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Fatty acid oxidation in macrophage polarization

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To the Editor

Recently, there has been considerable interest in the role of intracellular metabolism as a regulator of the fate and function of cells of the immune system[1,2]. In macrophages, the classical M1 activation program has been noted to rely on glycolysis, while the M2 program seems to require the induction of fatty acid oxidation (FAO)[1–4]. Mitochondria-dependent β-oxidation of long-chain fatty acids requires the carnitine palmitoyltransferase (CPT) system, which consists of one transporter and two mitochondrial membrane enzymes: CPT1 and CPT2 (ref. 5). This system facilitates the transport of long-chain fatty acids into the mitochondrial matrix, where they can be metabolized to produce ATP (Supplementary Fig. 1a). A simple genetic strategy involving *lox*P-flanked alleles encoding CPT2 (*Cpt2*$^{fl/fl}$) has provided the means with which to disrupt FAO within specific mouse cell types[6]. This is potentially important, because much of the evidence indicating that FAO is required for M2 macrophage polarization has relied on pharmacological approaches, particularly the use of the epoxide etomoxir, which is thought to function as a specific inhibitor of CPT1.

To directly assess the role of FAO in macrophage function, we crossed *Cpt2*$^{fl/fl}$ mice with mice transgenically expressing Cre from the myeoid cell–specific gene encoding LysM (*Lyz2*-Cre) to generate mice with myeloid linage–specific deletion of *Cpt2* (*Cpt2*$^{fl/fl}$*Lyz2*-Cre; called ‘CPT2 M-KO’ here). We obtained bone marrow–derived macrophages (BMDMs) from *Cpt2*$^{+/+}$*Lyz2*-Cre (control) mice and CPT2 M-KO mice. We confirmed the fidelity of macrophage-specific deletion of *Cpt2* by this approach (Fig. 1a,b). In *Cpt2*$^{+/+}$*Lyz2*-Cre (control) BMDMs, the addition of fatty acids resulted in a significant increase in the basal oxygen consumption rate that was blocked by the addition of the pharmacological inhibitor etomoxir (Fig. 1c). In contrast, BMDMs derived from CPT2 M-KO mice did not augment their metabolic rate when provided a fatty acid substrate, and

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

AUTHOR CONTRIBUTIONS

M.N. and J.L. performed the experiments; M.N., I.I.R., E.G.-H., J.L., M.J.W. and T.F. analyzed the data; and M.N. and T.F. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

etomoxir had no effect on respiration (Fig. 1c). Thus, BMDMs derived from the CPT2 M-KO mice did not express CPT2 and, as expected, did not seem to be capable of performing FAO.

Next we assessed the M2 polarization of *Cpt2*$^{+/+}$*Lyz2*-Cre (control) and CPT2 M-KO BMDMs. Following *in vitro* stimulation with interleukin 4 (IL-4), there was no apparent difference in their M2 polarization, as assessed by expression of the M2 markers CD301 and CD206 (Fig. 1d). Similar findings were obtained after injection of a complex of IL-4 and monoclonal antibody to IL-4 and assessment of the *in vivo* polarization of peritoneal macrophages (Fig. 1e). As has been noted before[4], we found that in *Cpt2*$^{+/+}$*Lyz2*-Cre (control) BMDMs, etomoxir was effective in inhibiting the expression of various genes encoding classical M2 markers that included a range of intracellular enzymes and surface markers (Fig. 1f). Indeed, while stimulation with IL-4 markedly induced expression of *Arg1*, *Mgl2* and *Retnla*, their expression was significantly inhibited by treatment with etomoxir (Fig. 1f). Nonetheless, etomoxir was equally effective in inhibiting these genes encoding M2-polarization markers in CPT2 M-KO BMDMs, which lacked the capacity for FAO (Fig. 1f).

Together our results suggested that the requirement for FAO in M2 polarization might be more complex than previously envisioned. In particular, consistent with the known requirement for CPT2 in FAO, we demonstrated that macrophages lacking CPT2 were unable to achieve β-oxidation of fatty acids yet still seemed to fully polarize toward an M2 state after stimulation with IL-4 *in vitro* and *in vivo*. While treatment with etomoxir potently blocks the M2 polarization of wild-type macrophages[3,4], our data suggested this occurred to an equal degree in CPT2-deficient macrophages. Of note, a published report has also shown that etomoxir does not affect the M2 polarization of human macrophages[7]. One possibility that might explain these divergent observations is that CPT1, the presumed target of etomoxir, participates in some additional, as-yet-unknown function that is independent of long-chain FAO. Alternatively, etomoxir, being an epoxide, might potentially react with a broad range of intracellular nucleophiles, and the inhibitory effects on macrophage polarization might be an off-target effect of the drug. The development of new models, particularly those with conditional deletion of alleles encoding CPT1A, would be helpful in distinguishing these possibilities. In the interim, it might be prudent to cautiously interpret observations based on the use of etomoxir and to re-assess whether FAO has a correlative role or causal role in macrophage polarization. Finally, while the growing field of immunometabolism has generated considerable enthusiasm, it is often difficult to know whether an observed metabolic shift is the cause or consequence of a change in the cellular phenotype. In this sense, the genetic approaches described here should be particularly useful for the field.

ONLINE METHODS

Mice and cells

To generate mice with myeloid lineage–specific deficiency in CPT2, C57BL/6 mice with *lox*P sites flanking exon 4 of the *Cpt2* gene[8] were crossed with C57Bl/6 Lyz2-Cre transgenic mice[9] obtained from Jackson Laboratory. Mice, both male and female, were used between

the ages of 4 and 12 weeks. All mice studies were done in accordance and approval of the National Heart, Lung and Blood Institute Animal Care and Use Committee.

To isolate bone marrow derived macrophages, cells were obtained by flushing the tibia and femur of mice with PBS. Cells were first cultured in growth medium (DMEM with 4.5 g l^{-1} glucose, L-glutamine, and sodium pyruvate (Corning), penicillin-streptomycin (Gibco), 10% FBS (Omega Scientific) and 10 ng/ml recombinant mouse macrophage colony-stimulating factor (M-CSF; eBioscience). At days 6–8, BMDMs were sub-cultured and cells were stimulated with IL-4 (10 ng ml^{-1}; eBioscience) for 24 h in the presence or absence of etomoxir (200 μM, 3 h pretreatment; R&D Systems).

Quantitative PCR

RNA was isolated using RNeasy Mini Kit (Qiagen) and cDNA was synthesized using iScript DNA Synthesis Kit (Bio-Rad). The cDNA was amplified by specific primers using FastStart Universal SYBR Green Master (Roche) and analyzed by the LightCycler 96 System (Roche). Expression levels in BMDMs and peritoneal macrophages were normalized to *Hprt*. The following primers were used for amplification: *Cpt2* forward, 5′-CAGACAGTGGCTACCTATG AATCCT-3′, and reverse, 5′-TGGTCAGCTGGCCATGGTATTT GGA-3′; *Arg1* forward, 5′-CTCCAAGCCAAAGTCCTTAGAG-3′, and reverse,5′-AGGAGCTGTCATTAGGGACATC-3′; *Mgl2* forward, 5′-GCATGAAGGCAGCTGCTATTGGTT-3′, and reverse,5′-TAGGCCCATCCAGCTAAGCACATT-3′; *Retnla* forward, 5′-CCAATCCAGCTAACTATCCCTCC-3′, and reverse,5′-ACCCAGTAGCAGTCATCCCA-3′; *Hprt* forward, 5′-TTTCCCTGGTTAAGCAGTACAGCCC-3′, and reverse, 5′-TGGCCTGTATCCAACACTTCGAGA-3′.

CPT2 protein expression

Mitochondria-enriched fraction was prepared as described previously[10]. BMDMs were cultured in a six-well plate (1.0×10^6 cells per well) and the cells were washed with PBS and re-suspended in 100 ml of mitochondrial isolation buffer (70 mM Tris, 0.25 M sucrose and 1 mM EDTA, pH 7.4), 100 ml of MES buffer (19.8 mM EDTA, 0.25 M D-mannitol and 19.8 mM MES, pH 7.4) and 4 ml of 10 mg ml^{-1} digitonin. After 10 min of incubation, the cells were scraped and collected from three wells and centrifuged at 900*g* for 2 min. The supernatant was collected and centrifuged at 13,000*g* for 5 min to pellet the mitochondria-enriched fraction. The pellet was washed in PBS and centrifuged at 16,000*g* for 5 min. This fraction was then directly re-suspended in 30 ml of 1× loading buffer and boiled. The samples were then loaded into 4–20% Mini-PROTEAN TGX Precast Protein Gel (Bio-Rad), and transferred to Trans-Blot Turbo Nitrocellulose Transfer Pack (Bio-Rad). Anti-CPT2 (1:1,000 dilution; ab181114; Abcam) and anti-COX4 (1:2,000 dilution; A21348; Thermo Scientific) were used as primary antibodies and the blots were acquired and analyzed by Odyssey CLx imager (LI-COR).

FAO assay

To assess the ability to oxidize exogenous fatty acids, the oxygen- consumption rate (OCR) of BMDMs was analyzed using the XF-24 Extracellular Flux Analyzer (Seahorse Bioscience). BMDMs were cultured in a 24-well Seahorse plate (0.25×10^6 cells per well) and the cells were incubated overnight with substrate-limited medium (XF Assay Medium Modified DMEM (Seahorse Bioscience) with 0.5 mM glucose, 1× GlutaMAX (Gibco), 0.5 mM carnitine (Sigma-Aldrich) and 1% FBS). The next day, the medium was changed for 30 min to FAO assay medium (111 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 2.0 mM $MgSO_4$, 1.2 mM Na_2HPO_4, 2.5 mM glucose, 0.5 mM carnitine and 5 mM HEPES). After 15 min of pre-treatment of etomoxir (40 μM), where appropriate, palmitate-BSA (200 μM palmitate conjugated with 34 μM BSA) or BSA (34 μM) (Seahorse Bioscience) were added and the OCR analyzed. Results were initially obtained as picomoles O_2 consumption per min per mg protein. The level of OCR for *Cpt2*$^{+/+}$*Lyz2*-Cre (control) macrophages in the presence of BSA was defined as an OCR of 1. Where appropriate, the cells were treated with the ATP synthase inhibitor oligomycin (1.0 μM), the chemical uncoupler FCCP (1.5 μM) and the electron transport inhibitor antimycin A (0.5 μM). Basal oxygen consumption was assessed before the addition of any mitochondrial inhibitor.

Flow cytometry

Nonspecific signals were blocked by Purified Rat Anti-Mouse CD16/CD32 (Mouse BD Fc BlockTM; 1:500 dilution; 553142; BD Pharmingen) and cell surface M2 markers were assessed with phycoerythrin-conjugated anti–mouse CD206 (MMR) (1:40 dilution; 141705; BioLegend) and phycoerythrin- Cy7–conjugated anti–mouse CD301 (MGL1/MGL2) (1:100 dilution; 145705; BioLegend). Data were acquired and analyzed with a FACSCanto (BD Biosciences).

Administration of the complex of IL-4 and antibody to IL-4

The complex of IL-4 and antibody to IL-4 (IL-4c) was administered as described[11]. Recombinant mouse IL-4 (PeproTech) was suspended at a concentration of 500 mg ml^{-1} and was mixed with monoclonal antibody (mAb) to mouse IL-4 (11b11; BioXcell) at a molar ratio of 2:1. IL-4c was suspended in saline to a concentration of 25 mg ml^{-1} IL-4 and 125 mg ml^{-1} of mAb to IL-4. Each mouse given intraperitoneal injection of 200 ml of IL-4c (5 mg IL-4 and 25 mg mAb to IL-4) or saline on days 0 and 2, and peritoneal macrophages were collected by washing of the cavity with 6 ml of PBS with 5% FBS on day 4. Erythrocytes were removed by incubation with 1× RBC lysis buffer (eBioscience). Analysis was performed in a blinded fashion with regard to genotype.

Statistical analysis

Statistical significance was calculated by the unpaired two-tailed Student's *t*-test (Fig. 1a), two-way analysis of variance with the Bonferoni correction (Fig. 1c) and one-way analysis of variance with the Tukey-Kramer comparison (Fig. 1e,f).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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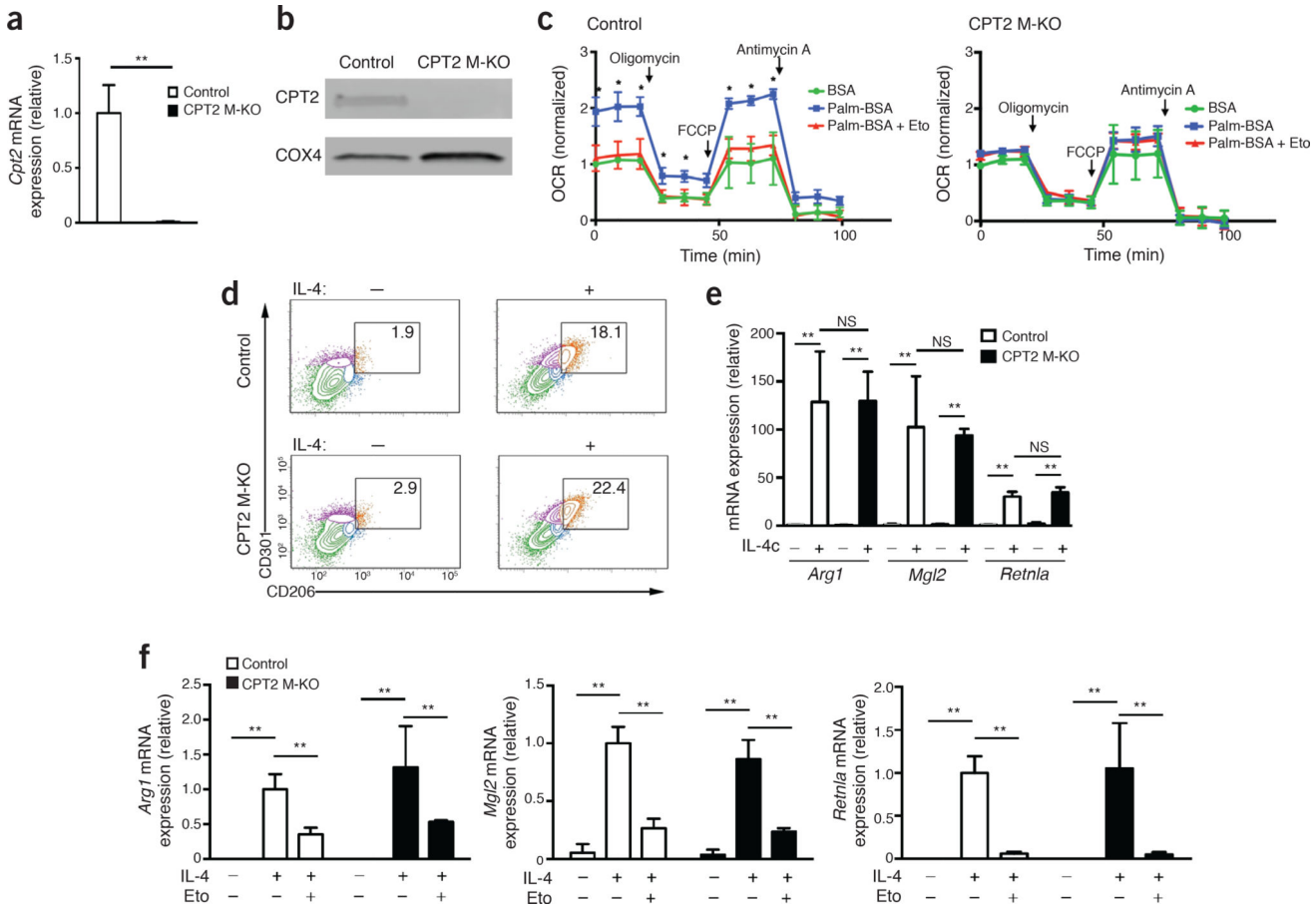

Figure 1.

Macrophage FAO is not required for M2 polarization. (**a**) Quantitative PCR analysis of *Cpt2* mRNA in *Cpt2*$^{+/+}$*Lyz2*-Cre (control) and CPT2 M-KO BMDMs; results are relative to those of *Cpt2*$^{+/+}$*Lyz2*-Cre cells, set as 1. (**b**) Immunoblot analysis of mitochondrial expression of CPT2 and COX4 (cytochrome *c* oxidase subunit 4; loading control) in *Cpt2*$^{+/+}$*Lyz2*-Cre (control) and CPT2 M-KO BMDMs. (**c**) Oxygen-consumption rate (OCR) of BMDMs treated sequentially with oligomycin, the chemical uncoupler FCCP and antimycin A (downward arrows) in the presence of bovine serum albumin alone (BSA) or conjugated to palmitate (Palm-BSA) or palmitate-BSA after pre-treatment with 40 μM etomoxir (Palm-BSA + Eto); results (measured as picomoles of O_2 per minute per microgram of protein) were normalized to those of control cells in the presence of BSA. (**d**) Flow cytometry analyzing the expression of CD301 and CD206 in *Cpt2*$^{+/+}$*Lyz2*-Cre (control) and CPT2 M-KO BMDMs. Numbers in outlined areas indicate percent CD301^{+}CD206^{+} cells. (**e**) Quantitative PCR analysis of mRNA encoding various M2 markers (below plot) in peritoneal macrophages isolated from *Cpt2*$^{+/+}$*Lyz2*-Cre (control) and CPT2 M-KO mice given injection of saline (IL-4c −) (*n* = 3 mice per genotype) or a complex of IL-4 and monoclonal antibody to IL-4 (IL-4c +) (*n* = 4 mice per genotype); results are relative to those of cells from saline-treated *Cpt2*$^{+/+}$*Lyz2*-Cre (control) mice.(**f**) Quantitative PCR analysis of mRNA encoding M2 markers as in **e**, in *Cpt2*$^{+/+}$*Lyz2*-Cre (control) and CPT2 M-KO BMDMs in the presence (+) or absence (−) of IL-4 or etomoxir (200 μM); results are

relative to the peak value of IL-4-treated *Cpt2*$^{+/+}$*Lyz2*-Cre (control) cells. **P* < 0.05 and ***P* < 0.01 (unpaired two-tailed Student's *t*-test (**a**), two-way analysis of variance with Bonferoni correction (**c**) or one-way analysis of variance with the Tukey-Kramer comparison (**e,f**)). Data are from one experiment representative of three independent experiments with similar results (**a,d,f**; mean and s.d. of triplicates in **a,f**), one experiment representative of four experiments with similar results (**b**), three independent experiments (**c**; mean ± s.d. of triplicates) or one experiment (**e**; mean and s.d.).