophil function and
430 metabolism including the bioenergetic demands of piecemeal degranulation and the
431 effects of mitochondrial DNA release on the metabolic status of eosinophils.
432 Eosinophils are a characteristic feature of type 2 immune responses linked to
433 immunopathology in asthma and other inflammatory disorders but also to tissue
434 defence and repair processes in helminthic parasite infection⁴⁵ and in other settings
435 including metabolic homeostasis in adipose tissue⁶. Greater understanding of the
436 regulation of eosinophil recruitment, retention and survival would provide mechanistic
437 insight and offer new metabolically targeted therapeutic approaches for respiratory and

438 other eosinophilic diseases. Cell-specific delivery systems of pharmacological agents
439 via for example Siglec-8 could be one route⁴⁶ or more general approaches to limiting
440 lacate in the tissue microenvironment during pathology might have broad effects on
441 multiple cells types³⁹, including eosinophils.

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486

487 **Authorship Contributions**

488 NJ, EEV, LCF, JGC, LMS performed the experiments; PSH, PL and CAT provided
489 intellectual discussion. NJ, EEV, PSH, PL and CAT designed the experiments. NJ, EEV,
490 PL and CAT wrote the manuscript. All authors critically revised and approved the
491 manuscript.

492

493 **Conflicts of Interest**

494 The authors declare no conflicts of interest.

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649 **Figure Legends**

650 **Figure 1. IL-3, IL-5 or GM-CSF stimulated eosinophils increase glycolytic
651 metabolism and production of lactate.** (A) Expression levels of glucose transporters
652 (*GLUT*) 1-4. (B) Representative flow cytometry plot of glucose uptake by eosinophils
653 activated for 1 h with IL-3, IL-5 or GM-CSF (10 ng/mL) using probe 2-(*N*-(7-nitrobenz-
654 2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG; 100 μ M). (C) Glycolytic
655 stress profile of eosinophils by measuring extracellular acidification rate (ECAR;
656 mpH/min) before and following addition of IL-3, IL-5, GM-CSF (10 ng/mL), glucose
657 (5.5 mM), oligomycin (1 μ M) and 2-DG (100 mM) at the time points indicated. (D)
658 Post-glucose ECAR calculated via the averaged ECAR values from measurement
659 points 7-9 minus the averaged ECAR values from measurement points 1-3. (E)
660 Schematic of uniformly labelled $^{13}\text{C}_6$ -glucose incorporation into pyruvate and lactate.
661 Eosinophils were activated with IL-3, IL-5, or GM-CSF (10 ng/mL) for 4 h. (F)
662 Relative abundance of ^{12}C and ^{13}C pyruvate. (G) Mass isotopologue distribution (MID)
663 of the pyruvate pool. (H) Relative abundance of ^{12}C and ^{13}C lactate including (I) MID
664 of the lactate pool. (J) Relative abundance of ^{12}C and ^{13}C extracellular lactate. (K)
665 Extracellular lactate production of eosinophils treated with IL-3, IL-5 or GM-CSF (10
666 ng/mL) for 4 h. Data are represented as mean \pm SEM of 7 (A), 4 (B), 3-5 (C-D, F-I), 2-
667 3 (J) and 2-4 (K) independent experiments with each data point representing an
668 individual donor. Statistical analysis was performed using a one-way ANOVA with
669 multiple comparisons to the control group (D, K) or a two-way ANOVA (F-J); * p \leq
670 0.05, ** p \leq 0.01, *** p \leq 0.001.

671

672 **Figure 2. Cytokine treatment induces mitochondrial-independent ROS
673 production** (A) Mitochondrial morphology of human eosinophils by TEM. (B)
674 Oxidative stress assay measured via oxygen consumption rate (OCR; pmoles/min)
675 before and following addition of IL-3, IL-5, GM-CSF (10 ng/mL), glucose (5.5 mM),
676 oligomycin (1 μ M) and 2-DG (100 mM) at the time points indicated. (C) Percentage
677 OCR increase in comparison to baseline. (D) Glycolytic stress and (E) oxidative stress
678 IL-3 dose response. Eosinophils were given IL-3 (10, 50 and 100 ng/mL), glucose (5.5
679 mM), oligomycin (1 μ M) and 2-DG (100 mM) at the time points indicated. (F)
680 Representative flow cytometry plot and (G) MitoSOX $^{+ve}$ population of eosinophils
681 activated with IL-3, IL-5 or GM-CSF (10 ng/mL) and incubated with MitoSOX for 15
682 min. (H) Representative flow cytometry plots of total intracellular ROS production

683 measured by CellROX from one donor and (I) CellROX^{+ive} population of eosinophils
684 stimulated with IL-3, IL-5 or GM-CSF (10 ng/mL) ± DPI (10 µM). Dotted line indicates
685 unstimulated controls in the presence of CellROX. Data expressed as mean ± SEM of
686 2 (A), 3-5 (B-C), 2 (D-E), 4-5 (G) and 6-8 (I) independent experiments with each data
687 point representing an individual donor. Statistical analysis was performed using a one-
688 way ANOVA with multiple comparisons to the control group (C) or an unpaired t test
689 (I); * p ≤ 0.05, *** p ≤ 0.001.

690

691 **Figure 3. IL-3, IL-5 or GM-CSF treatment induces the production of TCA cycle**
692 **intermediates.** (A) Schematic of uniformly labelled ¹³C₆-glucose incorporation into
693 TCA cycle intermediates. Eosinophils were activated with IL-3, IL-5 or GM-CSF (10
694 ng/mL) for 4 h. Relative abundance of ¹²C and ¹³C (B) citrate, (C) succinate, (D) malate
695 and (E) fumarate. (F) Mass isotopologue distribution (MID) of m+2 citrate, succinate
696 and malate. (G) Relative abundance of ¹²C and ¹³C glutamate including the (H) MID
697 distribution. All data are from 3-6 independent experiments with each data point
698 representing an individual donor. Data expressed as mean ± SEM. Statistical analysis
699 was performed using a two-way ANOVA; * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001.

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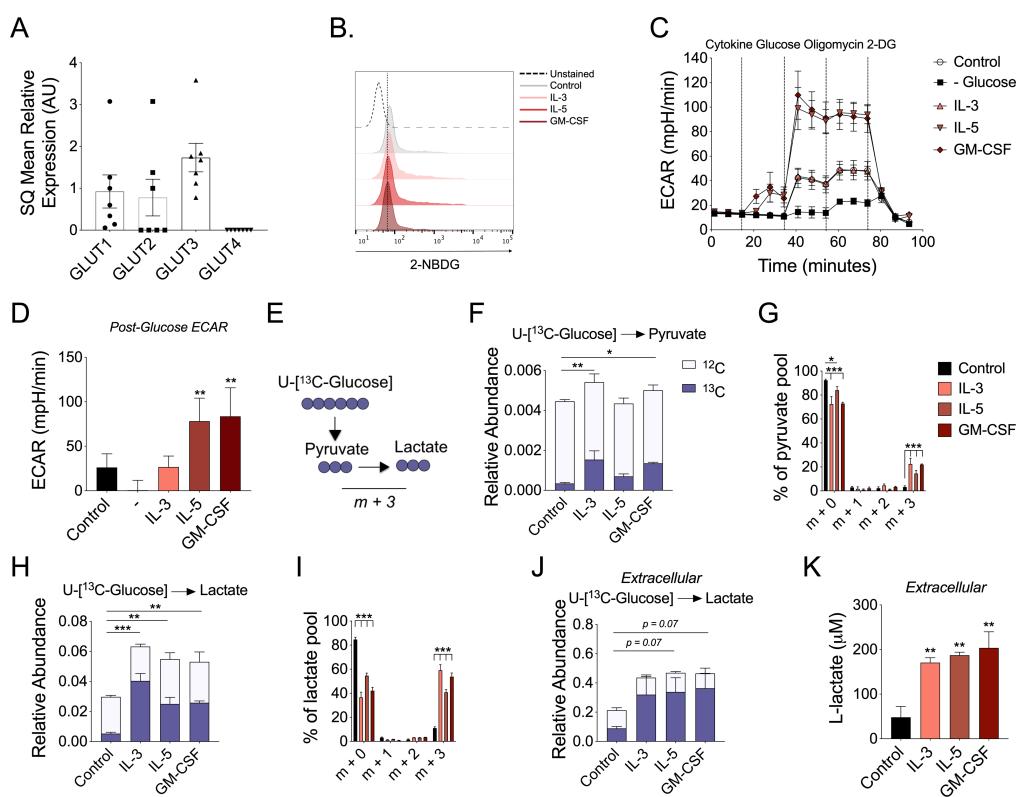
701 **Figure 4. The STAT5/PI3K/Akt axis is responsible for the metabolic switch in IL-**
702 **5 treated eosinophils.** (A) Representative immunoblot of eosinophils treated for 15
703 min with IL-3, IL-5 or GM-CSF (10 ng/mL) for pSTAT5⁶⁹⁴ and β-actin. Corresponding
704 densitometry analysis of pSTAT5 normalised to β-actin. (B) ECAR and (C) OCR
705 before and following addition of a STAT5 inhibitor (STAT5i; N'-((4-oxo-4H-chromen-
706 3-yl)methylene)nicotinohydrazide; 100 µM), IL-5 (10 ng/mL) and 2-DG (100 mM),
707 including ‘pre-cytokine’ activation and ‘post-cytokine’ activation pooled OCR and
708 ECAR data. (D) ECAR and (E) OCR before and following addition of a PI3K inhibitor
709 (LY294002; 10 µM) or Akt1/2 inhibitor (1,3-Dihydro-1-(1-((4-(6-phenyl-1H-
710 imidazo[4,5-g]quinoxalin-7-yl)phenyl)methyl)-4-piperidinyl)-2H-benzimidazol-2-one
711 trifluoroacetate salt hydrate; 10 µM), IL-5 (10 ng/mL) and 2-DG (100 mM). Data
712 expressed as mean ± SEM of 5 (A), 2-3 (B-C) and 4 (D-E) independent experiments
713 with each data point representing an individual donor. Statistical analysis was
714 performed using a Friedman test with Dunn’s multiple comparisons (A) or a two-way

715 ANOVA with Sidak's multiple comparison test (B-E); * p ≤ 0.05, ** ≤ p 0.01, *** p ≤
 716 0.001.

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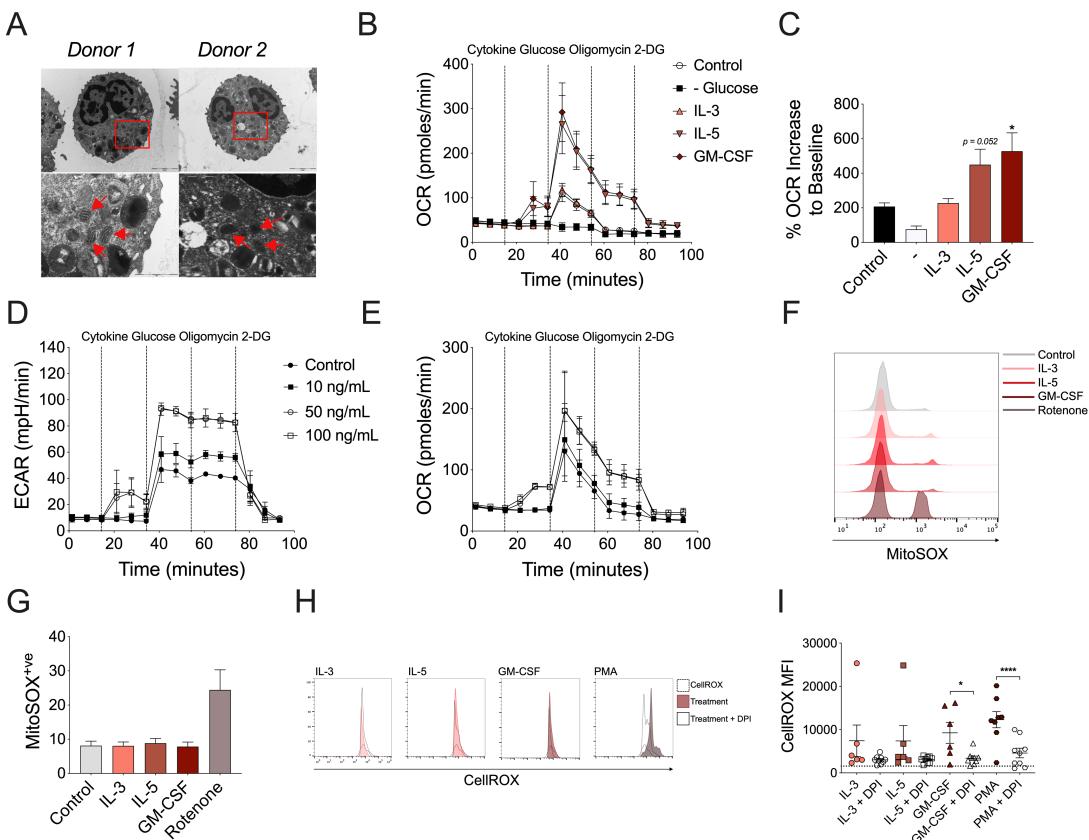
718 **Figure 5. DPI inhibits oxidative metabolism in IL-5 stimulated eosinophils.** (A)
 719 ECAR (mpH/min) and (B) OCR (pmoles/min) of eosinophils treated with IL-5 (10
 720 ng/mL) ± DPI (100 nM), glucose (5.5 mM), oligomycin (1 μM) and 2-DG (100 mM).
 721 Eosinophils were activated with IL-5 (10 ng/mL) ± DPI (100 nM) for 4 h in the presence
 722 of ¹³C-glucose. (C) Relative abundance of ¹²C and ¹³C and (D) mass isotopologue
 723 distribution (MID) of glycolytic intermediates pyruvate and lactate. (E) Relative
 724 abundance of ¹²C and ¹³C and (F) MID of TCA cycle intermediates citrate, succinate,
 725 fumarate and malate. (G) Relative abundance of ¹²C and ¹³C and (H) MID of amino
 726 acids glutamate and aspartate. Data expressed as mean ± SEM of 4 (A-B) and 3 (C-H)
 727 independent experiments with each data point representing an individual donor.
 728 Statistical analysis was performed using an unpaired t test (B) or a two way ANOVA
 729 (C-H); * p ≤ 0.05, ** ≤ p 0.01, *** p ≤ 0.001.

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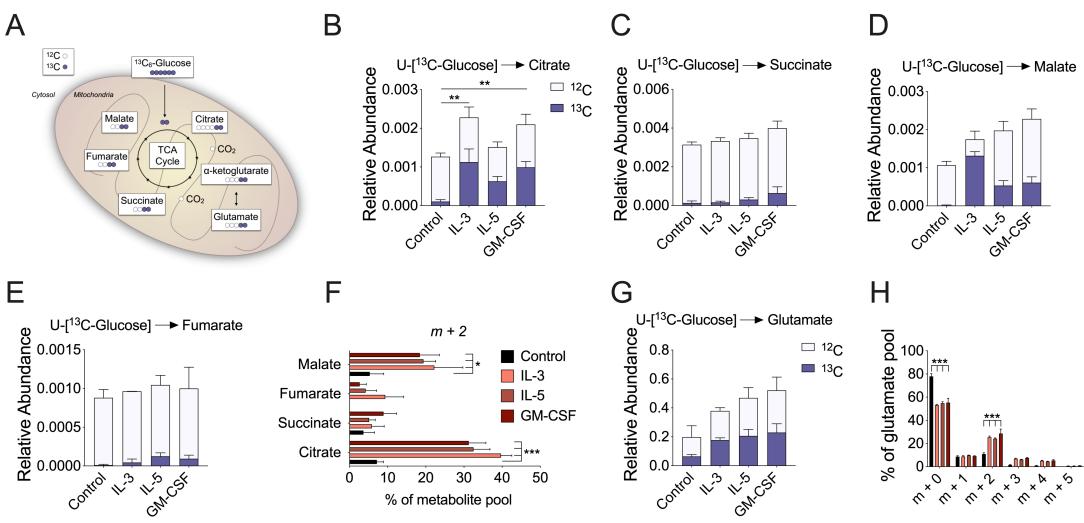
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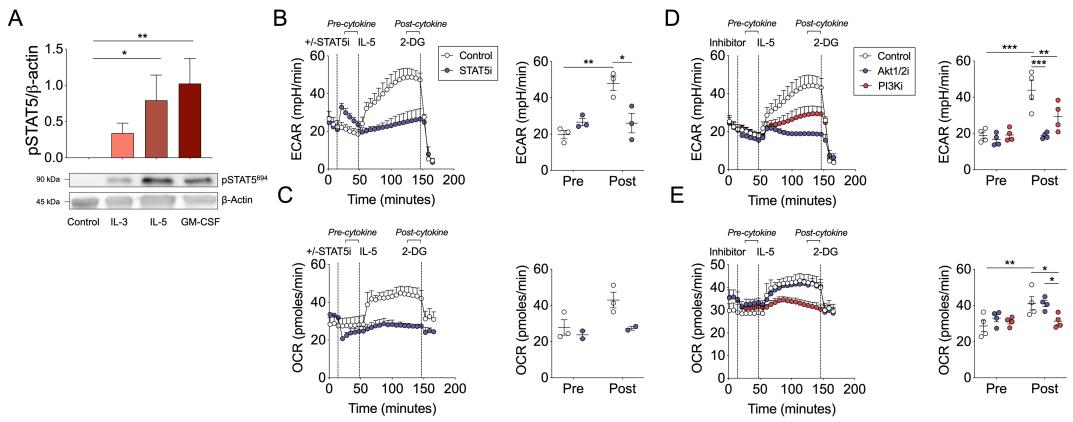
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