Oxidative phosphorylation selectively orchestrates tissue macrophage homeostasis

Graphical abstract

Highlights
- Tissue macrophages differentially use and need OXPHOS in the steady state
- OXPHOS dysfunction causes lipid accumulation and ER stress in specific macrophages
- Tissue macrophages require OXPHOS for fatty acid oxidation and cholesterol efflux
- OXPHOS loss impairs inflammatory adipose tissue macrophages and ameliorates obesity

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In brief
Wculek et al. uncover OXPHOS metabolism as a distinguishing feature among tissue macrophages in different organs in homeostasis. OXPHOS metabolism is required for maintenance of macrophage populations with high lipid- and cholesterol-handling activity. Thus, OXPHOS dysfunction impairs pro-inflammatory adipose tissue macrophages in obesity, improving metabolic syndrome and hepatosteatosis.
Oxidative phosphorylation selectively orchestrates tissue macrophage homeostasis

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SUMMARY

In vitro studies have associated oxidative phosphorylation (OXPHOS) with anti-inflammatory macrophages, whereas pro-inflammatory macrophages rely on glycolysis. However, the metabolic needs of macrophages in tissues (TMFs) to fulfill their homeostatic activities are incompletely understood. Here, we identified OXPHOS as the highest discriminating process among TMFs from different organs in homeostasis by analysis of RNA-seq data in both humans and mice. Impairing OXPHOS in TMFs via \( \text{Tfam} \) deletion differentially affected TMF populations. \( \text{Tfam} \) deletion resulted in reduction of alveolar macrophages (AMs) due to impaired lipid-handling capacity, leading to increased cholesterol content and cellular stress, causing cell-cycle arrest \( \text{in vivo} \). In obesity, \( \text{Tfam} \) depletion selectively ablated pro-inflammatory lipid-handling white adipose tissue macrophages (WAT-MFs), thus preventing insulin resistance and hepatosteatosis. Hence, OXPHOS, rather than glycolysis, distinguishes TMF populations and is critical for the maintenance of TMFs with a high lipid-handling activity, including pro-inflammatory WAT-MFs. This could provide a selective therapeutic targeting tool.

INTRODUCTION

Macrophages originate from embryonic progenitors or incoming monocytes and colonize the majority of organs in the body. Those macrophages in tissues (TMFs) not only contribute to innate immunity but perform distinct activities to maintain local and systemic homeostasis in the complex environment of tissues.1 For instance, alveolar macrophages (AMs) remove excess surfactant for optimal gas exchange in the lung, splenic red pulp macrophages (RPMs), and hepatic Kupffer cells (KCs) recycle iron from erythrocytes and facilitate lipid homeostasis, whereas lean white adipose tissue macrophages (WAT-MFs) aid adipogenesis and thermogenesis.1–4

The emerging field of immunometabolism is uncovering the relationship between metabolic features and functionality of immune cells, including macrophages. Cells adapt the use of metabolites derived from sugars, amino acids, or lipids for either anabolic or catabolic purposes, for energy production or synthesis of cellular components, respectively. The main bioenergetic processes in eukaryotic cells are lactate production following glycolysis in the cytosol and oxidative phosphorylation (OXPHOS) in the mitochondria. This mitochondrial respiration can be fueled by the degradation of various nutrients beyond glucose that enter the tricarboxylic acid (TCA) cycle, such as glutaminolysis or fatty acid oxidation (FAO).5 In vitro, exposure to specific stimuli induces metabolic reprogramming of bone marrow-derived macrophages (BMDMs) that underlies their activities. Enhanced glycolysis and a broken TCA cycle are associated with pro-inflammatory M1-like macrophage polarization, whereas anti-inflammatory M2-like macrophages are characterized by glutamine and FA-fueled mitochondrial respiration.6–8 However, TMFs are metabolically plastic cells that can distinctly tailor their cellular metabolism to facilitate their immune-promoting or resolving functions. For example, large peritoneal macrophages (LPMs) use a glutamate-driven mitochondrial metabolism for adequate microbial killing;9 however, AMs rely on glycolysis for proper induction of type 2 inflammatory responses,10 but not bacterial-induced inflammation,11 and the inflammation-resolving function of cardiac macrophages requires FA oxidation and mitochondrial respiration.12 Nevertheless, the specific metabolic requirements for maintenance of TMF
Moreover, non-infectious pathologic conditions can alter the tissue microenvironment and impact TMFs’ metabolism and function. For instance, hypertrophy of WAT upon overnutrition causes excess lipid-load and adipocyte death in obese individuals. This results in the adoption of an inflammatory state by a population of obese WAT-MFs that subsequently contribute to the development of metabolic syndrome, insulin resistance, and lipid accumulation in the liver. Notably, this pathologic functional change of obese WAT-MFs is accompanied by a profound metabolic remodeling and bioenergetic activation.17–20

Hence, understanding potentially distinct metabolic features of TMFs and the effect on their function can uncover specific vulnerabilities of disease-promoting TMFs.

Here, we uncovered OXPHOS as the biological pathway that most differs at the transcriptional level when comparing TMFs from different organs in homeostasis, both in human and mice. We revealed a distinct homeostatic function-dependent susceptibility of TMFs in 8 organs to genetic interference with mitochondrial respiration in mice. This vulnerability is determined by a high extracellular cholesterol and lipid-handling activity of specific TMF populations and could be rescued by ex vivo culture or simvastatin treatment in vivo. Pro-inflammatory lipid-exposed WAT-MFs in obesity also became sensitive to OXPHOS impairment, which ameliorated overnutrition-associated pathologies. Collectively, our study demonstrates the diversity of the energy metabolism of homeostatic TMF populations and uncovers a function-dependent metabolic vulnerability of selected TMFs that can be exploited for therapeutic purposes.

RESULTS

Expression of OXPHOS-related genes is a main distinctive characteristic of both human and mouse tissue macrophage populations in homeostasis

To identify differences among TMF populations from different organs in homeostasis, we interrogated publicly available single cell RNA sequencing (RNA-seq) data of human organs from the Human Cell Landscape database.21 First, pre-identified TMF clusters of the 10 organs containing sufficient macrophage numbers were aggregated by their origin to build pseudo-bulk samples. Then, we performed a principal component analysis (PCA) and found that TMFs from different organs clustered depending on their location (Figures 1A, 1B, and S1A–S1D). To

See also Figures S1 and S2.
identify which biological processes explained the differences in gene expression between TMFs that was detected by the PCA, we performed a gene set-enrichment analysis of the genes defining PC1 and PC2 loading vectors. This analysis identified OXPHOS-related pathways as the main source of variance that separates human TMFs among each other depending on their organ location (Figures 1C, 1D, and S1E–S1H). Next, we performed a similar PCA-based gene set-enrichment analysis on bulk RNA-seq data of 10 mouse TMF populations from healthy mice from the Immunological Genome Project.22 This analysis revealed a location-dependent clustering of TMFs and OXPHOS as a distinguishing biological process also when comparing mouse TMF populations located in different organs with each other (Figures 1E–1G and S2A–S2E). OXPHOS is the main catabolic and cellular energy-producing pathway in eukaryotic cells.5 The oxidation of nutrients in the TCA cycle in mitochondria allows electrons to enter the electron transport chain (ETC) via complex I or complex II and their transport through complex III and IV generates a proton gradient across the inner mitochondrial membrane. This membrane potential drives ATP generation by the ATP synthase as final step of mitochondrial respiration and OXPHOS. Mitochondria harbor their own genome that encodes several proteins of the ETC complexes.23–26 Notably, mitochondrially encoded (mt-) genes displayed the highest log-fold changes of OXPHOS-related genes among distinct mouse TMF populations (Figures 1H and S2E). This finding indicates that a distinct catabolic OXPHOS metabolism distinguishes TMFs in homeostasis from each other based on their organ location, in both mouse and human.

OXPHOS targeting differentially impacts tissue macrophages in the steady state

To study the relevance of mitochondrial respiration in TMF function in vivo, we deleted the mitochondrial transcription factor A (Tfam) in TMF populations by crossing Tfamf/f mice with ItgaxCretgaxCre (Itgax.Tfam) or Lyz2Cre (Lyz2.Tfam) mice. Tfam is nuclear-encoded and follows a similar expression pattern in mouse TMFs as mt-genes (Figure S2F). Tfam controls the replication and transcription of mt-DNA and mt-encoded genes that include components of complex I, III, IV, and V of the ETC. Hence, Tfam depletion simultaneously targets several ETC complexes that underlie functional OXPHOS and Tfam loss was previously shown to strongly reduce mitochondrial respiration in different immune cell subsets.24–28 By using distinct macrophage-targeting Cre drivers, we achieved Tfam mRNA deletion (Figure 2A) and consequently depletion of mt-DNA (Figure 2B), a functional readout for Tfam loss which underlies OXPHOS impairment, in several different TMFs. Notably, Tfam deletion largely reduced the numbers of AMs, LPMs, Langerhans cells (LCs), KCSs, and RPMs, whereas kidney macrophage (KM) or lean inguinal and epididymal WAT-MF (iWAT-MF and eWAT-MF) numbers were unaltered (Figures 2C and 2D). Reduction in numbers of the TMF populations significantly correlated with their expression of mt-genes (Figures 1H, 2C, and 2E), the latter being an indication for their mitochondrial activity. Moreover, TMFs that are more sensitive to OXPHOS interference also increased their autofluorescence (AF) (Figure 2F), which is often a sign of phenotypic alterations in macrophages. Overall, our data identify OXPHOS as a distinguishing feature between TMF populations with functional relevance for maintenance of specific TMF populations.

OXPHOS-impaired AMs undergo cell-cycle arrest and apoptosis despite normal amounts of ATP

To understand the reasoning behind the distinct use of OXPHOS by homeostatic TMFs in different organs, we further analyzed AMs, which are profoundly depleted upon OXPHOS interference (Figure 2C). Initial colonization of the lung by control Tfamf/f and Itgax.Tfam AMs at postnatal days 2 and 11 was comparable; however, Tfam-deficient AM numbers started declining 3 weeks after birth (Figures 3A and S3A). Phenotypic alterations of Itgax.Tfam AMs also appeared progressively, such as the deregulation of S (single) F and CD11b, indicative of impaired maturation, as well as AF, cell size, and forward and side scatter profiles (FSC and SSC) (Figures 3B, 3C, S3B, and S3C). A similar reduction in AM presence and maturity was confirmed in Lyz2.Tfam mice, an alternative Cre-line to target AMs, compared with their control littermates (Figure S3D). In line with the progressive reduction of AM numbers, Itgax.Tfam mice developed pulmonary alveolar proteinosis (PAP), that worsened over time. PAP was demonstrated by presence of protein, debris, dead cells, and immune cell infiltrates in the bronchoalveolar lavage (BAL) (Figures 3D and S3E). Notably, Tfam deficiency caused a decreased proliferation and an increased apoptosis of AMs, especially in 5-week-old mice (Figures 3E, 3F, and S3F), which explains the decline of the population. Itgax.Tfam AMs displayed decreased respiratory rates (Figures 3G and S3G), a slight reduction in mitochondrial content (Figure S3H) and a lower mitochondrial membrane potential (Figures 3I and S3I) compared with Tfamf/f littermates. Consistently, Tfam targeting in AMs showed a mild functional impairment of OXPHOS. This is likely explained by the fact that Tfam is the upstream factor that controls transcription of mt-encoded components of ETC complexes along with the accumulation of ETC complexes before Cre expression. Importantly, the partial effect in OXPHOS offered the advantage to study the modulation of OXPHOS activity in Itgax.Tfam AMs rather than a complete loss of mitochondria or their function, which could be more artefactual and aggressive. We next explored whether a bioenergetic deficiency could explain the limited maintenance of AMs upon Tfam loss. However, total ATP amounts were equal in Tfamf/f and Itgax.Tfam AMs (Figures 3I and S3J), despite lower production of mitochondrial ATP (Figure S3G). Notably, ATP concentrations of Tfam-deficient, but not control, AMs were sensitive to the glycolysis inhibitor 2-deoxy-D-glucose (Figure 3I). Tfam-deficient AMs also secreted more lactate (Figure 3J) and showed a higher extracellular acidification rate (ECAR) (Figure 3K). These results indicate a rewiring of the central carbon metabolism and bioenergetics toward lactic fermentation by Itgax.Tfam AMs to prevent an energetic crisis. Nevertheless, OXPHOS impairment upon Tfam deficiency causes apoptosis and reduces proliferation of AMs.

A deregulated TCA cycle associates with impaired numbers and phenotype of ETC-deficient AMs

To dissect the underlying bioenergetics-independent mechanisms causing the decline of the AM population upon OXPHOS interference, we performed an RNA-seq analysis of AMs from...
3-week-old ItgaxΔTfam and control mice as well as gas chromatography and mass spectrometry (GC-MS) metabolomics of adult AMs (Figures S4A–S4C). The transcriptomic analysis confirmed a reduction of OXPHOS and deregulated cell proliferation as well as apoptosis in Tfam-deficient AMs (Figures S4B and S4D–S4F). Metabolically, glucose and its derivatives were more abundant in ItgaxΔTfam than Tfamf/f AMs together with the upregulation of genes involved in glucose uptake and metabolism, such as Slc2a1 (Figures S4G–S4I), which is in line with enhanced glycolysis. Notably, we observed a profound deregulation of expression of genes regulating the TCA cycle and amino acid metabolism as well as enhanced concentrations of TCA cycle metabolites and glucogenic amino acids in Tfam-deficient AMs (Figures 3L, 3M, S4J, and S4K). Individual TCA cycle metabolites play distinct roles in cellular metabolism, for example, fumarate causes hypoxia-inducible factor 1α stabilization and reactive oxygen species (ROS) signaling.33 However, the global deregulation of numerous TCA cycle-associated enzymes and intermediates, as well as of TCA cycle-fueling metabolites such as amino acids, pointed toward a broad adaption of cellular catabolic metabolism in Tfam-deficient AMs. All catabolic processes in eukaryotic cells converge into the TCA cycle and its functioning is closely intertwined with mitochondrial OXPHOS. Complex III and IV are always required for the functioning of...
Figure 3. Impaired proliferation and increased apoptosis in Tfam-deficient AMs is not caused by a bioenergetic crisis but is linked to a TCA cycle deregulation

2-day-old, 11-day-old, 3-week-old, or adult (6–8 weeks old) Tfam<sup>f/f</sup> and Itgax<sup>f/f</sup> and Tfam<sup>f/f</sup> mice or their AMs in the lung were analyzed.

(A–C) Flow cytometric quantification of numbers (A, n = 4–9), relative median fluorescence intensity (MFI) of autofluorescence (AF), Siglec F or CD11b (B, n = 4–8) as well as representative plots of F4/80<sup>+</sup> CD11c<sup>+</sup> cells in the lungs of adult mice (C, gated on CD45+).

(D) BAL turbidity evaluated visually (photograph, top) and by optical density (OD, bottom) (n = 7–8).

(E and F) Flow cytometric quantification and representative plots of frequency of Ki67+ (E) or activated caspase 3+ cells (F) in CD11c+ cells in BAL of 3-week-old mice (n = 5–7).

(I) ATP concentration in F4/80<sup>+</sup> CD11c<sup>+</sup> cells from the lung of 3-week-old mice (n = 6–8).

(K) Extracellular acidification rate (ECAR) of CD11c<sup>+</sup> cells from BALs of adult mice (n = 3 merged from 5 to 10 mice).

(N and O) Flow cytometric quantification of numbers (N) and relative MFI of Siglec F (O, left) or CD11b (O, right) of CD45<sup>+</sup> F4/80<sup>+</sup> CD11c<sup>+</sup> cells in the lungs of adult Itgax<sup>f/f</sup>, Itgax<sup>ΔUqcrq</sup>, or Itgax<sup>ΔCox10</sup> mice compared with their control littermates (n = 4–8).

Data are merged from at least 2 independent experiments and presented as mean ± SEM. Dots represent individual data points. Statistical analysis by unpaired (A–H, J, and L–O) or paired (K) Student’s t test or one-way ANOVA with Tukey correction (I). *p < 0.05, **p < 0.01, ***p < 0.001.

See also Figures S3 and S4.

the ETC, while electrons may enter the ETC either via complex I or complex II. Importantly, the ETC complex II is also known as succinate dehydrogenase (SDH) and an enzyme within the TCA cycle, whereas complex I, III, and IV only participate in the ETC and OXPHOS. Nevertheless, a deficiency of complex III also deregulates the TCA cycle and the abundance of its metabolites. Hence, to understand the relevance of a TCA cycle deregulation for the maintenance of Tfam-deficient AMs, we individually deleted components of either complex I, II, III, or IV in those cells. First, Itgax<sup>ΔUqcrq</sup> (depleted of Uqcrq, mitochondrial
complex III gene) and Itgax−Cox10 (depleted of Cox10, mitochondrial complex IV gene) mice displayed a reduction in numbers of AMs and an altered AM phenotype compared with their control littersmates (Figures 3N and 3O), reminiscent of the phenotype of Itgax−Tfam mice (Figures 3A–3C). Moreover, Itgax−Sdhb (depleted of Sdhb, mitochondrial complex II gene) mice also exhibited reduced AM numbers and an impaired AM phenotype compared with their control littersmates, similar to complex III and IV deficiency (Figures 3N and 3O). Of note, AM numbers were comparable in Ndufs4−/− mice and their control littermates (Figures 3N and 3O), reminiscent of the phenotype of Sdhb−/− mice or their AMs in the lung were analyzed. These results indicate the importance of the mitochondrial ETC which potentially rescues this function of complex I. Altogether, these results indicate the importance of the mitochondrial ETC driven by complex II and, hence, the correct functioning of the TCA cycle for AM proliferation and survival.

Cellular stress and reduced proliferation in OXPHOS-impaired AMs depend on the tissue microenvironment

As a potential molecular mechanism for induction of cell cycle arrest and apoptosis,33,34 we detected induction of activating transcription factor 4 (Atf4) and corresponding cellular stress-related genes in Tfam-deficient AMs (Figures 4A and 4B). Atf4 is the effector of the integrated stress response (ISR) located downstream of the eukaryotic initiation factor (eIF) 2α. The ISR is induced upon different stress stimuli via activation of four distinct known kinases that phosphorylate eIF2α to re-establish cellular homeostasis.35 Double-stranded RNA-dependent protein kinase (PKR) and general control non-depressible protein (GCN) 2 were unlikely to be activated in Itgax−Tfam AMs. They usually respond to stresses related with viral infection or amino acid deprivation, whereas Tfam-deficient AMs were analyzed in homeostasis and harbored elevated amino acid concentrations compared with controls (Figure S4K). The PKR-like kinase (PERK)/eIF2α/Atf4 signaling pathway is generally induced upon endoplasmic reticulum (ER) stress and can regulate the immunosuppressive function of macrophages.36 However, Atf4 induces apoptosis in situations of prolonged ER stress via activation of C/EBP homologous protein.
Glycerophospholipids and cholesterol. The predominant function of AMs is the removal of the lipid-rich surfactant. The TCA cycle, which is deregulated in Tfam-deficient AMs, is vital for lipid and FA catabolism and a deregulation of cholesterol content is a well-known inducer of ER stress in Tfam-deficient AMs. Moreover, *Itgax*/*Tfam* AMs displayed enhanced expression of genes associated with mitochondrial stress compared with *Tfam*−/− AMs (Figure S5A), which can activate the ISR via heme-regulated eIF2α kinase (HRI). We also observed increased generation of ROS and signs of oxidative stress, such as activation of genes involved in glutathione synthesis and arachidonic acid expression and release (Figures S5B–SSD).

Given the tissue location-dependent effects of *Tfam* deletion in TfMs (Figure 2), we interrogated potential environmental-cell-extrinsic causes of cellular stress in *Itgax*/*Tfam* AMs. To this end, we harvested AMs from 3-week-old mice by BAL and cultured them for 48 h. Notably, ex vivo cultured *Itgax*/*Tfam* AMs normalized their *Atf4* expression to that of *Tfam*+/− AMs (Figure 4D). This rescue was accompanied by a recovery of the proliferative capacity and expression of cell cycle and apoptotic genes in Tfam-deficient AMs (Figures 4E, 4F, and S5E). This finding indicated an environmental extrinsic trigger of cellular stress that interferes with the maintenance of Tfam-deficient AMs. Oxidative or mitochondrial stress is unlikely to solely account for the activation of the ISR in *Itgax*/*Tfam* AMs because this type of cellular stress is most probably a result of the genetic interference with mitochondrial respiration upon Tfam loss and therefore cell intrinsic. Notably, culturing of AMs under presence of PERK inhibition already ameliorated the *Atf4* expression in *Itgax*/*Tfam* AMs after only 6 h of culture (Figure S5F). This PERK blockade-mediated acceleration of the ISR reduction upon ex vivo culture suggests the involvement of ER stress in the induction of *Atf4* in Tfam-deficient AMs in vivo, likely additional to Tfam loss-induced mitochondrial stress. In conclusion, the tissue microenvironment is inducing the cellular and ER stress in *Itgax*/*Tfam* AMs that, in turn, causes their impaired proliferation.

**Altered extracellular lipid- and cholesterol-handling ability causes the loss of Tfam-deficient AMs in vivo**

In homeostasis, most AMs are located in the lung alveoli and exposed to pulmonary surfactant, that is mainly composed of glycerophospholipids and cholesterol. The predominant function of AMs is the removal of the lipid-rich surfactant. The TCA cycle, which is deregulated in Tfam-deficient AMs (Figures 3L and 3M), is vital for lipid and FA catabolism and a deregulation of cholesterol content is a well-known inducer of ER stress in macrophages. Hence, we hypothesized that a deregulated lipid-handling capacity causes the reduced proliferation and enhanced apoptosis in OXPHOS-impaired AMs. Indeed, *Itgax*/*Tfam* AMs accumulated more intracellular lipids compared with *Tfam*−/− AMs (Figures 5A and S6A). By transmission electron microscopy, we observed large spiral-like structures in Tfam-deficient AMs, which were absent from controls (Figure 5B). Those structures were highly reminiscent of pulmonary surfac-
Elevated intracellular cholesterol amounts can cause cellular and/or ER stress and Atf4 activation in macrophages. To understand if lipids, mainly cholesterol, are the extracellular factors driving the progressive decline of Tfam-deficient AMs, we aimed to reduce the abundance of those lipids in vivo. Simvastatin is a clinically approved cholesterol synthesis inhibitor that reduces cholesterol concentration in BAL as well as in AMs of mice with PAP and improves the disease upon systemic administration. Treatment with simvastatin mildly decreased systemic cholesterol and triglycerides in serum and total cholesterol content in BAL of Itgax−/−Tfam−/− mice without ameliorating other readouts of PAP, such as BAL turbidity or protein content (Figures S5H, S6I, and S6J). Nevertheless, simvastatin treatment significantly increased the numbers of Tfam-deficient AMs in the lung and enhanced their proliferation (Figures 5I and S6K). Although we cannot exclude a contribution of additional mechanisms, those findings indicate that the inability of processing of extracellular lipids by Tfam-deficient AMs causes enhanced intracellular concentrations of cholesterol and other lipids. This induces cellular and ER stress and Atf4 activation in AMs and culminates in their apoptosis, reduced proliferation, and progressive loss of the population in vivo.

High cholesterol-handling activity determines the vulnerability of tissue macrophages to OXPHOS impairment

Next, we hypothesized that a similar mechanism could affect other TMF populations that are vulnerable to OXPHOS interference. To address this, we investigated lipid-handling and cellular stress in selected TMFs whose presence is reduced upon Tfam deletion (Figure 2C). Similar to AMs, numbers of LCs and RPMs were decreased by impairment of ETC complex II, III, and IV, whereas they remained unaltered by complex I dysfunction compared with control littersmates (Figure 6A). ATP content was unchanged in Tfam-deficient RPMs and LPMs, and both accumulated more intracellular lipids and increased the expression of Atf4 (Figures 6B–6D). Notably, simvastatin treatment also enhanced the presence of RPMs in Itgax−/−Tfam−/− mice (Figure 6E). Moreover, the expression of OXPHOS-related genes not only significantly correlated with genes involved in lipid and FA catabolism, but especially genes controlling cholesterol handling processes in human and mouse TMFs (Figures 6F and 6G). Overall, our data suggests that OXPHOS dysfunction selectively affects TMFs with a high lipid and cholesterol-processing activity by impairing their ability to oxidize and efflux extracellular lipids. This results in
cholesterol accumulation that drives cellular and ER stress, cell cycle arrest and apoptosis of lipid-handling TMFs (Figure 6H).

**OxPHOS impairment depletes pro-inflammatory, but not anti-inflammatory, eWAT-MFs and ameliorates obesity-related pathologies**

We hypothesized that the alteration of the tissue microenvironment toward an increased lipid content could enhance the vulnerability of TMFs to OxPHOS interference. We found that lean WAT-MF populations, which are metabolically quiescent, are largely undisturbed by Tfat deletion in homeostasis (Figure 2). However, during overnutrition-induced obesity, eWAT hypertrophy causes adipocyte death and the release of lipids into the microenvironment. This triggers recruitment of monocyte-derived eWAT-MFs, that also proliferate locally, form crown-like structures (CLSs) around dying adipocytes, become pro-inflammatory and participate in handling those excess extracellular lipids. Hence, we reasoned that OxPHOS impairment could selectively affect the maintenance and proliferation of those pro-inflammatory eWAT-MFs during obesity and fed Tfat and Lyz2ΔTfat mice with a high fat diet (HFD). Notably, HFD-fed Lyz2ΔTfat mice gained less fat...
mass than Tfam<sup>−/−</sup> animals, whereas their lean mass was unaltered (Figures 7A and S7A). The tibiae length, food and drink intake, energy expenditure, consumed O<sub>2</sub> volume, and respiratory exchange ratio of obese Lyz2<sub>−/−</sub>Tfam mice was similar to that of Tfam<sup>−/−</sup> controls; however, the released CO<sub>2</sub> volume was increased (Figures S7B–S7D). In line with our hypothesis, pro-inflammatory CD11c<sup>+</sup> and/or CD9<sup>+</sup> eWAT-MFs upregulated Atf4 expression and reduced their proliferation upon Tfam deficiency, which resulted in their reduced numbers (Figures 7B, 7C, and S7E). Notably, similar to lean WAT-MFs, the numbers, Atf4 expression and proliferation of anti-inflammatory CD206<sup>+</sup> and/or MerTK<sup>+</sup> eWAT-MFs remained unaltered in obese Lyz2<sub>−/−</sub>Tfam mice compared with Tfam<sup>−/−</sup> controls (Figures 7B, 7C, and S7E). The largely unaltered expression of genes associated with anti-inflammatory macrophage functions in eWAT (Figure S7F) supported this finding. This is likely due to a lower lipid burden of those anti-inflammatory compared with pro-inflammatory CD11c<sup>+</sup> and/or CD9<sup>+</sup> eWAT-MFs, as the latter predominates in CLS. Of note, the numbers and phenotype of circulating monocytes, which can give rise to eWAT-MFs in obesity, were unaffected in Lyz2<sub>−/−</sub>Tfam mice (Figure S7G). In accordance with decreased pro-inflammatory eWAT-MF presence, obese Lyz2<sub>−/−</sub>Tfam mice displayed a lower number of CLS and reduced inflammatory Tnfa and Ccl2 cytokine amounts in eWAT, but unaltered adipocyte size, as well as lower systemic leukocyte numbers in plasma compared with Tfam<sup>−/−</sup> mice (Figures 7D–7F).
and S7H). Moreover, the eWAT of HFD-fed Lyz2ΔTfam mice displayed an elevated expression of genes involved in lipid catabolism, lipolysis, ketogenesis, and mitochondrial respiration than that of Tfamf/f mice, but not lipogenesis (Figures 7G and 7H). This indicates a more metabolically active state and a higher lipid oxidation in the eWAT,52 which appears independent of eosinophil-mediated alternative macrophage activation and adipose tissue b impeig (Figures S7F, S7I, and S7J).53 HFD-fed Lyz2ΔTfam mice also showed higher glucose tolerance and insulin sensitivity compared with Tfamf/f mice (Figures 7I and S7K).

Low-density lipoprotein (LDL) and alanine aminotransferase (ALT) amounts in plasma, liver weight, and hepatic lipid accumulation were also notably reduced in obese Lyz2ΔTfam vs. Tfamf/f mice (Figures 7J and S7L–S7O).

To corroborate those data, we next analyzed obese ItgaxΔUqcrq mice, where ETC complex III and thereby OXPHOS dysfunction is limited to pro-inflammatory eWAT-MFs expressing CD11c. HFD-fed ItgaxΔUqcrq mice gained less weight and harbored lower pro-inflammatory eWAT-MF numbers than Uqcrq−/− mice (Figures 7K and S7P–S7T). Their cholesterol and ALT amounts in plasma as well as their hepatic lipid content was also reduced (Figures S7S–S7U). Notably, HFD-fed ItgaxΔUqcrq mice displayed elevated brown adipose tissue (BAT) thermogenesis (Figure 7L). Together with the higher expression of lipid catabolism–related genes in eWAT and enhanced released CO2 volume of HFD-fed Lyz2ΔTfam vs. Tfamf/f mice (Figures 7G, 7H, and S7C), this observation suggests that a decrease of pro-inflammatory eWAT-MFs due to OXPHOS interference in obesity causes improved eWAT functionality, removal of excess lipids by oxidation, and heat production via the BAT.92,94 In summary, OXPHOS dysfunction specifically reduces pro-inflammatory TMFs in obese eWAT, which handle high amounts of lipids, by inducing cellular stress and impairing their proliferation. This results in mitigation of inflammation, metabolic dysfunction, and hepatosteatosis.

**DISCUSSION**

Overall, we have uncovered a metabolic diversity in both human and mouse TMFs in vivo during homeostasis, and OXPHOS as the main distinguishing feature among TMFs located in different organs. The high contribution of OXPHOS to the bioenergetics of AMs and LPMs compared with BMDMs is established,2,9–11,55 and AMs do not enhance glycolysis upon lipopolysaccharide stimulation,11 whereas opposing effects of Tfam deletion in AMs are reported.28,56 Our study revealed OXPHOS as an important determinant for identity and maintenance of specific TMF populations in the steady state. We detected the induction of cellular and ER stress in affected Tfam-deficient TMFs, demonstrated by activation of Atf4, that likely accounts for their increased apoptosis and reduced proliferation. ATP amounts in OXPHOS-dependent Tfam-deficient TMFs were unaltered, but, at least in AMs, became dependent on glycolysis to fulfill their bioenergetic demands. However, an enhanced glycolytic metabolism per se in AMs does not phenotype the cellular stress and apoptosis caused by Tfam deletion.95 Regarding OXPHOS, the adoption of the usage of ETC complex I and II upon bacterial sensing regulates the functions of BMDMs57 and the complex I protein Ndufs4 controls inflammatory macrophage and osteo-

clast polarization.58 In contrast to complex II, III, and IV, complex I was the only ETC complex that is dispensable for the maintenance of Tfam deletion–sensitive TMFs. This points to an essential role of the correct functioning of the TCA cycle and a complex II–driven ETC for the activity and maintenance of AMs and other OXPHOS-dependent TMFs. The normalization of Atf4 expression and proliferation upon culture of Tfam-deficient AMs *ex vivo*, suggested a microenvironment–triggered effect that determines the vulnerability of certain TMFs to OXPHOS impairment during homeostasis.

Efferocytosis of dying cells by macrophages provides FAs and fuels mitochondrial respiration during tissue injury. However, this induces anti-inflammatory functions, not cellular stress and apoptosis, in cultured BMDMs or cardiac macrophages.12 Homoeostatic Tfam-deficient AMs did not accumulate free FAs, but larger lipid species reminiscent of extracellular surfactant and free cholesterol. An elevated lipid content, especially of cholesterol, is a potent cause of cellular stress and Atf4 activation in macrophages.33,34,43 Notably, we showed that a reduction of systemic lipid and cholesterol amounts in ItgaxΔTfam mice improves AM and RPM numbers. Our analysis reveals a significant correlation of OXPHOS and cholesterol handling–related gene expression across human and mouse TMF populations. Hence, we uncovered the need for a functional ETC and OXPHOS in TMFs for handling of extracellular lipids and cholesterol to maintain homeostasis. In line with this, the TMFs that are most affected by Tfam deficiency; AMs, LPMs, RPMs, and KCs; display a high activity and/or expression of genes required for lipid metabolism and handling.2,23 Moreover, the loss of peroxisome proliferator-activated receptor (PPAR) γ and/or LXRα and β, the master regulators of lipid catabolism and intracellular cholesterol handling, causes a comparable phenotype as Tfam deletion in AMs, RPMs, and/or KCs.42,59

Finally, we also revealed the relevance of OXPHOS for the maintenance of extracellular lipid-handling TMFs in the pathologic setting of overnutrition–induced obesity. In contrast to lean WAT, adipocytes die in obese eWAT and release lipids that change the microenvironment. eWAT-MFs become bioenergetically activated7,26 and form CLS around adipocytes to clear the released lipids. Those obese eWAT-MFs in CLS express CD11c and CD9, accumulate lipids and become pro-inflamma-

matory,2,14,19,51 Interference with increases in intracellular ROS can impair pro-inflammatory macrophage activation.60 Although we observed elevated ROS amounts at baseline in Tfam-deficient AMs, we cannot entirely exclude a potential contribution of alterations of ROS amounts to the reduction of pro-inflammatory Lyz2ΔTfam eWAT-MFs. Fgr kinase deletion in BM cells imbalances complex I/II usage by the ETC and alters the polarization of eWAT-MFs, decreasing pro-inflammatory and increasing anti-inflammatory macrophage presence.61 Notably, Tfam deficiency only diminished the numbers of pro-inflammatory, lipid-handling eWAT-MFs in obese WAT by specifically inducing cellular stress and decreasing their local proliferation, but not that of anti-inflammatory eWAT-MFs or lean WAT-MFs. Although we focused on analysis of embryo–derived TMFs in the steady state as they represent stable populations, this finding suggests a vital role of mitochondrial respiration, not glycolysis, for the maintenance of pro-inflammatory monocyte–derived macrophages in obesity. In line with this, cultured M1
BMDMs accumulate more cholesterol than M2 BMDMs and the lipid profile of obese C57B16 mice is distinct from that of other recruited WAT-MFs.62 Pro-inflammatory C57B16 WAT-MFs can induce an inflammatory phenotype in WAT19 and are important drivers of obesity-associated pathologies.15,16 Thus, we found that HFD-fed Lyz2ΔTfam mice show reduced signs of inflammation and are markedly protected from insulin resistance and hepatosteatosis.

In conclusion, we propose a selective function of OXPHOS for the maintenance of homeostatic TMFs with a high demand of lipid-handling activity. This metabolic dependency on mitochondrial respiration also affects pro-inflammatory TMF populations that promote pathologies. Hence, pharmacological interference with OXPHOS in such TMFs, for example, via specific targeting of mitochondrial inhibitors, holds promise for therapeutic exploration to ameliorate metabolic syndrome.

Limitations of the study
In this study, we report the significantly different expression of OXPHOS-related genes in TMF populations, depending on their homing organ. Moreover, we show a distinct vulnerability of TMFs to OXPHOS interference and reveal impaired lipid and cholesterol handling as underlying cause. To block OXPHOS for our mechanistic analysis, there are no entirely OXPHOS-related genes in TMF populations, depending on their requirements. Lipid-handling activity. This metabolic dependency on mitochondrial inhibitors, holds promise for therapeutic exploration to ameliorate metabolic syndrome.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.immuni.2023.01.011.

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We are grateful to N.-G. Larsson, F. Sánchez-Madrid, G. Sabio, R.D. Palmiter, E. Gottlieb, C.T. Moraes, and M.A. del Pozo for sharing essential reagents. We thank S. Iborra, his team, M. Sánchez-Alvarez, I. Nikolic, and members of the D.S. laboratory for discussions and critical reading of the manuscript. We thank the staff at the CNIC technical units; foremost the animal, cellomics, histology, metabolomics, genomics, microscopy, and bioinformatics facilities; and the Siddi of the Universidad Autonoma de Madrid for technical support. This project was supported by the “la Caixa” Foundation (ID 100010434) Postdoctoral Junior Leader Fellowship code LCF/BO/PR20/117/0008 (S.K.W.); “la Caixa” Foundation (ID 100010434) INPhINIT Fellowship code LCF/BO/I17/11620074 (I.H.-M.); Spanish Ministry of Education FPU fellowship code FPU20/01418 (M.G.); Ministerio de Ciencia e Innovacio´ n PID2019-104233RB-100/AEI/10.1309/ 501100011033 (S.L.); and NIH grants P01AG049665-08, RO1A148190, and P01HL154998 (N.S.C.). The J.A.E. laboratory is supported by the CNIC; by the “la Caixa” Foundation (ID 100010434) INPhINIT Fellowship code LCF/BO/I17/11620074 (I.H.-M.); Spanish Ministry of Education FPU fellowship code FPU20/01418 (M.G.); Ministerio de Ciencia e Innovacio´ n PID2019-108157RB-100/AEI/10.1309/ 501100011033 (S.L.); and NIH grants P01AG049665-08, RO1A148190, and P01HL154998 (N.S.C.). The J.A.E. laboratory is supported by the CNIC and a grant by Ministerio de Ciencia, Innovacio´ n y Universidades (MCIN); Agencia Es- tatal de Investigacio´ n (AEI) and Fondo Europeo de Desarrollo Regional (FEDER) (RT2018-099357-B-I00); the Biomedical Research Networking Center on Frailty and Healthy Ageing (CIBERFES-ISCii-CIB15/10/00289); and the HFSP agency (RGP0016/2018). Work in the D.S. laboratory is funded by the CNIC; by the European Union’s Horizon 2020 research and innovation program under grant ERC-2016-Consolidator grant 725091; by Spanish Ministerio de Ciencia e Innovacio´ n PID2019-108157RB-100/AEI/10.1309/ 501100011033; by Comunidad de Madrid (P2022/BMD-733 IMMUNOVAR- CM); and by “la Caixa” Foundation (LGF/PRHR20/00075 and LCF/PR/HR22/ 00253). The CNIC is supported by the Instituto de Salud Carlos III (ISCIII), the MCI/NIN, and the European Foundation for the Alexander M. Karpov and Florencio A. G. Ballestar. The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

DECLARATION OF INTERESTS
The authors declare no competing interests.


### Key Resources Table

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### Critical commercial assays

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### Experimental models: Organisms/strains

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### Oligonucleotides

All primers used in this study are listed in Table S1.

### Software and Algorithms

- Analysis code for the study "Oxidative phosphorylation selectively orchestrates tissue macrophage homeostasis" available at:
  - https://zenodo.org/
  - https://doi.org/10.5281/zenodo.7041482

- R version 4.1.2
- R package dplyr version 1.0.7
- R package Matrix version 1.4.1
- R package data.table version 1.14.2
- R package ggplot2 version 3.3.5
- R package ggpubr version 0.4.0

### Additional Resources

- R package ComplexHeatmap version 2.10.0
- R package Seurat version 4.0.6
- R package scater version 1.22.0
- R package edgeR version 3.36.0
- R package limma version 3.50.0
- R package fgsea version 1.20.0
- R package gage version 2.44.0
- R package gageData version 2.32.0
- R package GO.db version 3.14.0
- R package org.Hs.eg.db version 3.14.0
- R package org.Mm.eg.db version 3.14.0
**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, David Sancho (dsancho@cnic.es).

**Materials availability**
The gene expression and metabolomics data generated and the code used in this study are available at Gene Expression Omnibus (GEO), Metabolomics workbench or Zenodo, respectively (details see [key resources table](#)). There are restrictions to the availability of the following mouse strains because they were obtained under a material transfer agreement: with N-G. Larsson (Max Planck Institute for Biology of Ageing, *Tfam*^f/f^), R.D. Palmiter (University of Washington, *Ndufs4*^f/f^), E. Gottlieb (Beatson Institute for Cancer Research, *Sdhb*^f/f^), and C. T. Moraes (University of Miami, *Cox10*^f/f^). This study did not generate other new unique reagents.

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**Other**

| Rodent Diet with 60% kcal Fat | Research Diets Inc | Cat# D12492 |
| Anti-F4/80 MicroBeads UltraPure, mouse | Miltenyi Biotec | Cat# 130-110-443 |
| CD11c MicroBeads UltraPure, mouse | Miltenyi Biotec | Cat# 130-125-835 |
| LS Columns | Miltenyi Biotec | Cat# 130-042-401 |
| MS Columns | Miltenyi Biotec | Cat# 130-042-201 |
| ProLong™ Gold Antifade Mountant | Life Technologies | Cat# P36930 |
| µ-Slide 8 Well | Ibidi GmbH | Cat# 80826 |
| Contour-Next Blood Glucose Test Stripes | Bayer | Cat# 161962.4 |
| BD Insyte™ Autoguard™ Shielded IV Catheter 18g x 1.16 in (1.3mm x 30mm) Green | BD Biosciences | Cat# 381444 |
| BD Insyte™ Autoguard™ Shielded IV Catheter 20g x 1.00 in (1.1mm x 25mm) Pink | BD Biosciences | Cat# 381433 |
Data and code availability
This paper analyzes existing, publicly available data. These accession numbers for the datasets are listed in the key resources table. Bulk RNA-seq data have been deposited at GEO and metabolomics data at Metabolomics workbench and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. All other data are available in the main text, STAR Methods and supplemental information or will be shared by the lead contact upon request.

Code used for transcriptomics analysis has been deposited at Zenodo and is publicly available as of the date of publication. DOIs are listed in the key resources table.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice
Mouse colonies were bred at the CNIC under specific pathogen-free conditions and on C57BL/6 background. Ttamf/f, Ndufs4f/f, and Sdhb+/− mice were kindly provided by Nils-Goran Larsson (Max Planck Institute for Biology of Ageing, Cologne, Germany), Richard D. Palmiter (University of Washington, Seattle, USA), and Eyal Gottlieb (Beatson Institute, Glasgow, UK), respectively. Ndufs4f/f mice were created by crossing floxed males with Zp3Cre females. All floxed mouse lines, as well as Uqcrq−/− and Cox10−/− mice, were crossed with ItgaxCre or Lyz2Cre mice. Mice were group-housed, have not been used in previous procedures and were fed standard chow except for experiments using high fat diet (as indicated and see below). Littermates of the same sex were randomly assigned to experimental groups. Male and female mice were used for all experiments, except experiments using high fat diet (as indicated and see below). Mice with the following ages were used for all experiments (as indicated): 2 days, 11 days, 3 weeks or 6–10-weeks (adult). The local ethics committee approved all animal studies. All animal procedures conformed to EU Directive 86/609/EEC and Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes, enforced in Spanish law under Real Decreto 1201/2005.

Primary cell culture
Primary cells were obtained from the above-mentioned male and female mice and the experimental methods are described in the method details section. Cells were cultured at 37°C with 5% CO2 in R10 medium [RPMI Medium 1640 (Gibco) with 10% FBS, 2 mM L-Glutamine, 100 U/mL Penicillin and Streptomycin (100 µg both Lonza) and 1 mM Sodium Pyruvate (HyClone)] on Fibronectin-coated cell culture plates or glass slides for up to 72 hours, as indicated for individual experiments. Culture media was supplemented with 0.1 ng/ml recombinant murine GM-CSF for experiments using cells from 3 week-old mice and inhibitors or small molecules were added as outlined in the method details section.

METHOD DETAILS

Animal procedures
Simvastatin treatment: 3 week-old mice received daily intraperitoneal injections of 10 mg/kg Simvastatin in 10% DMSO in PBS for 8 weeks before all analyses, except for AM proliferation and apoptosis which were analysed after 3 weeks.

High fat diet experiments: 7–8 week-old males were changed to Rodent Diet with 60% kcal Fat (D12492, Research Diets Inc) for 9 weeks before perfusion and analysis. At week 7–9 after diet change, the following tests were performed:

For glucose or insulin tolerance tests, mice were fasted for 16 hours before intraperitoneal injection of 2 g/kg glucose (Sigma) or 0.75 U/kg insulin (Humulina regular), respectively. Blood glucose concentration were measured by bleeding from the tail vein using a handheld glucose meter (Contour Next, Bayer) at indicated times.

Food and water intake, consumed O2 volume, expired CO2 volume, respiratory exchange ratio and energy expenditure of mice were determined by an indirect calorimetry system (TSE LabMaster, TSE Systems, Germany) for 48-72 hours after a 2-day acclimatization and analyzed using METABOLISM Software (Panlab).

Biomedical Imaging was conducted at the Advanced Imaging Unit of the CNIC and this project used the ReDIB ICTS infrastructure TRIMA@CNIC, Ministerio de Ciencia e Innovación. Body, fat, and lean mass were quantified by magnetic resonance imaging (MRI) and analyzed using FLIR R&D software.

For determination of maximal and average BAT-adjacent interscapular temperature, the skin around the BAT area of mice was shaved 24 hours before taking 5 thermographic images per animal using a FLIR T430sc Infrared Camera (FLIR Systems, Inc., Wilsonville, OR), which were analyzed using FLIR R&D software.

Tissue dissociation for cell isolation
Lungs were minced and incubated for 20 min in HBSS (Gibco); ear skin layers were separated, minced and digested for 30 min in HBSS; kidneys were minced and incubated for 30 min in FACS buffer [3% heat-inactivated Fetal Bovine Serum (FBS), 0.5mM EDTA (both Sigma) in PBS (Gibco)] and spleens were digested for 10 min in R10 medium. Digestions were done with 0.25 mg/ml Liberase TL and 50 µg/ml DNase1 with shaking at 37°C. Minced adipose tissue (eWAT and iWAT) was incubated for 20 min in 50:50 PBS/HBSS with 0.5% BSA (Sigma) and 2 mg/ml collagenase type 2 and miniced livers were digested for 20 min in HBSS with 0.5% BSA, 1 mg/ml Collagenase A and 50 µg/ml DNasel with shaking at 37°C. To obtain single cell suspensions, ear skin
was passed 5 times through a 18g syringe, adipose tissue was trituated 5 times with a 10 ml pipette and all tissues were squeezed through a 70 or 100 µm cell strainer (Corning). Peritoneal cells were collected by lavage of the peritoneum with 8-10 ml PBS. Bronchoalveolar lavage (BAL) was performed by inserting a venal catheter (BD) into the trachea and 3-10 washes with 0.3-1 ml FACS buffer to harvest BAL cells. Blood was harvested by bleeding from the submaxillary vein or cardiac puncture. All cell suspensions were subjected for 1-5 min (blood 3 times) to Red Blood Cell Lysing Buffer, washed with FACS buffer and re-filtered through a 40 µm cell strainer and either directly analyzed or further processed.

Flow cytometry and cell sorting

Single cell suspensions were incubated for 20 min at 4°C in PBS with 2% heat inactivated-FBS and 0.5 mM EDTA (Sigma) with FcR block anti-mouse CD16/CD32 antibody and a mix of fluorochrome-conjugated antibodies as indicated in the key resources table. DAPI was used to exclude dead cells. Autofluorescence was determined using the UV or blue laser.

After antibody incubation, cells were stained with Nile-Red (1 µg/ml in PBS) for 10 min at room temperature (RT) or with Dihydroethidium (DHE, 20 µM in R10 without Phenol Red or FBS) for 1 hour at 37°C, for ROS determination. For intracellular staining related to apoptosis, the Active Caspase-3 Apoptosis Kit was used according to manufacturers’ instructions in combination with anti-active Caspase-3 (clone C92-605) and/or Ki67 (clone SolA15) antibodies. The LSRFortessa or FACSsymphony cell analyzers running FACSDiva software and FlowJo Version 10 software were used to record and analyze data.

For FACS cell sorting: lung or spleen single cell suspensions were incubated with mouse CD11c MicroBeads UltraPure or Anti-F4/80 MicroBeads UltraPure, respectively, loaded onto LS columns and the positive fraction collected according to manufacturers’ instructions before fluorescent staining. Immune cells from kidney cell suspensions were enriched using a density gradient centrifugation (Biocell cell separation solution, 700 xg centrifugation for 30 min at RT). Cells were flow-sorted using the FACSaria II cell sorter running FACSDiva software.

TMF populations were identified as follows: F4/80+ CD64+ CD206+ or CD9+ cells in iWAT and eWAT (iWAT-MFs, eWAT-MFs); CD45+ F4/80+ CD64+ cells in kidney (KMs); F4/80+ CD11blow MHCIImi low cells in spleen (RPMs); CD45+ Siglec F- Ly6G- Ly6C- F4/80+ cells in liver (KCs); CD64+ MHCIImi low CD11b+ CD24+ cells in CD45+ cells in skin (LCs); CD11b+ MHCIImi low F4/80high cells in peritoneal lavage (LPMs) and CD45+ F4/80+ CD11c+ cells in lung (AMs).

For magnetic MACS cell sorting of BAL: AMs were purified using mouse CD11c MicroBeads UltraPure and MS columns according to manufacturers’ instructions.

Analysis of single cell RNA sequencing

All RNA-seq-related analyses were performed in R (version 4.2.1). Single-cell RNA-seq raw counts data from the Human Cell Landscape project were downloaded from https://figshare.com/articles/HCL_DGE_Data/7235471. After selecting datasets based on the health status of the donors and absence of inflammatory cell infiltrates to ensure homeostatic samples (Figure S1A), cells annotated as TMFs were independently analyzed using Bioconductor packages and Seurat. After ruling out cells with poor quality metrics (counts ≥ 400 and ≤ 3000, detected genes ≥ 200 and ≤ 1250, and % of mitochondrial genes ≤ 15), further analysis of resulting TMFs were performed at the pseudo-bulk level by aggregating cells according to the tissue of origin. Single-cell profiles were summarized by averaging counts per million (CPM) in order to force all cells to contribute with the same extent. The number of pseudo-replicates for every tissue was determined by keeping a similar number of detected genes per pseudo-bulk sample. Finally, pseudo-bulk samples were transformed with logarithm to the base 2 for further analysis.

Log2(CPM) pseudo-bulk samples were scaled and used as input for principal component analysis (PCA). Genes were ranked according to the loading vectors of each PC. Resulting ranks were used as input for the FGSEA algorithm along with the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) (www.geneontology.org) databases in order to identify the main sources of variability detected by PCA. Only gene sets with a number of genes ≥ 15 and ≤ 500 were considered for PCA-based enrichment analysis.

Gene set analysis was performed using log-fold changes as per gene statistics as previously described. In summary, log-fold changes for every gene were calculated comparing each TMF population to the background (rest of TMFs). Then, gene set enrichment scores in each TMF population were calculated as T-scores (two-sample T-test) comparing the corresponding mean log-fold changes of each gene set with all genes (background). Finally, Pearson’s correlations between T-scores of different gene sets were calculated using R.

Bulk RNA sequencing and analysis

Analysis of bulk RNA-seq data was performed in R (version 4.1.2) using the Bioconductor package Limma for normalization (using TMM method) and differential expression testing (moderated t-test and Benjamini–Hochberg correction with adjusted p-value ≤ 0.05 as criterion for differentially expressed genes), taking only those genes into account that were expressed with at least 1 CPM. For the differential analysis of TMFs, raw counts generated by the Immunological Genome Project were downloaded from Gene Expression Omnibus (GEO, accession code: GSE109125). Samples used in the analysis are specified in Figure S2A. PCA and gene set analysis was performed similar to that of the scRNAseq data. Briefly, normalized counts were used as input for the PCA. Genes were ranked according to the leading vector of each PC and used as input for the FGSEA algorithm along with KEGG and GO databases.
For the analysis of AMs, total RNA was isolated from CD45^+ F4/80^+ CD11c^+ FACS-sorted cells from the lung of 3 week-old Tbam^{−/−} and Itgax^{−/−}Tbam mice and subjected to RNAseq. Next generation sequencing experiments were performed in the Genomics Unit of the CNIC on the Illumina HiSeq 4000 System. Reads were mapped against reference transcriptome GRCm38.99, quantified using RSEM using expected expression counts and normalized as previously indicated. Genes were ranked according to log-Fold change and gene set enrichment analysis was performed using FGSEA algorithm and KEGG and GO databases. Heatmaps display deregulated genes (Adj. p-value ≤ 0.05) of the specified gene set.

**GC-MS untargeted metabolomics**

Samples were prepared for the gas chromatography and mass spectrometry (GC-MS) untargeted metabolomics analysis by optimizing methods previously described. Briefly, 1 ml of cold MeOH:H2O (9:1, v:v) was added to each sample containing 10^6 CD45^+ F4/80^+ CD11c^+ FACS-sorted AMs from the BAL of adult Tbam^{−/−} and Itgax^{−/−}Tbam mice. Samples were subjected to two freeze-thaw cycles for metabolism quenching and complete metabolite extraction, specifically by placing the samples at -80°C for 15 min and thawing them on ice for 10 min with brief vortex-mixing. The samples were then centrifuged at 20,000 x g at 4°C for 10 min and the supernatant collected. The supernatant was evaporated to dryness (SpeedVac Concentrator, Thermo Fisher Scientific, Waltham, MA, USA) and derivatized with 10 μl O-methoxyamine hydrochloride (15 mg/mL) in pyridine and 10 μl N,O-bis(trimethylsilyl)trifluoroacetamide in 1% trimethylchlorosilane. Finally, 100 μl of heptane containing 10 ppm of 4-nitrobenzoic acid (IS) was used as internal standard to monitor sample injection. For data acquisition, 7250 GC/Q-TOF using the electron ionization (EI) source was used; separation was carried out using a J&W guard column (10 m x 0.25 mm, 0.25 μm film, Agilent Technologies). Metabolite deconvolution and identification were carried out using Agilent MassHunter Unknowns Analysis version B.07.00, then, data was aligned in Agilent Mass Profiler Professional version B.12.1 and exported to Agilent MassHunter Quantitative Analysis version B.07.00. Metabolites were identified by comparing their retention time, retention index and mass fragmentation patterns with those available in an in-house library including both the NIST mass spectral database (version 2017) and Fiehn RTL library (version 2008). Samples were normalized by IS as reference feature. The result was a matrix with the compounds in the samples sorted by their characteristic retention time and target ion, and the relative abundance of each compound for each sample.

Quality Control (QC) samples (n=4) were prepared by pooling equal volumes of cell extracts from each sample by following protocols mentioned above. QC samples were injected at the beginning, at the end and every six samples in order to assess the reproducibility of both sample preparation and data acquisition. Raw data from all samples were processed as described above. Data quality was assured by using the QC samples as reference by filtering the data matrix. Specifically, metabolites present in 50% of the QC samples with a coefficient of variation below 50% were retained and the resulting data matrix underwent principal component analysis (PCA) on Metaboanalyst website (http://www.metaboanalyst.ca/) to assess the analytical reproducibility.

Cubed root transformed metabolites’ abundancies from AMs from Tbam^{−/−} and Itgax^{−/−}Tbam mice were compared using Student’s t test and the p-values adjusted using the Benjamini–Hochberg method correcting for false discovery rate (FDR, q=0.05). Differences were considered statistically significant when p ≤ 0.05. Individual samples (n=3 per each genotype) were generated by merging FACS-sorted AMs to 10^6 cells/sample from >10 independent experiments of more than 13-30 animals/genotype.

**Nucleic acid analysis and quantitative PCR**

From FACS-sorted TMF cells, total DNA was isolated using the QIAamp DNA Mini Kit. Tissue was homogenized using an IKA Ultra-Turrax T-10 disperser and total RNA of tissue and TMFs was extracted with the RNeasy Micro or Mini Kit and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit with random hexamers following manufacturer’s instructions. Quantitative PCR was performed using the GoTaq® qPCR Master Mix in a 7900HT Fast Real-Time PCR System (Applied Byosystem®). Please find all sequences of primers used in Table S1 in the supplementary information. 2^-ΔCt mRNA expression values of mouse genes were calculated relative to expression of 18S rRNA and mt-Co2 (mt-DNA) amounts were calculated relative to Sdh (nuclear DNA). For detection of Xbp1 splice isoforms, the PCR product was purified using the MinElute PCR Purification Kit and digested with the PstI-High-Fidelity restriction enzyme for 60 min at 37°C. Then, DNA bands were separated by agarose gel electrophoresis and imaged following standard procedures and quantified using ImageJ/Fiji.

**In vitro treatments and lactate measurement**

10^6 MACS-purified BAL-AMs from pools of 3 week-old mice were analyzed directly or cultured for 48 hours before staining and mRNA extraction. 0.2-1x10^6 MACS-purified BAL-AMs from pools of adult mice were cultured for 6 hours or 24 hours, respectively, under presence or not of PERK inhibitor GSK2606414, LXR agonists 1 μM T0901317 or 0.1 μM GW3965 before extraction of mRNA. 3x10^5 MACS-purified BAL-AMs from pools of adult mice were cultured for 3 days to collect supernatant for lactate measurement using the colorimetric Lactate Assay Kit following manufacturers' instructions.

**Analysis of proteinosis**

A vein catheter (BD) was inserted into the trachea and the first wash was performed with PBS following 2-9 washes with 0.3-1 ml FACS buffer to harvest BAL cells. The first BAL-wash with PBS was centrifuged for 5min at 500 x g and the supernatant used for measurement of optical density at 600 nm using a spectral photometer (after a 1:3 dilution). Protein concentration were determined with the Pierce BCA Protein Assay Kit and total cholesterol content using the Cholesterol Quantitation Kit according to manufacturers' instructions.
Measurement of FA oxidation rates was performed as previously described. 1.5x10⁵ MACS-purified BAL-AMs from pools of adult mice were seeded onto Fibronectin-coated 48 well plates and cultured in R10 overnight. Then, AMs were incubated in 500 µl of media containing 0.3% BSA/100 µM palmitate/1 mM L-Carnitine/0.4 µCi/ml ¹⁴C-palmitate at 37°C for 3 hours. Each sample was assayed in duplicate. The reaction was stopped by the addition of 200 µl of 1 M perchloric acid. The rate of palmitate oxidation was measured as released ¹⁴CO₂ trapped in a filter paper disk with 20 µl of 1 M NaOH on the top of sealed vials. ¹⁴C products were counted in an LS6500 liquid scintillation counter (Beckman Coulter). Scintillation values were converted to nmol ¹⁴CO₂ by multiplying the specific activity and normalized to DNA content (determined with the CyQUANT NF Cell Proliferation Assay Kit following the manufacturers’ instructions).

**Metabolic flux analysis**

For metabolic flux analysis (Mitostress test), 1.25x10⁵ MACS-purified BAL-AMs from pools of adult mice were plated per well onto Fibronectin-coated Seahorse plates (Agilent Technologies, Seahorse Bioscience) and cultured in R10 overnight before changing to Seahorse media [DMEM (Gibco) supplemented with 100 U/mL Penicillin and Streptomycin and either 2 mM L-Glutamine, 1 mM Sodium Pyruvate and 25 mM Glucose or 5 mM L-Carnitine and 50 µM Palmitoyl-CoA (all Sigma) at pH 7.4] and incubation at 37°C without CO₂ for 30 min. Real-time oxygen-consumption rate (OCR) and extracellular acidification rate (ECAR) in AMs were determined with an XF-96 Extracellular Flux Analyzer (Agilent Technologies, Seahorse Bioscience). Three measurements were performed at the basal state and, for some experiments, after the sequential addition of the following inhibitors every 15 min (Mitostress test): 1 µM Oligomycin, 1 µM Carbonyl cyanide 3-chlorophenylhydrazone (CCCP), and 1 µM Rotenone with 1 µM Antimycin A. Basal respiratory rate (BRR), maximal respiratory rate (MRR), spare respiratory capacity (SRC), respiration linked to mitochondrial ATP (mt-ATP) production and ECAR were calculated according to manufacturers’ instructions.

**ATP measurements**

1-5x10⁵ FACS-sorted TMF cells were incubated or not for 10 min at RT with 10 mM 2-deoxy-D-glucose in PBS. Intracellular ATP content was determined using the Luminescent ATP Detection Assay Kit following the manufacturers’ instructions.

**Cholesterol uptake and efflux measurements**

10⁴ MACS-purified BAL-AMs from pools of adult mice were allowed to adhere in 100 µl R10 medium overnight. Then, measurement of cholesterol uptake and efflux was performed using the Cholesterol Efflux Assay Kit with the acceptor Lipoprotein (High Density Lipoprotein, human plasma, solution) according to the manufacturers’ instructions.

**Fluorescence microscopy**

MACS-purified BAL-AMs from pools of 3 week-old or adult mice were allowed to adhere overnight in R10 media on Fibronectin-coated μ-Slide 8 wells. Then, cells were incubated with 250 nM Tetramethylrhodamine methyl ester (TMRM), 100 nM MitoTracker Green FM and APC-conjugated anti-CD11c antibody for 30 minutes in a humidified incubator at 37°C in staining medium [RPMI without phenol red (Gibco), supplemented with 100 U/mL Penicillin and Streptomycin, 2 mM L-Glutamine, 1 mM Sodium Pyruvate, 25 mM Glucose, 50 µM [β-Mercaptoethanol (Sigma), 0.1 mM NEAA, and 1 mM HEPES (both from HyClone)]. Hoechst 33342 dye was added for 5 minutes, cells washed and immediately imaged in staining media containing 25 nM TMRM with the Nikon A1R confocal microscope (60x objective). Alive cells were kept at 37°C at all times. Automatic image analysis was carried out with ImageJ/Fiji and cell area was determined using CD11c staining area and positive TMRM or MitoTracker Green pixels were quantified within each cell.

Alternatively, AMs were seeded onto Fibronectin-coated class coverslips and cultured overnight in R10 media. Then, cells were stained with 1 µg/ml Nile-Red in PBS for 30 min at RT, fixed for 20 min with 4% paraformaldehyde (PFA, Sigma) and incubated with DAPI for 10 min. For epidermal ear sheet preparations, skin layers of ears from adult mice were separated using tweezers and incubated floating on 3.8% ammonium thiocyanate (Sigma)/PBS for 25 min at 37°C. Then, epidermis was carefully separated from the dermis and fixed in 4% PFA overnight and stained with DAPI for 20 min at RT. After mounting with ProLong Gold Antifade mounting media, AMs or epidermal sheets were imaged using the Nikon ECLIPSE Ti-TimeLapse microscope and Langerin+ cells, which mark Langerhans cells in the epidermis, quantified using ImageJ/Fiji software.

**Transmission electron microscopy**

Pellets of CD45+C. F4/80+/CD11c+ FACS-sorted AMs from the BAL of adult Tfam-/- and Itgax ΔTfam mice were fixed for 4 hours at 4°C in 0.1 M cacodylate buffer with 5% PFA and 4% glutaraldehyde, washed 3 times with PBS and incubated in 1% osmium tetroxide for 1 hour. Then, samples were treated for 10 min with 0.5% uranyl acetate and dehydrated with increasing concentrations of ethanol and acetate before the infiltration with epoxy resin Durcupan (all Sigma). After polymerization, 60 nm sections were cut using an
ultra-microtome (Leica Reichert ultracut), contrast-stained with 2% uranyl acetate and Reynolds’s lead and imaged using a transmission electron microscope [Jeol Jem 1010 (Japan)] equipped with a digital camera [Gatan Orius 200 SC (Pleasanton-CA)].

**Histology and image analysis**

Adipose and liver tissue samples were fixed in 10% formalin (Sigma) for 48 hours, processed and embedded in paraffin. Sections (5 μm) were prepared and mounted on slides for staining with hematoxylin and eosin (Sigma-Aldrich and Thermo Scientific) or immunohistochemistry. Immunohistochemistry was performed using the primary rat monoclonal anti-F4/80 antibody (Abcam ab6640, clone A3-1) and secondary antibodies HRP-conjugated rabbit anti-rat antibody (Agilent P045001) and HRP-conjugated goat anti-rabbit polymer (EnVision® K4003, Agilent). DAB (3,3’- diaminobenzidine) was used for visualization and nuclei were counterstained with hematoxylin. All the immunohistochemical procedures were performed using an automated autostainer (Autostainer Plus®, Dako). Alternatively, liver tissue samples were rehydrated in 30% sucrose (Sigma) for 3 days and embedded in OCT compound (Tissue-Tek). Sections (8 μm) were stained with Oil red-O stain (American Master Tech Scientific, 0.7% in propylene glycol) for lipid staining.

For lipid content, crown-like structures (CLS) and adipocyte size quantification, slides were digitalized, analyzed with NDP.view2 viewing (Hamamatsu), and quantified with ImageJ/Fiji software to evaluate the Oil red-O-positive area versus the total area (manual quantification). CLS per field-of-view were counted manually. Adipocyte size was automatically quantified with ImageJ/Fiji software using the Cellpose72 algorithm for cellular segmentation.

**Blood and plasma analysis**

Blood was collected by bleeding from the submaxillary vein or cardiac puncture in EDTA-containing tubes (Sarstedt) and white blood cell (WBC) count determined immediately using an ABX Pentra XL 80 (HORIBA Medical). Plasma was obtained by centrifugation at 1000 xg for 30 min at 4°C and snap-frozen in liquid nitrogen. Plasma enzymes or biochemical parameters (ALT/GPT, total cholesterol, free cholesterol, HDL, LDL and triglycerides) were analyzed with a Dimension RxL Max automated analyzer.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Data analyses employed GraphPad Prism version 7.0c. Data are presented as mean ± standard error of the mean or individual values and were analyzed as indicated in the legends or the dedicated STAR Methods section. All experiments were repeated at least twice and pooled data from several experiments are shown as indicated in the legends. Mice were allocated randomly in different experimental groups, but no blinding or randomization strategy was used. No animals were excluded from analysis, unless they had wounds from fighting or over-grooming. All n values represent biological replicates (different mice, primary cell preparations or in vitro experiments). Differences were considered significant when P ≤ 0.05 and are indicated as ns, not significant, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.