C13orf31 (FAMIN) is a central regulator of immunometabolic function

M Zaeem Cader1, Katharina Boroviak2, Qifeng Zhang3, Ghazaleh Assadi4, Sarah L Kempster1, Gavin W Sewell1, Svetlana Saveljeva1, Jonathan W Ashcroft1, Simon Clare2, Subhankar Mukhopadhyay2, Karen P Brown5, Markus Tschurtschenthaler1, Tim Raine1, Brendan Doe2, Edwin R Chilvers6, Jules L Griffin7, Nicole C Kaneider1, R Andres Floto5,6, Mauro D’Amato4,8, Allan Bradley2, Michael J O Wakelam3, Gordon Dougan2 & Arthur Kaser1

Single-nucleotide variations in C13orf31 (LACC1) that encode p.C284R and p.I254V in a protein of unknown function (called ‘FAMIN’ here) are associated with increased risk for systemic juvenile idiopathic arthritis, leprosy and Crohn’s disease. Here we set out to identify the biological mechanism affected by these coding variations. FAMIN formed a complex with fatty acid synthase (FASN) on peroxisomes and promoted flux through de novo lipogenesis to concomitantly drive high levels of fatty-acid oxidation (FAO) and glycolysis and, consequently, ATP regeneration. FAMIN-dependent FAO controlled inflammasome activation, mitochondrial and NADPH-oxidase-dependent production of reactive oxygen species (ROS), and the bactericidal activity of macrophages. As p.I254V and p.C284R resulted in diminished function and loss of function, respectively, FAMIN determined resilience to endotoxin shock. Thus, we have identified a central regulator of the metabolic function and bioenergetic state of macrophages that is under evolutionary selection and determines the risk of inflammatory and infectious disease.

Immunological and inflammatory diseases arise from complex environment–gene interactions. While the external triggers remain unknown, human genetic studies have revealed the genomic-risk ‘landscapes’ of these conditions. Genes encoding proteins with established immunological function have been predictably identified as plausible candidates at various risk loci. The mechanistic contribution of their polymorphic variants to disease pathophysiology has been addressed experimentally for a few of these genes, although this is currently based largely on conjecture for most. Furthermore, for a large number of risk loci and genes, it remains entirely unclear how their products are mechanistically involved in disease, including some for which no biological function is known yet at all. We hypothesized that studying uncharacterized gene products singled out by genetic disease association might reveal novel biological mechanisms that are important not only for human health but also for mammalian biology in general.

Two coding polymorphisms in C13orf31 (LACC1), an open reading frame that encodes a protein of unknown function, render this gene and its product particularly notable. First, homoallelic carriage of a rare missense mutation at g.43,883,879T>C, which leads to substitution of arginine for cysteine at position 284 (p.C284R), has been linked in consanguineous families to systemic juvenile idiopathic arthritis (sJIA) (a periodic fever syndrome) and to early-onset Crohn's disease (CD) (an inflammatory bowel disease)1,2. Second, the haplotype identified by the common single-nucleotide polymorphism (SNP) rs3764147 (g.43,883,789A>G), which leads to substitution of valine for isoleucine at position 254 (p.I254V), has been associated with increased risk for leprosy (an infection caused by Mycobacterium leprae) and CD3,4.

Here we found that the protein encoded by that open reading frame served as ‘rheostat’ for the synthesis of endogenous fatty acids and their mitochondrial oxidation and thereby controlled glycolytic activity and overall ATP regeneration. As a consequence, the product of this polymorphic gene determined the mitochondrial and nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase-dependent production of reactive oxygen species (ROS), bactericidal activity and inflammasome activation in macrophages. These findings prompted us to call this protein ‘FAMIN’ (‘fatty acid metabolism–immunity nexus’).

RESULTS
Interaction of FAMIN with fatty acid synthase
C13orf31 mRNA has its highest expression in macrophages and is predicted to encode a cytoplasmic protein of 430 amino acids that lacks similarity to other mammalian protein families5,6. We performed

1Division of Gastroenterology and Hepatology, Department of Medicine, Addenbrooke’s Hospital, University of Cambridge, Cambridge, UK. 2Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, UK. 3Signalling Programme, Babraham Institute, Babraham Research Campus, Cambridge, UK. 4Department of Biosciences and Nutrition, Karolinska Institutet, Stockholm, Sweden. 5Cambridge Institute for Medical Research, University of Cambridge, Cambridge Centre for Lung Infection, Cambridge, UK. 6Division of Respiratory Medicine, Department of Medicine, Addenbrooke’s and Papworth Hospitals, University of Cambridge, Cambridge, UK. 7Department of Biochemistry, University of Cambridge, Cambridge, UK. 8BioDonostia Health Research Institute San Sebastian and Ikerbasque, Basque Foundation for Science, Bilbao, Spain. Correspondence should be addressed to A.K. (ak729@cam.ac.uk).

Received 9 May; accepted 1 July; published online 1 August 2016; doi:10.1038/ni.3532
Figure 1 FAMIN interacts with fatty acid synthase and localizes to peroxisomes. (a) Immunoblot analysis (IB) of FAMIN and FASN (right margin) in primary human macrophages (MΦ) derived from peripheral blood mononuclear cells and in U937 and THP-1 macrophages differentiated with PMA (below blots), assessed after immunoprecipitation (IP) with antibody to FAMIN (anti-FAMIN), mouse immunoglobulin G (IgG; control) or anti-FASN (above lanes) or without immunoprecipitation (Input; far right). Left margin, molecular size, in kilodaltons (kDa). (b) PLA of FAMIN and FASN (yellow) in THP-1 macrophages also stained with the DNA-binding dye DAPI (blue throughout). (c) Immunoblot analysis of FAMIN or GAPDH (loading control) in lysates of HEK293 cells transfected to express human FAMIN(p.254I) or FAMIN(p.254V) or with vector alone (above lanes), assessed with (top blot) or without (bottom two blots) immunoprecipitation with anti-FASN. (d) Immunofluorescence analysis of the co-localization of FAMIN (red) with PMP70 (green) in primary human macrophages (enlarged from areas outlined in Supplementary Fig. 2a); far right, enlargement of the area outlined at left. (e) PLA of FAMIN and PMP70 (red) in THP-1 macrophages. (f) Immunofluorescence analysis of the co-localization of FASN (red) with catalase (green) in mFamin+/+ and mFamin−/− M1 macrophages (M1Φ) and M2 macrophages (M2Φ). (g) Quantification of the results in f. (h) PLA of FASN and catalase (yellow) in mFamin+/+ and mFamin−/− M1 and M2 macrophages. (i) Quantification of the results in h. Scale bars (b,d,f,h), 5 μm. Data are representative of three independent experiments (a–e) or one experiment with three mice and six cells (f,g) or ten cells (h,i) imaged per sample (f–i; mean ± s.e.m.).

Paragraph...

an unbiased proteomics search for interaction partners to begin to elucidate FAMIN’s function. After discounting proteins involved in the synthesis, folding and degradation of proteins or cytoskeletal organization, we found that fatty acid synthase (FASN; EC 2.3.1.85) was the most abundant peptide detected with FAMIN tagged with Strep-tag at the amino or carboxyl terminus, expressed as ‘bait’ in human monocytes and THP-1 cells. Immunofluorescence analysis revealed nearly perfect co-localization of FAMIN with the peroxisome markers PMP70 and catalase (Fig. 1d and Supplementary Fig. 2a–c), while it had little co-localization with other organelle markers (Supplementary Fig. 2d,e). Co-localization between FAMIN and PMP70 was confirmed by PLA (Fig. 1c and Supplementary Fig. 2f); this confirmed the peroxisomal localization of FAMIN.

Peroxisomes are organelles involved in lipid metabolism that are closely associated with lipid droplets and mitochondria. Macrophages exhibit metabolic and functional plasticity, epitomized by in vitro culture under non-polarizing (M0), classical (M1) or alternative (M2) activation conditions. While aerobic glycolysis is a defining characteristic of ‘inflammatory’ M1 macrophages and is their chief mechanism for the generation of ATP, ‘regenerative’ M2 macrophages rely on mitochondrial fatty-acid oxidation (FAO; also called β-oxidation) to fuel oxidative phosphorylation. To study FAMIN’s function, we generated mice in which we knocked out alleles of the mouse homolog of the human gene C13orf31; called ‘mFamin’ here) (Supplementary Fig. 3). Similar to FAMIN, FASN does not have a peroxisomal localization motif and has been shown to form a complex with PMP70 on the cytoplasmic side of peroxisomes. Consistent with that, we found that a portion of FAMIN co-localized with PMP70 in THP-1 cells (Supplementary Fig. 3).
Figure 2  FAMIN promotes glycolysis and flux of glucose into de novo lipogenesis.  
(a–d) Quantification of 13C-labeled C16:0 fatty acyl-CoA (a,c) and C18:1 fatty acyl-CoA (b,d) in mFamin+/+ and mFamin−/− M1 macrophages (a,b) and M2 macrophages (c,d) after a 24-hour pulse with 1,2-13C-glucose.  
(e,f) Quantification of total fatty acyl CoA in mFamin+/+ and mFamin−/− M1 macrophages (e) and M2 macrophages (f).  
(g,h) Quantification of 13C-labeled C16:0 fatty-acyl carnitine (FaCN) (g) and total fatty-acyl carnitine (h) in mFamin+/+ and mFamin−/− M2 macrophages pulsed as in a–d. (i–k) ECAR of mFamin+/+ and mFamin−/− M0 macrophages (i), M1 macrophages (j) and M2 macrophages (k) given sequential treatment (dotted vertical lines) with oligomycin (Oligo) and 2-deoxyglucose (2-DG).  
(l) Quantification of citrate in mFamin+/+ and mFamin−/− M1 and M2 macrophages; results are presented relative to those of mFamin+/+ M1 macrophages, set as 1. Each symbol (a–h,i) represents an individual mouse; small horizontal lines indicate the mean (±s.e.m.). *P < 0.05, **P < 0.01 and ***P < 0.001 (unpaired, two-tailed Student’s t-test; Grubb’s test outlier exclusion). Data are from one experiment with six mice (a–h) or seven mice (i) or one experiment with three mice representative of three independent experiments (i–k; mean ± s.e.m.).

Control of carbon flux via DNL by FAMIN

FASN catalyzes all steps in the synthesis of long-chain saturated fatty acids (LCFAs) (predominantly C16:0 palmitic acid)15. This enzymatic reaction utilizes cytoplasmic acetyl–coenzyme A (acyetyl-CoA), malonyl-CoA (generated from acetyl-CoA by carboxylation) and NADPH15. Cytoplasmic acetyl-CoA is itself generated by ATP citrate lyase after the export of mitochondrial citrate formed during glucose- or amino-acid-fueled Krebs-cycle activity16. In consequence, FASN function allows the storage of excess energy, derived from glucose and amino-acid oxidation, in lipid droplets after the activation of LCFAs and its esterification to LCFA-CoA and thence to triacylglycerol17. FASN also synthesizes fatty-acyl moieties of membrane lipids and provides the precursors of many other cellular and signaling lipids. Given the diverse functions of lipid moieties that are dependent on FASN function, precise channeling of its products into distinct metabolic pathways is essential15,17.

To assess FASN activity in situ, we pulse labeled mouse bone-marrow-derived macrophages with [13C1,2]-glucose (Supplementary Fig. 1). Uptake of [13C] into total LCFAs and palmitic acid was below the limit of detection in mFamin+/+ and mFamin−/− M1 and M2 macrophages (data not shown), consistent with limited turnover and exogenous uptake of the large pools of free fatty acids. However, we did observe incorporation of [13C] into C16:0 and C18:1 fatty-acyl-CoA esters (the activated forms of LCFAs used for lipid synthesis and mitochondrial oxidation) in mFamin+/+ M1 and M2 macrophages (Fig. 2a–d). Together these data indicated that newly synthesized fatty acids were immediately esterified to CoA for subsequent use and thus did not determine the abundance of the free-fatty-acid pool. Furthermore, we discovered that the incorporation of [13C] into C18:1-CoA in M1 macrophages and into C16:0-CoA and C18:1-CoA in M2 macrophages was much lower in mFamin−/− cells than in mFamin+/+ cells (Fig. 2a–d). The abundance of total LCF-CoA was also significantly lower in mFamin−/− M1 and M2 macrophages than in their mFamin+/+ counterparts (Fig. 2e,f), while total fatty-acid content, including palmitic acid, was predictably indistinguishable in cells of these genotypes (Supplementary Table 2). Together these data indicated that FAMIN determined, via DNL, the availability of fatty-acyl-CoA for processes such as FAO.

FAO requires the transport of CoA-activated LCFAs across mitochondrial membranes as acyl-carnitine esters18. The exchange of CoA for carnitine by the cytoplasmic carnitine palmitoyltransferase CPT1a is the rate-limiting step of FAO18. Consistent with the diminished specific availability of fatty-acyl-CoA, the incorporation of [13C]glucose-derived [13C] into fatty-acyl carnitine species (total and C16:0) was much lower in mFamin−/− M2 macrophages than in mFamin+/+ M2 macrophages (Fig. 2g,h), while its incorporation was below the limit of detection in M1 macrophages (data not shown). Notably, while the incorporation of glucose-derived [13C] into acyl-CoA was substantial after a 24-hour pulse (~20–30% of C16:0-CoA and C18:1-CoA in mFamin+/+ M2 macrophages), it was low
for acyl carnitine species (~4% of total acyl carnitine in mFamin+/+ M2 macrophages) at the same time point (Fig. 2g,h). Collectively, these results indicated FAMIN directly controlled the flux of glucose-derived carbon into DNL and onward into acyl carnitine, with the latter possibly involving an intermediary step via triacylglycerols12.

**Control of glycolysis by FAMIN via DNL**

To determine whether loss of FAMIN consequently influenced glycolytic metabolism in macrophages, we measured the extracellular acidification rate (ECAR), an indicator of glycolysis19, in wild-type and mutant macrophages. We found that the ECAR was much lower in mFamin+/− M1 macrophages than in mFamin+/+ M1 macrophages, with a similar proportional decrease from the expected lower baseline in M0 and M2 macrophages (Fig. 2i–k). The difference in the ECAR was further enhanced after blockade of the F1γ subunit of mitochondrial ATP synthase with oligomycin A (Fig. 2i–k), which stimulates the alternative generation of ATP via glycolysis20. This observation indicated that FAMIN-dependent glycolysis was maximized under conditions in which mitochondrial respiration was uncoupled from ATP synthesis. Differences between mFamin+/+ macrophages and mFamin+/− macrophages in their ECAR were abolished after inhibition of glycolysis with 2-deoxy-D-glucose (Fig. 2i–k). Similarly, measurement of ECAR in the presence of exogenous pyruvate, which bypasses glycolysis, showed that the ECAR of mFamin+/− macrophages was indistinguishable from that of mFamin+/+ macrophages (Supplementary Fig. 5b). Together these data demonstrated that FAMIN determined the baseline and maximal glycolytic capacity of macrophages. Notably, the intracellular concentration of citrate, which itself inhibits glycolysis at the level of phosphofructokinase21, was substantially higher in mFamin+/− macrophages differentiated under M1 conditions than in their mFamin+/+ counterparts (Fig. 2l). The abundance of Krebs-cycle intermediates other than citrate in mFamin+/− M1 and M2 macrophages remained indistinguishable from that in their mFamin+/+ counterparts (Supplementary Fig. 5c), which indicated an absence of direct effects of FAMIN on Krebs-cycle activity. Collectively, these results indicated that the impairment in glycolysis might have been a consequence of defective DNL.

**Promotion of mitochondrial FAO by FAMIN**

Since we had observed that the extent of carbon flux through DNL into acyl carnitine was determined by FAMIN, we hypothesized that FAMIN might control mitochondrial FAO. As expected, mFamin+/+ M2 macrophages had high basal oxidative phosphorylation, as reflected by their high oxygen-consumption rate (OCR), whereas M1 macrophages had a low OCR that was refractory to treatment with oligomycin and 2-deoxyglucose (Fig. 3a–h). Basal respiration (as OCR) of mFamin−/− M1 and M2 macrophages remained indistinguishable from that in their mFamin+/+ counterparts (Supplementary Fig. 5c), which indicated an absence of direct effects of FAMIN on Krebs-cycle activity. Collectively, these results indicated that the impairment in glycolysis might have been a consequence of defective DNL.
Given our finding that FAMIN controlled the flux of carbon from glucose into acyl-CoA, we hypothesized that FAMIN might facilitate the conversion of fatty acids into acyl-CoA. Thus, we examined how FAMIN might be involved in the oxidative metabolism of endogenously synthesized lipids. Mice deficient in FAMIN had defective FAO of endogenously synthesized lipids. Deletion of FAMIN decreased FAO in M2 macrophages, while silencing FAMIN for 1 hour reduced the flux of carbon from glucose into acyl-CoA.

The concentration of cellular phosphocreatine, a rapidly mobilizable energy store, was similarly lower in mFamin−/− M1 and M2 macrophages than in their wild-type counterparts (a decrease of 30% ± 12% and 26% ± 6% (mean ± s.e.m.), respectively; Fig. 4f). Transmission electron microscopy demonstrated morphological changes in the mitochondria of mFamin−/− macrophages, characterized by elongation and narrowing (consistent with adaptation in response to ATP depletion). The mitochondria of mFamin−/− macrophages were smaller and more abundant than those of the wild-type macrophages. Moreover, the mitochondrial ROS (mROS) was lower in mFamin−/− M1 and M2 macrophages than in their wild-type counterparts (Fig. 4g), indicating that FAMIN maintained mitochondrial integrity.

In summary, FAMIN is essential for the oxidative metabolism of endogenously synthesized lipids. Its deletion leads to decreased FAO and mitochondrial dysfunction, which in turn affects cellular energy stores and ROS production. These findings highlight the importance of FAMIN in maintaining mitochondrial health and energy homeostasis in macrophages.
Figure 5  FAMIN-deficient macrophages have impaired mROS production and exhibit features of mitochondrial injury and remodeling. (a) Transmission electron microscopy of mFamin+/+ and mFamin−/− M1 and M2 macrophages; arrowheads indicate mitochondria. N, nucleus. Scale bars, 1 μm. (b) Mitochondrial length and width in cells as in a. (c,d) mROS in unstimulated mFamin+/+ and mFamin−/− M1 macrophages (c) and M2 macrophages (d) stained with a red fluorescent mitochondrial superoxide indicator; results are presented in relative fluorescence units (RFU). (e–i) eROS production in mFamin+/+ and mFamin−/− M1 macrophages left untreated or treated for 16 h with 20 μM C75 (e), for 1 h with 40 μM etomoxir (f) or for 1 h with a scavenger of mitochondrial superoxide (500 μM mitoTEMPO) (g), or with control siRNA (g,i) or Cpt1a-specific siRNA (g) or Cybb-specific siRNA (siCybb) (i), all followed by stimulation with zymosan; results are presented as relative light units (RLU) (left) or as the area under the curve (AUC) (right, e,f,h). Each symbol (c,d) represents an individual mouse; small horizontal lines indicate the mean ± s.e.m. *P < 0.05 and **P < 0.01 (Mann–Whitney U-test) or unpaired, two-tailed Student’s t-test (c,f,h).

counterparts, as assessed by a red-fluorescent indicator of mitochondrial superoxide (Fig. 5c,d). The abundance of cytoplasmic ROS was also lower in mFamin−/− M1 macrophages than in mFamin+/+ M1 macrophages, as measured with the cytosolic ROS indicator CM-H2DCFDA, although this parameter did not differ between these genotypes in M2 macrophages (Supplementary Fig. 6a,b). Stimulation with the Toll-like receptor 2 agonist zymosan resulted in significantly less production of extracellular ROS (eROS) in mFamin+/− M1 and M2 macrophages than in their mFamin+/+ counterparts (Fig. 5e and Supplementary Fig. 6c). Inhibition of FASN with C75 or silencing of Fasn through the use of small interfering RNA approximately halved the production of eROS in mFamin+/+ macrophages polarized under M1 or M2 conditions and abrogated the mFamin-associated differences (Fig. 5e and Supplementary Fig. 6c–e). Inhibition of CPT1a with etomoxir diminished the zymosan-elicited generation of eROS in mFamin+/+ macrophages to levels observed in mFamin−/− cells, regardless of whether the latter were treated with etomoxir or not (Fig. 5f and Supplementary Fig. 6f). A similar pattern of eROS production was observed after silencing of Cpt1a (Fig. 5g and Supplementary Fig. 6g) and, notably, after scavenging of mROS (Fig. 5h and Supplementary Fig. 6h). As expected, the production of eROS was dependent on the gp91phox NADPH oxidase25, since silencing the gene encoding gp91phox (Cybb) largely ablished the mFamin-genotype-related differences (Fig. 5i and Supplementary Fig. 6i). Notably, mFamin−/− macrophages exhibited diminished generation of eROS relative to that of mFamin+/+ macrophages despite greater availability of NADPH in mFamin−/− macrophages than in mFamin+/+ macrophages (Supplementary Fig. 6j). In contrast to results obtained for macrophages, the production of eROS elicited by the phorbol ester PMA in mouse neutrophils was independent of FAMIN (Supplementary Fig. 6k), consistent with absence of mFamin expression in mouse neutrophils6 (data not shown). In summary, FAMIN controlled mROS production via a DNL- and FAO-dependent mechanism and mROS, in turn, determined the capacity of the NADPH oxidase system to elicite eROS in macrophages.

We next investigated whether the metabolic mechanisms elucidated above were affected by genetic variation in mFamin. Wild-type C57BL/6N mice have the human ‘risk’ nucleotide guanine that encodes valine at the corresponding amino acid position 254 (called mFamin\(^{254V}\)) here. Using the CRISPR-Cas9 gene-editing system, we introduced single-nucleotide exchanges and generated mice homozygous for the nucleotide (adenosine) that is protective against leprosy and CD and that encodes isoleucine at amino acid position 254 (mFamin\(^{254I}\)) \(^{28}\) (Supplementary Fig. 3). We also generated mice homozygous for the nucleotide (cytosine) at position g.43,883,879 that encodes arginine at amino acid position 284 (the rare mFamin\(^{284R}\) variant linked to monogenic sJIA and early-onset CD in consanguineous families) instead of the highly conserved cytosine at that position (mFamin\(^{284C}\)) (Supplementary Fig. 3). Metabolic flux was equivalent in mFamin\(^{284C}\) macrophages and mFamin\(^{–/–}\) macrophages, while mFamin\(^{254I}\) macrophages exhibited the highest ECAR, basal OCR and MU-OCR, and mFamin\(^{254V}\) macrophages had intermediate levels of these (Fig. 6a–d). Consistent with such diminished function and loss of function for FAMIN(p.I254V) and FAMIN(p.C284R), respectively, zymosan elicited greater production of eROS from mFamin\(^{254I}\) macrophages than from mFamin\(^{254V}\) cells, while eROS production in mFamin\(^{284R}\) was diminished similar to its decrease in mFamin\(^{–/–}\) macrophages (Fig. 6e and Supplementary Fig. 7a). The genotype-related differences between mFamin\(^{254I}\) macrophages and mFamin\(^{254V}\) macrophages in zymosan-elicited production of eROS were abolished by the inhibition of FASN with C75 (Fig. 6f and Supplementary Fig. 7b). Overexpression of human FAMIN(p.254I) under control of a cytomegalovirus promoter in HEK293T cells yielded FAMIN expression indistinguishable from that of cells transfected with a construct encoding FAMIN(p.254V) (Fig. 6g), while expression of FAMIN(p.284R) was low and FAMIN(p.284R) localized together with calreticulin (Fig. 6h,i), indicative of retention in the endoplasmic reticulum. Together our results demonstrated that the substitution p.C284R...
caused complete loss of FAMIN function, while the single methyl
group change in p.1254V resulted in diminished FAMIN function.

We next obtained peripheral blood from healthy humans
homozygous for the haplotype linked to greater risk of CD and
leprosy (rs3764147G/G) and the haplotype that is protective against
leprosy and CD (rs3764147A/A). We isolated primary neutrophils
and monocytes from the donors and differentiated the latter,
produced eROS than that of rs3764147 A/A M2 macrophages
in supernatants of M1 macrophages given no pretreatment or pre-treated for 2 h with 100 µM etomoxir (horizontal axis) and stimulated with zymosan (200 µg/ml). (g-i) Concentration of TNF (g),
CXCL1 (h), and IL-6 (i) in supernatants of M1 macrophages pre-treated with etomoxir or not as in f (horizontal axes) and stimulated with LPS (1 µg/ml). *P < 0.05 and **P < 0.01 (one-way analysis of variance with post-hoc Bonferroni’s test (a,b) or unpaired, two-tailed Student’s t-Test (c-f,i)). Data are from one experiment with three mice representative of three independent experiments (a-d,i); mean ± s.e.m. in a and mean ± s.e.m. in f-i or are from one experiment with six independent samples per group (b,c; mean ± s.e.m.).

Control of bactericidal and inflammasome activity by FAMIN

Finally, we turned our attention to the consequences of the profound
metabolic derangement described above on immunological function.
mROS can directly augment the bactericidal activity of macrophages
directed against intracellular pathogens39. mFamin+/− M0 macrophages
expressed significantly less intracellular killing of a luminescent
Mycobacterium bovis bacillus Calmette-Guérin (BCG) strain30
than that of mFamin+/− M0 macrophages (Fig. 7a). Silencing FAMIN
in human macrophages resulted in diminished bactericidal activity
toward luminescent M. bovis BCG (Fig. 7b), similar to results obtained
for mouse macrophages. Finally, more Salmonella enterica
serovar Typhimurium were recovered from infected mFamin−/−
M0 macrophages than from infected mFamin+/+ M0 macrophages
regardless of whether exogenous lipids were provided in serum or not
(Fig. 7c); these results collectively demonstrated that FAMIN deter-
mined the capacity of macrophages to kill intracellular pathogens.

mROS also have a critical role in the caspase-1-mediated matu-
ration of interleukin 1β (IL-1β) by the NLRP3 inflammasome31.
The inflammasome assembles on mitochondria-associated mem-
branes after triggering of cells by pathogen-associated molecular
patterns31. The activation of mFamin−/− M1 macrophages by the
NLRP3 inflammasome via treatment with lipopolysaccharide (LPS)
plus ATP resulted in lower levels of cleaved active (p20) caspase-1
than those in mFamin+/− cells activated similarly. Consequently, there
was barely detectable cleaved (p17) IL-1β in the lysates and super-
natants of mFamin−/− M1 macrophages, in contrast to the readily
detectable IL-1β in mFamin+/+ M1 macrophages (Fig. 7d). Together
these results demonstrated that FAMIN determined the capacity for
triggering the NLRP3 inflammasome in M1 macrophages for secretion
of the host innate danger signal IL-1β. Moreover, FAMIN-dependent
inflammasome activation required intact FAO, as inhibition of FAO...
Figure 8  FAMIN deficiency causes dysregulated response to endotoxin in vivo. (a–d) Concentration of IL-1β (a), TNF (b), CXCL1 (c) and IL-6 (d) in serum from mFamin+/+ and mFamin−/− mice 2 h after intraperitoneal injection of LPS (2 mg per kg body weight). (e,f) Clinical scores for sepsis severity (maximum score, 15) (e) and concentration of IL-1β in serum (f) of mFamin+/+ and mFamin−/− mice 3 h after intraperitoneal injection of LPS (10 mg per kg body weight). (g) Concentration of IL-1β in 5-week-old homozygous mFaminp254V−284C and their homozygous mFaminp254V−284C control littermates or homozygous mFaminp254V−284C mice and their homozygous mFaminp254V−284C control littermates (key) treated as in e.f. (h,i) Clinical scores of sepsis severity (as in e) in mFaminp254I−284C and mFaminp254V−284C littermates (h) or mFaminp254V−284C and mFaminp254I−284C littermates (i) treated as in e.f. Each symbol (a–d,g) represents an individual mouse; small horizontal lines indicate the mean (± s.e.m.). *P < 0.05 and **P < 0.01 (Unpaired, two-tailed Student’s t-test (a–d), or linear mixed model (e,h,i)). Data are from one experiment with four mice per group (a–d), three mice per group (e,f) or seven mice (mFaminp254V−284C), four mice (mFaminp254V−284C or mFaminp254V−284C) or five mice (mFaminp254V−284C) (g–i; mean ± s.e.m.).

with etomoxir in mFamin+/+ M1 macrophages stimulated with LPS plus ATP resulted in less secretion of cleaved IL-1βp17 (Fig. 7c), similar to results obtained for zymosan-stimulated M1 macrophages (Fig. 7f); no additive effect of the inhibition of FAO was observed for mFamin−/− M1 macrophages (Fig. 7e). A similar pattern of FAO dependence was observed in LPS-stimulated M1 macrophages for the cytokine TNF and the chemokine CXCL1, while IL-6 secretion remained unaffected (Fig. 7g–i). Notably, intraperitoneal injection of LPS (2 mg per kg body weight) resulted in lower concentrations of IL-1β, CXCL1 and TNF in serum from mFamin−/− mice than in that from mFamin+/+ mice, while the concentration of IL-6 remained unaffected (Fig. 8a–d). Notably, this pattern of LPS-induced regulation of serum cytokines is reminiscent of the inhibition of glycolysis in vivo with 2-deoxyglucose.11

After being stimulated with LPS or with LPS plus ATP in vitro, mononuclear cells from patients with sJIA exhibit much less secretion of IL-1β than do cells from healthy donors, although it has remained unclear whether this is cause or consequence of active disease or is related to therapeutics.32 Given that the rare SNP variant resulting in the substitution FAMIL(p.C284R) is the only firmly established genetic cause of sJIA so far,33 impaired induction of IL-1β upon stimulation with LPS and ATP in vitro would be consistent with our results obtained for mFamin−/− M1 macrophages. Nonetheless, blockade of IL-1 is therapeutic in the vast majority of patients with sJIA and leads to swift normalization of their fever, rash and acute-phase reactants.32,34–35. We therefore hypothesized that the profound impairment in ‘energetic reserves’ in the absence of FAMIN function (reflected by low ATP and phosphocreatine, mitochondrial injury, and diminished FAO and glycolysis) might limit an organism’s capacity to withstand serious injury. We injected a high dose of LPS (10 mg per kg body weight) intraperitoneally into mFamin+/+ and mFamin−/− mice and continuously assessed the mice for clinical signs of sepsis over a 3-hour period. mFamin+/+ mice had higher sepsis scores than those of mFamin+/− mice, and these findings were particularly apparent during the first 90 min of observation (Fig. 8e). Moreover, two of seven mFamin−/− mice also showed signs of epistaxis (consistent with a bleeding disorder), a feature not observed in mFamin+/− mice (data not shown). The higher sepsis scores of mFamin−/− mice were accompanied by a twofold higher concentration of IL-1β in serum from mFamin−/− mice than in that of mFamin+/+ mice at the conclusion of the experiment (Fig. 8f). In analysis of ‘SNP-mutant’ mice, treatment with LPS (at a dose of 10 mg per kg body weight) resulted in the highest concentration of IL-1β in serum from mFaminp254V−284C mice, an intermediate concentration in mFaminp254V−284C mice and the lowest concentration in mFaminp254V−284C mice (Fig. 8g). Correspondingly, mFaminp254V−284C mice had higher clinical sepsis scores than those of mFaminp254V−284C mice, and mFaminp254V−284C mice had higher clinical sepsis scores than those of mFaminp254I−284C mice (Fig. 8h,i). Collectively, these results demonstrated that in the absence of FAMIN function, a high dose of LPS led to catastrophic activation of IL-1β. We speculate that the impaired bioenergetic reserves of mFamin−/− mice might tip the balance of sepsis-associated inflammasome activation toward a pyroptotic, pro-death response, as has been reported in other contexts.36

DISCUSSION

At the level of the organism, FAO and glucose oxidation are reciprocal metabolic pathways referred to as ‘the Randle cycle’.37 Similarly, coupling of DNL and FAO is not energetically efficient in terms of ATP production but nonetheless are engaged in certain immune and non-immune cells.38–40. In macrophages, FAMIN promotes flux through DNL to concomitantly drive high levels of both FAO and glycolysis. FAMIN thereby profoundly determines the maximum bioenergetic capacity of macrophages. The closest orthologs of FAMIN belong to a new class of proteins (the DUF152 ‘domain of unknown function 152’ family), and those with a reported function are bacterial proteins that exhibit atypical laccase (phenol-oxidoreductase) activity.41,42. We purified recombinant eukaryotically expressed human FAMIL(p.254I) and FAMIL(p.254V) and did not observe any laccase activity toward a series of prototypical substrates (data not shown). At this stage, the precise biochemical mechanism whereby FAMIN enhances DNL-dependent FAO remains unclear.

Intriguingly, FAMIN-dependent FAO occurred even when it was uncoupled from ATP synthesis, as in M1 macrophages, and might be of greatest importance in this setting for supporting glycolysis. ‘Uncoupled’ FAO has been suggested to have a critical role in cancer
cells engaged in aerobic glycolysis. Moreover, inhibition of DNL at the level of the acetyl-CoA carboxylase ACC1 (the enzyme that synthesizes the malonyl-CoA required for FASN function) has been reported to impair glycolysis in helper T cells. Here we identified a critical role for FAO in M1 macrophages that was required for IL-1β activation and ROS production. Glycolysis in M1 macrophages is well known to be critically required for IL-1β production. In contrast, the importance of β-oxidation has received less attention, even though FAO, not glycolysis, has been reported to be the sole driver of bactericidal mROS production in Salmonella-infected macrophages. Moreover, FAMIN-dependent mROS production also determines the maximal capacity of the NADPH oxidase system, the main source of bactericidal ROS in M1 macrophages.

While metabolic pathways in immune cells are emerging as important determinants of immunological function, we found here that a core metabolic regulator in immune cells was affected by genetic variation that results in a predisposition for inflammatory diseases and infection. Patients with systemic JIA, a condition demonstrated to mycobacterial experiments; E.R.C. helped with metabolic-flux assays and ROS experimentation; and A.K. devised and coordinated the project and, together with M.Z.C. and G.D., and with contributions from all authors, designed experiments, interpreted data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.


ONLINE METHODS

Mice. Ear-biopsy genomic DNA was used for routine genotyping of all mice. Primer sequences are shown in Supplementary Table 3. C57BL/6N mice were bred and maintained in specific pathogen-free conditions at the Central Biomedical Services (CBS) facility, University of Cambridge and at the Wellcome Trust Sanger Institute. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted, and all were conducted with approval of the UK home office. mFamin+/-, mFamin284R and mFamin254I mice generated as described below littered at Mendelian ratios and developed normally and no spontaneous disease emerged under specific pathogen free (SPF) conditions. Six- to twelve-week-old mice that were age and sex matched as described in the relevant methods sections were used for all experiments unless otherwise stated.

mFamin+/- allele. mFamin+/- mice were generated by disruption of the mouse homolog (9030625A04Rik, Lacc1) of human C13orf31 (LACCI) by homologous recombination in C57BL/6N-A/a embryonic stem (ES) cells (ES cells (Supplementary Fig. 3) to generate mice on a pure C57BL/6NTac background (ES cell clone ID EDD0538_1_D06 (International Knockout Mouse Consortium))23.

CRISPR-Cas9 target sites and vector construction. CRISPR target sites were identified using the CRISPR design website (http://crispr.mit.edu/) as described28 and are shown in Supplementary Table 4. The strategy to generate mouse lines carrying the p.254I and p.284R FAMIN variants (corresponding residue numbers are identical for human C13orf31 and mouse 9030625A04Rik) is shown in Supplementary Figure 3. In brief, wild-type C57BL/6NTac mice carry p.254V (human ‘risk variant’) and p.284C. For introduction of the ‘non-risk’ p.254I allele, guide RNA (gRNA) ‘line7’ along ‘oligo 7’ or ‘oligo 7 wobble’ were used, the latter introducing two additional synonymous nucleotide changes (Supplementary Table 4 and Supplementary Fig. 3). The corresponding strategy for introduction of the ‘Mendelian variant’ p.284R with gRNA ‘line9 along ‘oligo 9’ or ‘oligo 9 wobble’ is shown in Supplementary Figure 3. Oligonucleotide tyrex2R (Supplementary Table 4) was co-injected with each of the four lines to target the Tyr gene to allow color-based selection. The selected gRNA sequences were cloned into a gRNA vector containing the gRNA backbone and a T7 promoter to facilitate RNA production (Bsal sites). Cas9 mRNA was produced using a previously described vector modified to contain a T7 promoter24. The integrity of all plasmids was confirmed by DNA sequencing. The targeting oligonucleotides were synthesized as desalted ssDNA oligonucleotides from Integrated DNA Technologies (IDT).

Cas9 and gRNA production. For Cas9 RNA production, the T7/Cas9 plasmid was linearized (EcoRI), purified with PCR purification kit (Qiagen) and in vitro transcribed using mMessage mMachine T7 Ultra kit (Life Technologies). For gRNA production, the plasmid was linearized (Dral), purified with PCR purification kit (Qiagen) and in vitro transcribed using the MEGAscript kit (Life Technologies). Both Cas9 mRNA and gRNA were purified using the MEGAclear kit (Life Technologies) and were eluted in RNase-free water. The quality of the RNA was analyzed using Agilent RNA 6000 Nano kit (Agilent Technologies, 2100 Bioanalyzer) and Qubit RNA BR assay kit (Thermo Fisher Scientific).

One-cell embryo injection. Twelve 4- to 5-week old C57BL/6NTac females were super-ovulated by intraperitoneal injection of 5 IU of pregnant mare’s serum (PMSG) at 12:00-13:00 h (on a 12 h light/dark cycle, on at 07:00/off at 19:00) followed 48 h later by an intraperitoneal injection of 5 IU human chorionic gonadotrophin (hCG) and were mated overnight with C57BL/6NTac stud males. The next morning the females were checked for the presence of a vaginal copulation plug as evidence of successful mating, and oviducts were dissected at approximately 21–22 h post hCG. Cumulus masses from these were released and treated with hyaluronidase, and the fertilized one-cell embryos were left in KSOM media ready for cytoplasmic injection, as previously described49. 50 ng/µl Cas9 mRNA, 25 ng/µl gRNA and 100 ng/µl oligonucleotide were mixed in RNase-free water and injected into the cytoplasm of fertilized one-cell embryos in FHM medium. One-cell injected embryos were transferred the same day by oviductal embryo transfer into 0.5 d post-coital pseudo-pregnant female B6C3F1 strain (CBA<br/>&<br;/J-C57BL/6F1–Jax F1) recipients, produced by estrus selection and overnight mating to vasectomized males49.

Cytoplasmic injection. Injections were performed using a new cytoplasmic injection technique. An injector using a positive balance pressure, such as an Eppendorf Femtotext, was connected to a microinjection tip filled with the CRISPR materials. The microinjection tip was steadily advanced toward the opposite side of the zygote, which was anchored by the holding pipette until the micropipette passed through the plasma membrane to break it. The pipette was then drawn back into the cytoplasm, the CRISPR mix was delivered and the micropipette was immediately withdrawn. A successful injection was indicated by visible movement in the cytoplasm.

SNP-mutant-mice breeding strategy. Illumina sequencing of the mFamin gene was used in founder and F1 generation mice, confirmed by Sanger sequencing, to assess for successful gene editing. Founder mice were crossed with wild-type C57BL/6NTac mice to generate mFamin254Ip254V and mFamin284Rp284C heterozygous mice, which were then intercrossed, and homozygous littermates were used for experiments. Mice generated with and without wobble bases in targeting oligonucleotides were maintained separately and were used in experiments alongside their littermate controls irrespective of the presence or absence of wobble bases. Offspring from several founders were used to populate genotype categories in experiments. Routine genotyping was performed via Sanger sequencing.

Genotype-selected human samples. Peripheral blood for human neutrophil and monocyte isolation was collected from healthy volunteers from a genotype-selectable biorepository of ~10,000 donors (Cambridge BioResource; http://www.cambridgebiobioresource.org.uk). Healthy age- and sex-matched donors identified as homozygous for the ‘risk haplotype’ (G/G) or ‘non-risk haplotype’ (A/A) at SNP rs3764147, encoding FAMIN(p.254V) and FAMIN(p.254I) respectively, were recruited to participate in the study. Nucleotide positions in text correspond to genome assembly GRCh38.p2. None of the volunteers had received corticosteroid or immunosuppressant therapy within 6 months of enrollment. Investigators were kept blinded to the genotype of samples until study completion, with all individual experimental procedures performed on ‘(blinded)’ genotype pairs. The study protocol has been reviewed and approved by the South Central Berkshire B Research Ethics Committee (15/SC/0068), and written informed consent was obtained from each subject.

Antibodies and reagents. The following antibodies were used for immunoblotting. Abcam: anti-PMP70 (1:1,000 dilution; ab34212), anti-FASN (1 µg/ml dilution; ab22759); Cell Signaling: anti-cytotoxic enzyme oxidase IV (1:250 dilution; 4850), anti-catalase (1:800 dilution; 12980), anti-centromere protein A (1:400 dilution; 2186), anti-calreticulin (1:200 dilution; 12238), anti-FAS (1:1,000 dilution; 13080); Santa Cruz Biotechnology: anti-C13orf31 (anti-FAMIN) (1:200 dilution; sc-374553; E7 and 1:500 dilution; sc-376231; E12), anti-caspase 1 p20 (1:250 dilution; sc-1218-R); R&D Systems: anti-IL-1B (1:250 dilution; AF-401-NA); Sigma: anti-β-actin (1:10,000 dilution; A5060). All antibodies used have validation profiles on either Antibodypedia or iDegreeBio.

The following reagents were used: M-CSF (Peprotech, 300–25), LPS (from Escherichia coli K12, InvivoGen, tlr-pekLps), human IFN-γ (Peprotech, 300–02), mouse IFN-γ (Peprotech, 315–05), human IL-4 (Peprotech, 200–04), mouse IL-4 (Peprotech, 214–14), ATP (Sigma-Aldrich, A2383), C75 (Sigma-Aldrich, C5490), tomatoxin (Sigma-Aldrich, E9065), MitoTEMPO (Sigma-Aldrich, SML0737), oligomycin (Sigma-Aldrich, O4876), FCCP (Sigma-Aldrich, C2920), rotenone (Sigma-Aldrich, R8875), antimycin A (Sigma-Aldrich, A6674), palmitate-BSA (Seahorse Bioscience, 102720–100), zymosan A (Sigma-Aldrich, Z4230), PMA (Sigma-Aldrich, P1585), HRP (Sigma-Aldrich, P8375), luminol (Sigma-Aldrich, A8511).

Mouse bone-marrow-derived macrophages and neutrophils. Bone marrow was flushed from mouse femurs and tibias, filtered through a 70 µm cell strainer and incubated in complete medium (RPMI-1640 containing 100 U/ml of penicillin-streptomycin, 1 mM HEPES pH 7.4) and 10% FBS. Bone-marrow-derived macrophages (BMDMs) were prepared by culturing cells for 6 d in the presence of 100 ng/ml of M-CSF. After harvesting and
re-seeding, macrophages were polarized for 16 h toward M1 or M2 with IFN-γ (50 ng/ml) plus LPS (20 ng/ml) or with IL-4 (20 ng/ml), respectively, and where indicated, in the presence or absence of C75 (20 µM) and etomoxir (100 µM). Bone-marrow neutrophils were prepared using a neutrophil isolation kit (Miltenyi Biotec) according to the manufacturer's instructions.

Human peripheral-blood-derived macrophages. For studies of human cells, blood received from healthy donors was used to isolate peripheral blood mononuclear cells and neutrophils by Lymphoprep (Axis-Shield) gradient centrifugation. Monocyte-derived macrophages were generated by resuspending cells in complete RPMI-1640 and plated on NuncIon surface tissue culture dishes (Nunc). After 2 h, non-adherent cells were discarded and the remaining monocytes cultured for 4 d in complete medium with 10% FBS and 40 ng/ml M-CSF. At day 4, macrophages were harvested and polarized into M1 and M2 as described for mouse macrophages. Neutrophils were concurrently isolated during Lymphoprep gradient centrifugation (Axis-Shield) by sedimentation of the red blood cell–neutrophil layer with 10% dextran, followed by hypotonic lysis of contaminating erythrocytes.

Cell lines. THP-1 and U937 cell lines were maintained in complete RPMI-1640 with 10% FBS. To polarize into M1 macrophages, cells were incubated overnight with 50 ng/ml IFN-γ plus 20 ng/ml LPS and for M2 macrophages, cells were treated with 1 µM of PMA for 6 h and then polarized with 50 ng/ml IL-4 and 50 ng/ml IL-13. HEK293 and HEK293T cells were maintained in DMEM with 10% FBS. All cell lines were purchase from ATCC, which guarantees cell-line authentication.

Plasmids. The gene encoding human FAMIN (C13orf31 (LACCI)); called 'FAMIN' below) was amplified from an IMAGE clone (MHS1010-7508636) using a forward primer containing a KpnI site followed by a Kozak sequence and start codon and reverse primer containing a BamHI site, excluding the and to introduce the p.284R mutation the following primers were used: Forward, 5′-AGACCTACATTCCAAATGACGCTGCTTTATGAGGAAAGG-3′; Reverse, 5′-TAAAGACTCATCATTCCAAATGACCTGCTTTATGAGGAAAGGAGGAG-AG-3′ and to introduce the p.284R mutation the following primers were used: Forward, 5′-CAAAAACTATCGGTATACGGTCTGCACCAAGAGCTGC-3′; Reverse, 5′-GACGACTCTTTGGTACGACCGTATACCGATATTGGTTTTG-3′. All constructs were sequence verified in both forward and reverse orientations.

Protein affinity-purification mass spectrometry. HEK293T cells were transfected with pEXPR-IBA103 vector only or plasmid constructs encoding an untagged FAMIN, N-terminal Strep-tag FAMIN or C-terminal Strep-tag FAMIN using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. 48 h following transfection, cells were washed twice in ice-cold PBS and harvested by gently scraping into 5 ml ice-cold lysis buffer containing 20 mM HEPES-KOH, pH 7.5, 150 mM KCl, 1% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture. Cells were incubated on ice for 10 min, then were homogenized by shearing 20 times through a 21-gauge needle. The protein lysate was centrifuged for 15 min at 4 °C at 3,000g and the protein supernatant was collected into a freshly chilled tube to remove cellular debris. 1 U/ml of avenin was added to block endogenous biotin and incubated on ice for 15 min and then re-centrifuged for a further 10 min at 4 °C. Supernatant containing Strep-tag labeled FAMIN protein was precipitated using a Strep-Tactin column (IBA Life Sciences). Strep-tagged FAMIN protein complexes were eluted using 2.5 mM desthiobiotin and eluted fractions were subjected to SDS-PAGE separation, followed by silver staining or immunoblot analysis. Gels were submitted to the Cambridge Centre for Proteomics, Department of Biochemistry and were excised manually. Proteins were reduced, carbamoyl methyalted, and then digested to peptides using trypsin on a MassPrepStation (Micromass). The resulting peptides were applied to liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) on a QToF (Micromass). Fragmentation data were used to search the National Center for Biotechnology Information database using the MASCOT search engine (http://www.matrixscience.com).

Lipid mass spectrometry. Day-6 macrophages were seeded at 5 × 10⁶ cells per 60 mm dish and polarized under M1 or M2 conditions. Cells were harvested, washed twice in ice-cold PBS and then flash-frozen in liquid nitrogen. For 1, 2-¹³C-glucose–tracing experiments, macrophages were polarized overnight and then cultured for 24 h in the presence of 10 mM 1,2-¹³C-glucose (Sigma). Cell pellets were washed twice with cold PBS and re-suspended in 1.5 ml methanol. After spiking with 40 µl lipid standards cocktail mix of 50 ng 17:0-FaCoA, 100 ng 17:0-FaCoA and 400 ng 17:0-FFA, 1.5 ml of LC-MS grade water and 3 ml chloroform were added in. The mixture was subjected to Folch extraction. After collection of the lower phase, the upper phase was re-extracted with 3 ml synthetic lower phase (chloroform/methanol/water at volume ratio of 2:1:1, using the lower phase for re-extraction of lipid). The lower phase from both extractions was combined and dried under vacuum at 20 °C with SpeedVac (Thermo) and re-dissolved in 100 µl chloroform. 7 µl were injected for LC/MS/MS analysis. A Thermo Orbitrap Elite system (Thermo Fisher) hyphenated with a five-channel online degasser, four-pump, column oven, and autosampler with cooler Shimadzu Prominance HPLC system (Shimadzu) was used for lipid analysis. In detail, lipid classes were separated on a normal-phase Cogent silica-C column (150 × 2.1 mm, 4 µm, 100 Å, MicoSolv Technology) with hexane/dichloromethane/chloroform/methanol/acetanilite/water/ethylamine solvent gradient based on the polarity of head group. High resolution (240k at m/z 400) accurate mass (with mass accuracy <5 ppm) were used for molecular species identification and quantification. The identity of lipid was further confirmed by reference to appropriate lipids standards. Orbitrap Elite mass spectrometer operation conditions were as follows. For positive ion analysis: heated ESI source in positive ESI mode; heater temperature, 325 °C; sheath gas flow rate (arb), 35; aux gas flow rate (arb), 5; sweep gas flow rate (arb), 0; 1 spray voltage, 3.5 kV; capillary temperature, 325 °C; and S-lens RF level, 60%. Orbitrap mass analyzer was operated as SIM scan mode with two events. Event 1: mass range, m/z 238–663; and mass resolution, 240 k at m/z 400. Event 2: mass range, m/z 663–1088; and mass resolution, 240 k at m/z 400. B. For negative ion analysis, heated ESI source in negative ESI mode; heater temperature, 325 °C; and S-lens RF level, 70%. Orbitrap mass analyzer was operated as SIM scan mode with two events. Event 1: mass range, m/z 218–628; and mass resolution, 240 k at m/z 400. Event 2: mass range, m/z 628–1038; and mass resolution, 240 k at m/z 400. All the solvents used for lipids extraction and LC/MS/MS analysis are LC-MS grade from FisherScientific.

Aqueous metabolites mass spectrometry. Metabolites were extracted from 5 × 10⁶ macrophages using methanol/chloroform. The organic and aqueous fractions were separated by the addition of water and chloroform. The aqueous layer was analyzed subsequently for metabolomics. Mass spectrometry was performed on an AB Sciex 5500 (Warrington) coupled to an Acquity ultra performance liquid chromatography (UPLC) system from Waters. For chromatography of Krebs-cycle intermediates, metabolites were separated on a BEH amide HILIC column (100 × 2.1 mm, 1.7 µm; Waters) in negative ion mode. After drying, the samples were analyzed on a HSS T3 column (100 × 2.1 mm, 1.7 µm; Waters). Cone voltage, collision energy and mass transitions were optimized for each metabolite for quantification. Data are presented in arbitrary units as the area ratio of metabolite peak relative to the internal standard.

ATP assay. ATP content in total cell lysates was measured using an ATP determination kit (Molecular probes, A20206). M1 and M2 macrophages were trypsinized after an overnight polarization and counted, and 2 × 10⁵ cells were lysed in 100 mM Tris pH 7.75, 4 mM EDTA and 1% Triton-X.
Protein concentration was determined by BCA (Pierce, 25225). Remaining lysates were boiled at 96 °C, spun down for 1 min at 1,000g and the assay was performed according to the manufacturer's protocol. The amount of ATP was determined from the standard curve and is presented as nM of ATP per mg of protein in the sample.

NADPH quantification. NADPH concentration was determined using the NADP/NADPH Assay kit (Abcam, ab65349) according to the manufacturer's protocol. 4 × 10^5 macrophages were harvested in extraction buffer and filtered through a 10-kDa Spin column (Abcam, ab93349). The NADPH cycling reaction was performed for 2 h, and the NADPH amount was determined from the standard curve and normalized to protein concentration.

Oxygen-consumption rate and extracellular acidification rate. Macrophages were plated in XF-96 cell culture plates (7.5 × 10^5 cells/well) and polarized to M1 and M2 in the presence or absence of 20 µM C75 for 16 h. Macrophages were then washed and incubated for 1 h in XF assay medium (unbuffered DMEM pH 7.4 with 10 mM glucose and 2 mM L-glutamine, with or without 2 mM sodium pyruvate) in a non-CO_2 incubator at 37 °C as per manufacturer's instructions (Seahorse Bioscience). Real-time measurements of macrophage extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were performed using an XF-96 Extracellular Flux Analyzer (Seahorse Bioscience). Three or more consecutive measurements were obtained under basal conditions and after the sequential addition of 1 mM oligomycin, to inhibit mitochondrial ATP synthase; 1.5 µM FCCP (fluoro-carbonyl cyanide phenylhydrazone), a protonophore that uncouples ATP synthesis from oxygen consumption by the electron-transport chain; and 100 nM rotenone plus 1 µM antimycin A, which inhibits the electron transport chain. SRC is calculated as the difference between basal OCR and maximal OCR after the addition of FCCP. To assess glycolysis, three or more consecutive ECAR measurements were obtained under basal conditions and after the sequential addition of 1 µM oligomycin, to elicit maximal glycolytic activity and 100 nM 2-DG (2-deoxyglucose) to inhibit glycolysis-dependent ECAR. Measurement of endogenous and exogenous fatty-acid oxidation (FAO) was performed according to the manufacturer's instructions (Seahorse Bioscience). In brief, macrophages were seeded and polarized toward M2 as described above. After 16 h, macrophages were cultured in substrate limited DMEM medium containing 0.5 mM glucose, 1 mM GlutaMAX, 0.5 mM carnitine and 1% FBS for 24 h. Macrophages were then washed and incubated for 1 h in FAO assay medium (111 mM NaCl, 4.7 mM KCl, 1.25 mM CaCl_2, 2 mM MgSO_4, 1.2 mM NaH_2PO_4) supplemented with 2.5 mM glucose, 0.5 mM carnitine, and 5 mM HEPES, pH 7.4 in the presence or absence of 40 µM etomoxir in a non-CO_2 incubator at 37 °C. 1 mM palmitate conjugated to 0.17 mM BSA or BSA control was added to respective wells immediately before OCR measurement on XF-96 analyzer (Seahorse Bioscience).

ROS measurement. For mitochondrial and intracellular ROS measurements, macrophages were seeded at 1 × 10^5 cells per well in a 96-well plate and polarized toward M1 and M2 as described above. Macrophages were then washed with warm PBS and incubated at 37 °C with 5 µM MitoSox (Invitrogen), mitochondrial superoxide indicator, or 10 µM CM-H2DCFDA (Invitrogen), cytosolic ROS indicator, in serum-free phenol red-free RPMI medium (111 mM NaCl, 4.7 mM KCl, 1.25 mM CaCl_2, 2 mM MgSO_4, 0.5 mM NaH_2PO_4) with protease (Thermo Fisher Scientific, 78407) and phosphatase inhibitors (Thermo Fisher Scientific, 78426). Protein concentration was determined by BCA assay (Thermo Fisher Scientific, 23225) and equal amounts of proteins loaded onto SDS polyacrylamide gels. The proteins were transferred onto nitrocellulose membrane using a Trans-Blot Turbo transfer system (Bio-Rad). Membranes were incubated overnight with primary antibody, followed by corresponding secondary antibody, and the membranes were developed using 20X LumiGLO Reagent (Cell Signaling, 7003S).

Immunoprecipitation. Cells were washed three times with ice-cold PBS and then directly lysed with Tris-Triton buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS) with protease (Thermo Fisher Scientific, 78410) and phosphatase inhibitors (Thermo Fisher Scientific, 78426). Protein concentration was determined by BCA assay (Thermo Fisher Scientific, 23225) and equal amounts of proteins loaded onto SDS polyacrylamide gels. The proteins were transferred onto nitrocellulose membrane using a Trans-Blot Turbo transfer system (Bio-Rad). Membranes were incubated overnight with primary antibody, followed by corresponding secondary antibody, and the membranes were developed using 20X LumiGLO Reagent (Cell Signaling, 7003S).

Pretreatment of IL-1β from supernatants. Equal volumes of supernatants were precipitated by adding the TCA-DOC method. 1/100 of 2% sodium deoxycholate was added to one volume of the supernatant, vortexed and left for 30 min at 4 °C. Next, 1/10 of trichloroacetic acid was added, then samples were vortexed and left overnight at 4 °C. Samples were then spun down for 15 min at 4 °C at 15,000g. Pellets were washed once with one volume of ice cold acetone, air dried, resuspended in 2x loading buffer (4% SDS, 120 mM Tris HCl pH 6.8, 10% glycerol, 100 mM DTT and 100 µg of bromphenol blue), boiled for 5 min and separated by SDS-PAGE for further analysis.
**Silver staining.** Polyacrylamide gels were fixed for 1 h at 20 °C in 40% ethanol with 10% acetic acid. Gels were washed overnight with distilled water and then sensitized with 0.02% sodium thiosulphate for 1 min, washed and then stained for 20 min at 4 °C with 0.1% silver nitrate with 0.02% formaldehyde. Gels were developed with 3% sodium carbonate with 0.05% formaldehyde and staining was terminated with 5% acetic acid for 5 min.

**Immunofluorescence staining.** Cells were seeded on glass coverslips and differentiated overnight as required. Cells were fixed with 4% paraformaldehyde containing either 0.2% or 1% Triton X-100 for 15 min at 20 °C or 100% methanol at −20 °C for 15 min. Fixed cells were washed with PBS and non-specific binding was blocked with either 5% normal serum or 0.5% milk before incubation with primary antibodies for 1 h at 20 °C. Unbound antibody was removed by washing with PBS and secondary antibody was bound for 30 min at 20 °C. Coverslips were then mounted with either Prolong or Mowiol mountant containing DAPI. Fluorescence was visualized with a Zeiss LSM 510 or a DeltaVision Spectris Deconvolution Microscope.

**Proximity-ligation assay.** Cells were fixed and stained with primary antibody as described above for immunofluorescence. Proximity-ligation assay (PLA) was performed as per the manufacturer’s instructions (Duolink In situ, Olink Bioscience). In brief, unbound antibody was removed by washing twice for 5 min with 1× wash buffer A and PLA probes were bound for 1 h at 37 °C. Unbound PLA probes were removed by washing twice for 5 min with 1× wash buffer A. Coverslips were incubated with Ligation-Ligase solution for 30 min at 37 °C. Ligation-Ligase solution was removed by washing twice for 2 min with 1× wash buffer A and coverslips were incubated with Amplification-Polymerase solution for 100 min at 37 °C. Amplification-Polymerase solution was removed first by washing twice for 10 min with 1× wash buffer B and second by washing 1 min with 0.01% wash buffer B. Coverslips were then mounted and fluorescence was visualized as described for immunofluorescence staining.

**Oil Red O staining.** Macrophages were seeded onto glass coverslips and allowed to differentiate overnight. Cells were washed twice with PBS and then incubated for 3 min in 100% propylene glycol (VWR) before staining with Oil Red O solution (0.5% Oil Red O [Amresco] in propylene glycol) for 10 min at 20 °C. Excess stain was removed by washing with 85% propylene glycol and washing with distilled water. Cells were counterstained with hematoxylin and mounted with Kaiser’s glycerol gelatine (Merck Millipore).

**Transmission electron microscopy.** Cells were fixed in 4% glutaraldehyde in 0.1 M HEPES (pH 7.4) for 12 h at 4 °C, washed with 0.1M HEPES 5 times and then treated with 1% osmium ferricyanide at 20 °C for 2 h. Cells were rinsed in water five times and treated with 2% uranyl acetate in 0.05 M maleate buffer (pH 5.5) for 2 h at 20 °C, rinsed and dehydrated in an ascending series of ethanol solutions (70% to 100%) and then were treated with two changes of dry acetonitrile before infiltration with Quetol epoxy resin. Images were taken in an FEI Tecnai G2 operated at 120 Kv using an AMT XR60B digital camera running Deben software.

**Quantitative real-time PCR.** Total RNA was isolated from cultured cells and tissues using RNAasy Mini Kit (QIAGEN) according to the manufacturer’s instructions. For RT-PCR, cDNA was synthesized using oligo(dT) primers and M-MLV reverse transcriptase followed by quantitative PCR analysis. The primers are shown in Supplementary Table 5.

**Statistical analysis.** Statistical significance was calculated as appropriate using an unpaired, two-tailed Student’s t-test, Mann-Whitney U-test or analysis of variance and Bonferroni’s test and Grubb’s test for outlier exclusion, as described in the figure legends. Data is represented as mean and s.e.m. (s.e.m.). A P value of <0.05 was considered significant. Data were analyzed with Graphpad Prism software (version 6).