Title: Oxidative phosphorylation selectively orchestrates tissue macrophage homeostasis

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Summary

In vitro studies associated oxidative phosphorylation (OXPHOS) with anti-inflammatory macrophages, while pro-inflammatory macrophages rely on glycolysis. However, the metabolic needs of macrophages in tissues (TMFs) to fulfil 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 RNAseq data, in both human and mouse. Impairing OXPHOS in TMFs via *Tfam* deletion differentially affected TMF populations. *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 in vivo. In obesity, *Tfam* depletion selectively ablated pro-inflammatory lipid-handling white adipose tissue macrophages (WAT-MFs), preventing insulin resistance and hepatosteatosis. Thus, 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.

Keywords

Tissue macrophages, Immunometabolism, Oxidative phosphorylation, Tfam, Cholesterol handling, Obesity, Proinflammatory macrophages

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 (Nobs and Kopf, 2021). 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, while lean white adipose tissue macrophages (WAT-MFs) aid adipogenesis and thermogenesis (Nobs and Kopf, 2021; Remmerie and Scott, 2018; Schneider et al., 2014; Wculek et al., 2022).

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 TCA cycle, such as glutaminolysis or fatty acid (FA) oxidation (O'Neill et al., 2016). In vitro, exposure to specific stimuli induces metabolic reprogramming of bone marrow-derived macrophages (BMDMs) that underlies their activities. Enhanced glycolysis and a broken tricarboxylic acid (TCA) cycle are associated with pro-inflammatory M1 macrophage polarization, while anti-inflammatory M2 macrophages are characterized by glutamine and FA-fueled mitochondrial respiration (Faas et al., 2021; Lauterbach et al., 2019; Russell et al., 2019). 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 (Davies et al., 2017), yet AMs rely on glycolysis for proper induction of type 2 inflammatory responses (Svedberg et al., 2019), but not bacterial-induced inflammation (Woods et al., 2020), and the inflammation-resolving function of cardiac macrophages requires FA oxidation and mitochondrial respiration (Zhang et al., 2019). Nevertheless, the specific metabolic requirements for maintenance of TMF populations and their functions in vivo during homeostasis are largely unknown (Caputa et al., 2019; Wculek et al., 2022).

Moreover, non-infectious pathologic conditions can alter the tissue microenvironment and impact TMFs metabolism and function (Nobs and Kopf, 2021; Wculek et al., 2022; Yang et al., 2020). For instance, hypertrophy of WAT upon overnutrition causes excess lipid-load and adipocyte death in obese individuals. This causes 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 (Korf et al., 2019; Lefere and Tacke, 2019). Notably, this pathologic functional change of obese WAT-MFs is accompanied by a profound metabolic remodeling and bioenergetic activation (Boutens et al., 2018; Dahik et al., 2020; Hill et al., 2018; Serbulea et al., 2018). 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/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 (RNAseq) data of human organs from the Human Cell Landscape database (Han et al., 2020). Firstly, 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 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 RNAseq data of 10 mouse TMF populations from healthy mice from the Immunological Genome Project (Yoshida et al., 2019). 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 (Russell et al., 2019). The oxidation of nutrients in the tricarboxylic acid (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 (Baixauli et al., 2015; Desdín-Micó et al., 2020; Latorre-Pellicer et al., 2019; Smeitink et al., 2001). 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 *Tfam^{f/f}* mice with *CD11c^{Cre}* (*CD11cΔTfam*) or *Lyz2^{Cre}* (*LysMΔTfam*) 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 (Baixauli et al., 2015; Desdín-Micó et al., 2020; Gao et al., 2022; Larsson et al., 1998; Smeitink et al., 2001). 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), KCs and RPMs, while kidney macrophages (KMs) or lean inguinal and epididimal WAT-MFs (iWAT-MFs or eWAT-MFs) 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 levels of autofluorescence (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 alveolar macrophages undergo cell cycle arrest and apoptosis despite normal ATP levels

To understand the reasons 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 *Tfam^f/f* and *CD11cΔTfam* AMs at postnatal day 2 and 11 was comparable, however *Tfam*-deficient AM numbers started declining 3 weeks after birth (Figures 3A and S3A). Phenotypic alterations of *CD11cΔTfam* AMs also appeared progressively, such as the deregulation of Siglec F and CD11b, indicative of impaired maturation, as well as autofluorescence, 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 *LysMΔTfam* mice, an alternative Cre-line to target AMs, compared with their control littermates (Figure S3D). In line with the progressive reduction of AM numbers, *CD11cΔTfam* 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 3 week-old mice (Figures 2E, 2F and S3F), which explains the decline of the population.

CD11cΔTfam AMs displayed decreased respiratory rates (Figures 3G and S3G), a slight reduction in mitochondrial content (Figure S3H) and a lower mitochondrial membrane potential (Figures 3H and S3I) compared with *Tfam^f/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 offers the advantage to study the modulation of OXPHOS activity in *CD11cΔTfam* 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 levels were equal in *Tfam^f/f* and *CD11cΔTfam* AMs (Figures 3I and S3J), despite lower production of mitochondrial ATP (Figure S3G). Notably, ATP levels 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 (Figure 3K). These results indicate a rewiring of the central carbon metabolism and bioenergetics towards lactic fermentation by

CD11cΔTfam 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 alveolar macrophages

To dissect the underlying bioenergetics-independent mechanisms causing the decline of the AM population upon OXPHOS interference, we performed an RNAseq analysis of AMs from 3 week-old *CD11cΔTfam* and control mice as well as GC/MS metabolomics of adult AMs (Figures S4A-C). The transcriptomic analysis confirmed a reduction of OXPHOS and deregulated cell proliferation as well as apoptosis in *Tfam*-deficient AMs (Figures S2B and S4D-F). Metabolically, glucose and its derivatives were more abundant in *CD11cΔTfam* than *Tfam^{f/f}* AMs together with the upregulation of genes involved in glucose uptake and metabolism, such as *Slc2a1* (Figures S4G-I), 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 levels 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 (Martínez-Reyes and Chandel, 2020). However, the global deregulation of numerous TCA cycle-associated enzymes and intermediates, as well as of TCA cycle-fueling metabolites such as amino acids, points towards 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 the ETC, while electrons may enter the ETC either via complex I or complex II (Martínez-Reyes and Chandel, 2020). 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 (Martínez-Reyes and Chandel, 2020). Nevertheless, a deficiency of complex III also deregulates the TCA cycle and the abundance of its metabolites (Weinberg et al., 2019). Hence, in order 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. Firstly, *CD11cΔUqcrq* (deficient in *Uqcrq*, mitochondrial complex III gene) and *CD11cΔCox10* (deficient in *Cox10*, mitochondrial complex IV gene) mice displayed a reduction in numbers of AMs and an altered AM phenotype compared with their control littermates (Figures 3N and 3O), reminiscent of the phenotype of *CD11cΔTfam* AMs (Figures 3A-C). Moreover, *CD11cΔSdhb* (deficient in *Sdhb*, mitochondrial complex II gene) mice also exhibited reduced AM numbers and an impaired AM phenotype compared with their control littermates, similar to complex III and IV deficiency (Figures 3N and 3O). Of note, AM numbers were comparable in *CD11cΔNdufs4* (deficient in *Ndufs4*, mitochondrial complex I gene whose deletion results in a complex I hypomorph) mice compared with their controls (Figures 3N and 3O). The ETC complex I does not directly participate in the TCA cycle, but oxidizes NADH to maintain a healthy NAD$^+$/NADH ratio. However, mitochondrial NADH oxidation also occurs during the folate pathway or through the activity of the Nicotinamide Nucleotide Transhydrogenase, for instance, which potentially rescue 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 alveolar macrophages depend on the tissue microenvironment

As a potential molecular mechanism for induction of cell cycle arrest and apoptosis (Feng et al., 2003; Yao et al., 2014), 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 (Pakos-Zebrucka et al., 2016). Double-stranded RNA-dependent protein kinase (PKR) and general control non-depressible protein (GCN) 2 were unlikely to be activated in *CD11cΔTfam* AMs. They usually respond to stresses related with viral infection or amino acid deprivation, while *Tfam*-deficient AMs were analyzed in homeostasis and harbored elevated amino acid levels compared with controls (Figure S4K). The PKR-like ER kinase (PERK)/eIF2α/ATF4 signaling pathway is generally induced upon endoplasmic reticulum (ER) stress and can regulate the immunosuppressive function of macrophages (Raines et al., 2022). However, ATF4 induces apoptosis in situations of prolonged ER stress via activation of C/EBP homologous protein (CHOP) (Nishitoh, 2012). In fact, the "intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress" and expression of the gene encoding CHOP (*Ddit3*) was significantly upregulated in AMs from 3 week-old *CD11cΔTfam* compared with control mice (Figures 4A and S4B). We also detected increased splicing of X-box binding protein (*Xbp*) 1 in those *CD11cΔTfam* AMs (Figure 4C), which is mediated by inositol-requiring enzyme (IRE) 1 upon ER stress independently of the PERK/eIF2α/ATF4 axis, further indicating the induction of ER stress in *Tfam*-deficient AMs. Moreover, *CD11cΔTfam* AMs displayed enhanced expression of genes associated with mitochondrial stress compared with *Tfam^{f/f}* AMs (Figure S5A), which can activate the ISR via heme-regulated eIF2α kinase (HRI) (Fessler et al., 2020; Guo et al., 2020). 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-D).

Given the tissue location-dependent effects of *Tfam* deletion in TMFs (Figure 2), we interrogated potential microenvironmental cell-extrinsic causes of cellular stress in *CD11cΔTfam* AMs. To this end, we harvested AMs from 3 week-old mice by BAL and cultured them for 48 hours. Notably, ex vivo cultured *CD11cΔTfam* AMs normalized their *Atf4* expression levels to that of *Tfam^{f/f}* 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 *CD11cΔTfam* 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* levels in *CD11cΔTfam* AMs after only 6 hours 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/ER stress in *CD11cΔTfam* AMs that, in turn, causes their impaired proliferation.

Altered extracellular lipid and cholesterol handling ability causes the loss of Tfam-*deficient alveolar macrophages 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 (Fessler and Summer, 2016). The predominant function of AMs is the removal of the lipid-rich surfactant (Nobs and Kopf, 2021; Wculek et al., 2022). The TCA cycle, which is deregulated in *Tfam*-deficient AMs (Figures 3L and 3M), is vital for lipid and FA catabolism (Remmerie and Scott, 2018) and a deregulation of cholesterol levels is a well-known inducer of ER stress in macrophages (Feng et al., 2003; Yao et al., 2014). Hence, we hypothesized that a deregulated lipid handling capacity causes the reduced proliferation and enhanced apoptosis in OXPHOS-impaired AMs. Indeed, *CD11cΔTfam* AMs accumulated more intracellular lipids compared with *Tfam^{f/f}* 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 surfactant (Botas et al., 1998) and the previously reported "cholesterol whorls" formed by cholesterol-laden BMDMs in vitro (Tabas, 2002). Despite unaltered abundance of free FAs (Figure S6B), *Tfam*-deficient AMs showed decreased oxidation of palmitate compared with controls as well as a downregulation of genes involved in FA oxidation (Figures 5C and 5D). Lipid catabolism, especially FA oxidation, the TCA cycle and OXPHOS are intricately linked processes. FA oxidation generates Acyl-CoA that enters in the TCA cycle, but also delivers electrons directly to the ETC and ubiquinone in the inner mitochondrial membrane forming ubiquinol. Re-oxidation of ubiquinol is usually performed by complex III and IV, and interference with this process can impair FA oxidation (Chokchaiwong et al., 2018). Hence, a defective OXPHOS metabolism frequently results in reduced FA oxidation in macrophages (O'Neill et al., 2016; Remmerie and Scott, 2018; Russell et al., 2019). In line, *Tfam*-deficient AMs also displayed a lower oxygen consumption and respiratory rate in media containing only FA fuels (Figure S6C).

Notably, cholesterol was strongly enriched in *CD11cΔTfam* vs *Tfam^{f/f}* AMs. This could not be ascribed to increased endogenous cholesterol biosynthesis as the controlling genes were even downregulated. Rather, signatures for cholesterol uptake, transport and storage were strongly deregulated and cholesterol efflux-related genes downregulated (Figures 5E, 5F and S6D). This includes the downregulation of the ABC transporter gene *Abcg1*, which is vital for AM functionality and cholesterol efflux (Thomassen et al., 2007). Notably, *Tfam*-deficient AMs imported more and exported less cholesterol than control AMs (Figure 5G). The deregulated expression of lipid/cholesterol handling-related genes and enhanced lipid accumulation occurred already in AMs of 3 week-old *CD11cΔTfam* mice (Figures 5D, 5E, S6A and S6D), when no elevation of cholesterol levels was detected in the BAL (Figure S6E). This suggests that the increased intracellular lipid/cholesterol levels in *Tfam*-deficient AMs are a result of their inability to process homeostatic amounts of lipids/cholesterol and not a reflection of elevated cholesterol levels in the lung. The nuclear receptor transcription factors liver X receptors (LXR) α and β are the master regulators of intracellular cholesterol handling (Remmerie and Scott, 2018). We found that numerous LXRα/β target genes are deregulated in *CD11cΔTfam* vs *Tfam^{f/f}* AMs (Figure S6F). Mitochondria are known to contribute to LXR activation. The mitochondrial proteins mitofusin 2 and optic atrophy 1, the mitochondrial inhibitor oligomycin, as well as mitochondria-derived ATP can regulate cholesterol handling and efflux in macrophages (Graham, 2015; Karunakaran et al., 2015). A current model for mitochondrial cholesterol transport involves a trafficking complex containing steroidogenic acute regulatory (STAR) proteins that delivers cholesterol to the mitochondrial enzyme sterol 27-hydroxylase (Cyp27a1). Cyp27a1 converts cholesterol into endogenous LXR ligands (Fu et al., 2001; Graham, 2015). We found transcripts of STAR proteins

to be deregulated and Cyp27a1 to be downregulated in *Tfam*-deficient AMs (Figures 5E and S6G). Notably, *CD11cΔTfam* AMs remained equally responsive to LXR activation as *Tfam^{f/f}* AMs, demonstrated by similar upregulation of the LXR target gene *Abcg1* upon treatment with LXR ligands (Figure S6H). Hence, limited activation of LXR signaling, likely due to decreased mitochondrial cholesterol transport and LXR ligand generation, and the reduced mitochondrial ATP production (Figure S3G) mechanistically contribute to the defective cholesterol handling and accumulation in *Tfam*-deficient AMs.

Elevated intracellular cholesterol levels can cause cellular/ER stress and ATF4 activation in macrophages (Feng et al., 2003; Yao et al., 2014). To understand if lipids, mainly cholesterol, are the extracellular factors driving the progressive decline of *Tfam*-deficient AMs, we aimed to reduce those lipid levels in vivo. Simvastatin is a clinically approved cholesterol synthesis inhibitor that reduces cholesterol levels in BAL as well as in AMs of mice with PAP and improves the disease upon systemic administration (McCarthy et al., 2018). Treatment with simvastatin mildly decreased systemic cholesterol and triglycerides in serum and total cholesterol levels in BAL of *CD11cΔTfam* mice without ameliorating other readouts of PAP, such as BAL turbidity or protein content (Figures 5H, 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). While 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 levels of cholesterol and other lipids. This induces cellular/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 as well as IV, while they remained unaltered by complex I dysfunction compared with control littermates (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 *CD11cΔTfam* mice (Figure 6E). Moreover, the expression of OXPHOS-related genes not only significantly correlated with genes involved in lipid and FA catabolism, but especially cholesterol handling processes in human and mouse TMFs (Figure 6F and 6G). Overall, our data suggests that OXPHOS dysfunction selectively affects TMFs with high lipid/cholesterol processing activity by impairing their ability to oxidize and efflux extracellular lipids. This results in cholesterol accumulation that drives cellular/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 towards an increased lipid content could enhance the vulnerability of TMFs to OXPHOS interference. We found that lean WAT-MF populations, which are metabolically quiescent (Boutens et al., 2018; Serbulea et al., 2018), are largely undisturbed by *Tfam* 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 (Amano et al., 2014), form crown-like structures (CLS) around dying adipocytes, become pro-inflammatory and participate in handling those excess extracellular lipids (Hill et al., 2018; Li et al., 2010; Wculek et al., 2022; Yang et al., 2020). Hence, we reasoned that OXPHOS impairment could selectively affect the maintenance and proliferation those pro-inflammatory eWAT-MFs during obesity and fed *Tfam^f/f* and *LysMΔTfam* mice with a high fat diet (HFD). Notably, HFD-fed *LysMΔTfam* mice gained less fat mass than *Tfam^f/f* animals, while their lean mass was unaltered (Figures 7A and S7A). The tibiae length, food and drink intake, energy expenditure, consumed O_2 volume as well as respiratory exchange ratio of obese *LysMΔTfam* mice was similar to that of *Tfam^f/f* controls, however the released CO_2 volume was increased (Figures S7B-7D). In line with our hypothesis, pro-inflammatory CD11c$^+$ and/or CD9$^+$ 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$^+$ and/or MerTK$^+$ eWAT-MFs remained unaltered in obese *LysMΔTfam* mice compared with *Tfam^f/f* controls (Figures 7B, 7C and S7E). The largely unaltered expression of genes associated with anti-inflammatory macrophage functions in eWAT (Figure S7F) supports this finding. This is likely due to a lower lipid burden of those anti-inflammatory compared with pro-inflammatory CD11c$^+$ and/or CD9$^+$ eWAT-MFs, as the latter predominate in CLS (Hill et al., 2018; Li et al., 2010; Yang et al., 2020). Of note, the numbers and phenotype of circulating monocytes, which can give rise to eWAT-MFs in obesity, were unaffected in *LysMΔTfam* mice (Figure S7G). In accordance with decreased pro-inflammatory eWAT-MF presence, obese *LysMΔTfam* mice displayed a lower number of CLS and reduced inflammatory *Tnfa* and *Ccl2* cytokine levels in eWAT, but unaltered adipocyte size, as well as lower systemic leukocyte numbers in plasma compared with *Tfam^f/f* mice (Figures 7D-7F and S7H). Moreover, the eWAT of HFD-fed *LysMΔTfam* mice displayed an elevated expression of genes involved in lipid catabolism, lipolysis, ketogenesis and mitochondrial respiration than that of *Tfam^f/f* mice, but not lipogenesis (Figures 7G and 7H). This indicates a more metabolically active state and a higher lipid oxidation in the eWAT (Bae et al., 2018), which appears independent of eosinophil-mediated alternative macrophage activation and adipose tissue beigeing (Figures S7F, S7I and S7J) (Rao et al., 2014). HFD-fed *LysMΔTfam* mice also showed higher glucose tolerance and insulin sensitivity compared with *Tfam^f/f* mice (Figure 7I and S7K). High-density lipoprotein (LDL) and alanine aminotransferase (ALT) levels in plasma, liver weight as well as hepatic lipid accumulation were also notably reduced in obese *LysMΔTfam* vs *Tfam^f/f* mice (Figures 7J and S7L-O).

To corroborate those data, we next analyzed obese *CD11cΔUqcrq* mice, where ETC complex III and thereby OXPHOS dysfunction is limited to pro-inflammatory eWAT-MFs expressing CD11c. HFD-fed *CD11cΔUqcrq* mice gained less weight and harbored lower pro-inflammatory eWAT-MF numbers than *Uqcrq^f/f* mice (Figures 7K, S7P-S7R). Their cholesterol and ALT levels in plasma as well as their hepatic lipid content was also reduced (Figures S7S-S7U). Notably, HFD-fed *CD11cΔUqcrq* 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 CO_2 volume of HFD-fed *LysMΔTfam* vs *Tfam^f/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 as well as heat production via the BAT (Bae et al., 2018; Matesanz et al., 2018). In summary, OXPHOS dysfunction specifically reduces pro-inflammatory TMFs in obese eWAT, which handle

394 high amounts of lipids, by inducing cellular stress and impairing their proliferation. This results in
395 mitigation of inflammation, metabolic dysfunction and hepatosteatosis.

Discussion

397 Overall, we uncovered a metabolic diversity in both human and mouse TMFs in vivo during
398 homeostasis, and OXPHOS as the main distinguishing feature among TMFs located in different
399 organs. The high contribution of OXPHOS to the bioenergetics of AMs and LPMs compared with
400 BMDMs is established (Davies et al., 2017; Izquierdo et al., 2018; Svedberg et al., 2019; Wculek
401 et al., 2022; Woods et al., 2020) and AMs do not enhance glycolysis upon LPS stimulation (Woods
402 et al., 2020), whereas opposing effects of *Tfam* deletion in AMs were reported (Gao et al., 2022;
403 Soberanes et al., 2019). Our study unambiguously reveals OXPHOS as an important determinant
404 for identity and maintenance of specific TMF populations in the steady state. We detected the
405 induction of cellular and ER stress in affected *Tfam*-deficient TMFs, demonstrated by activation
406 of Atf4, that likely accounts for their increased apoptosis and reduced proliferation. Interestingly,
407 ATP levels in OXPHOS-dependent *Tfam*-deficient TMFs are unaltered, but, at least in AMs,
408 become dependent on glycolysis to fulfil their bioenergetic demands. However, an enhanced
409 glycolytic metabolism per se in AMs did not phenocopy the cellular stress and apoptosis caused
410 by *Tfam* deletion (Izquierdo et al., 2018). Regarding OXPHOS, the adaption of the usage of ETC
411 complex I and II upon bacterial sensing regulates the functions of BMDMs (Garaude et al., 2016)
412 and the complex I protein Ndufs4 was shown to control inflammatory macrophage and osteoclast
413 polarisation (Jin et al., 2014). Interestingly, in contrast to complex II, III, and IV, complex I is the
414 only ETC complex that is dispensable for the maintenance of *Tfam* deletion-sensitive TMFs. This
415 points to an essential role of the correct functioning of the TCA cycle and a complex II-driven
416 ETC for the activity and maintenance of AMs and other OXPHOS-dependent TMFs. The
417 normalization of *Atf4* levels and proliferation upon culture of *Tfam*-deficient AMs ex vivo,
418 suggested a microenvironment-triggered effect that determines the vulnerability of certain TMFs
419 to OXPHOS impairment during homeostasis.

420 Efferocytosis of dying cells by macrophages provides FAs and fuels mitochondrial respiration
421 during tissue injury. Yet, this induces anti-inflammatory functions, not cellular stress and
422 apoptosis, in cultured BMDMs or cardiac macrophages (Zhang et al., 2019). Homeostatic *Tfam*-
423 deficient AMs did not accumulate free FAs, but larger lipid species reminiscent of extracellular
424 surfactant and free cholesterol. Elevated lipid levels, especially of cholesterol, is a potent cause of
425 cellular stress and Atf4 activation in macrophages (Feng et al., 2003; Tabas, 2002; Yao et al.,
426 2014). Notably, we show that a reduction of systemic lipid and cholesterol levels in *CD11cΔTfam*
427 mice improves AM and RPM numbers. Our analysis reveals a significant correlation of OXPHOS
428 and cholesterol handling-related gene expression across human and mouse TMF populations.
429 Hence, we uncovered the need for a functional ETC and OXPHOS in TMFs for handling of
430 extracellular lipids/cholesterol to maintain homeostasis. In line with this, the TMFs that are most
431 affected by *Tfam* deficiency; AMs, LPMs, RPMs, and KCs; display a high activity and/or
432 expression of genes required for lipid metabolism and handling (Remmerie and Scott, 2018;
433 Wculek et al., 2022). Moreover, the loss of peroxisome proliferator-activated receptor (PPAR) γ
434 and/or LXRα and β, the master regulators of lipid catabolism and intracellular cholesterol
435 handling, causes a comparable phenotype as *Tfam* deletion in AMs, RPMs and/or KCs (Schneider
436 et al., 2014; Schuster et al., 2002).

437 Finally, we also revealed the relevance of OXPHOS for the maintenance of extracellular lipid-
438 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 activated (Boutens et al., 2018; Serbulea et al., 2018) 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-inflammatory (Hill et al., 2018; Li et al., 2010; Wculek et al., 2022; Yang et al., 2020). Interference with increases in intracellular ROS can impair proinflammatory macrophage activation (Mills et al., 2016). While we observed elevated ROS levels at baseline in *Tfam*-deficient TMFs, we cannot entirely exclude a potential contribution of alterations of ROS levels to the reduction of proinflammatory *LysMΔTfam* eWAT-MFs. Fgr kinase deletion in bone marrow cells imbalances complex I/II usage by the ETC and alters polarization of eWAT-MFs, decreasing pro-inflammatory and increasing anti-inflammatory macrophage presence (Acín-Pérez et al., 2020). 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. While 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 CD9^{+} WAT-MFs is distinct from that of other recruited WAT-MFs (Morgan et al., 2021). Pro-inflammatory CD9^{+} WAT-MFs can induce an inflammatory phenotype in WAT (Hill et al., 2018) and are important drivers of obesity-associated pathologies (Dahik et al., 2020; Korf et al., 2019). Thus, we find that HFD-fed *LysMΔTfam* 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.

Author contributions:

Conceptualization: SKW, IHM, DS
Methodology, investigation, analysis, visualization, and validation: SKW, IHM, AM, DM, MG, SL, VM, AC
Essential reagents and support: CB, JAE, NSC
Writing of original draft: SKW, DS
Funding acquisition, supervision, and project administration: SKW, DS
Editing of draft: all authors

Declaration of interests: Authors declare that they have no competing interests.

Figure legends

Figure 1. Mitochondrial OXPHOS metabolism is a main distinctive signature of different TMF populations.

(A) Schematic representing the workflow used in the pseudo-bulk-based analysis of human TMF clusters.

(B) PCA of scRNAseq data from the Human Cell Landscape of previously identified TMF clusters of indicated human organs. Dots represent individual pseudo-bulked samples.

(C and D) Pre-ranked gene set-enrichment analysis of the genes defining PC1 in the analysis shown in (B) using Gene Ontology (GO) biological processes (C) and gene set-enrichment plot of OXPHOS-related genes (KEGG reference pathway hsa00190, D). NES, normalized enrichment score; Adj., adjusted with Benjamini–Hochberg correction.

(E) PCA of RNAseq data from the Immunological Genome Project of indicated mouse TMF populations. Dots represent individual samples.

(F and G) Pre-ranked gene set-enrichment analysis of the genes defining PC1 in the analysis shown in (E) using GO biological processes (F) and gene set-enrichment plot of OXPHOS-related genes (KEGG reference pathway mmu00190, G).

(H) Heatmap of log-Fold change (FC) of detected mt-genes (calculated comparing each individual tissue with the mean of all tissues) and their average in the mouse TMF population RNAseq data analysis shown in (E). *$p < 0.05$, by Moderated t-test. See also Figure S1 and S2.

Figure 2. OXPHOS impairment has different effects on distinct TMF populations in homeostasis.

(A to D) *Tfam* mRNA levels (A) mt-DNA levels (B) flow cytometric quantification of numbers (C) and frequency (D) of the indicated TMF populations in lean adult *CD11cΔTfam* or *LysMΔTfam* relative to *Tfam^f/f* mice (n=3-9). Flow cytometric identification of TMFs is detailed in methods and Langerin$^+$ cell numbers (LCs, n=8-11) in (C) were quantified by fluorescence microscopy of epidermal sheets.

(E) Pearson's correlation of the average percentage of number reduction in organs of *CD11cΔTfam* or *LysMΔTfam* relative to *Tfam^f/f* mice (C) and average levels of detected mt-gene expression (Figure 1H) of the indicated mouse TMF populations.

(F) Relative median intensity of autofluorescence determined by flow cytometry of the indicated TMF populations in lean adult *CD11cΔTfam* or *LysMΔTfam* relative to *Tfam^f/f* mice (n=3-9).

Data in (A to D and F) were normalized to the respective *Tfam^f/f* controls for each TMF population to facilitate comparisons. For orientation, OXHPOS-interference insensitive and sensitive TMFs are separated by a gap in the graphs. Data are merged from at least 2 independent experiments and presented as mean ± SEM. Dots represent individual data points. Statistical analysis by unpaired Student's t- test (A-D and F) or Pearson's correlation test (E). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$; ns, not significant.

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 (wk)-old or adult (6-8 week-old) *Tfam^f/f* and *CD11cΔTfam* mice or their AMs in the lung were analyzed.

(A to 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$^+$ CD11c$^+$ 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).

(G) Oxygen consumption rate (OCR) of CD11c$^+$ cells from BAL of adult mice (n=3 merged from 5-10 mice).

(H) Quantification of mitochondrial membrane potential ($\Delta\Psi$m) and representative images of fluorescence microscopy of tetramethylrhodamine, methyl ester (TMRM, red), CD11c (green) and Hoechst 33342 (blue) staining of CD11c$^+$ cells from BAL of adult mice (scale bar: 20µm, n=3 [mean of 54-231 cells/mouse]).

(I) ATP levels in F4/80$^+$ CD11c$^+$ cells from the lung of 3 week-old mice (n=6-8).

(J) Lactate levels and picture of the supernatant of CD11c$^+$ cells from BALs of adult mice cultured for 3 days (n=4, merged from 5-10 mice).

(K) Extracellular acidification rate (ECAR) of CD11c$^+$ cells from BALs of adult mice (n=3 merged from 5-10 mice).

(L) Relative abundance of the indicated TCA cycle intermediates in F4/80$^+$ CD11c$^+$ cells from BAL of adult mice (n=3 merged from 13-30 mice).

(M) Heat map of expression levels of deregulated (Adj. p-value <0.05) TCA cycle-related (KEGG mmu00020) genes detected in the RNAseq of FACS-sorted CD45$^+$ F4/80$^+$ CD11c$^+$ lung cells from 3 week-old *Tfam$^{f/f}$* and *CD11cΔTfam* mice.

(N and O) Flow cytometric quantification of numbers (N) and relative MFI of Siglec F (O, left) or CD11b (O, right) of CD45$^+$ F4/80$^+$ CD11c$^+$ cells in the lung of adult *CD11cΔNdufs4* mice, *CD11cΔSdhb* mice, *CD11cΔUqcrq* or *CD11cΔCox10* 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 Figure S3 and S4.

Figure 4. Extrinsic tissue microenvironmental factors induce cellular stress and a cell cycle block in OXPHOS-impaired AMs.

3 week (wks)-old or adult *Tfam$^{f/f}$* and *CD11cΔTfam* mice or their AMs in the lung were analyzed.

(A) Heat map of expression levels of deregulated genes included in the "intrinsic apoptotic signaling pathway in response to endoplasmic reticulum (ER) stress" (GO process GO:0070059) pathway detected to be significantly deregulated in the RNAseq of FACS-sorted CD45$^+$ F4/80$^+$ CD11c$^+$ lung cells from 3 week-old mice.

(B) *Atf4* expression levels detected in RNAseq data (n=4) of CD45$^+$ F4/80$^+$ CD11c$^+$ lung cells from 3 week-old mice.

(C) Quantification and representative gel electrophoresis of *Xbp1* splice isoforms detected by PstI-restriction of a PCR product amplified from cDNA of FACS-sorted CD45$^+$ F4/80$^+$ CD11c$^+$ lung cells from 3 week-old mice (n=5-7).

(D to F) CD11c$^+$ cells were magnetically purified from BAL of 3 week-old mice and either directly analyzed (Day 0) or cultured for 48h before analysis of *Atf4* (D) or *Cdk1* and *Ccnb2* mRNA levels (E) or flow cytometric analysis of Ki67$^+$ cells (F, representative plot and quantification) (n=4 merged from 2-4 mice).

Data are merged from at least 2 independent experiments and presented as mean ± SEM. Dots represent individual data points. Statistical analysis by unpaired Student's t-test. *p≤0.05, **p≤0.01, ***p≤0.001. See also Figure S5.

Figure 5. Environmental lipids and cholesterol accumulate in *Tfam*-deficient AMs and cause

594 **the decline of their population.**

595 3 week (wks)-old or adult *Tfam^{f/f}* and *CD11cΔTfam* mice or their AMs in the lung were analyzed.

596 (A) Flow cytometric quantification of relative MFI (n=7), representative plots and images of

597 fluorescence microscopy (scale bar: 20µm) of Nile-Red staining of CD11c$^+$ cells from BAL of

598 adult mice.

599 (B) Representative images of transmission electron microscopy of and quantification of spiral-like

600 structures in FACS-sorted F4/80$^+$ CD11c$^+$ cells from BAL of adult mice (n=16-24 cells from 4-9

601 mice).

602 (C) Radiolabeled palmitate-derived CO_2 after incubation of CD11c$^+$ cells from BAL of adult mice

603 with ^{14}C-palmitate (n=4 merged from 5-10 mice).

604 (D and E) Heat map of expression levels of selected FA degradation- (D) and cholesterol handling-

605 related genes (E) detected in RNAseq of CD45$^+$ F4/80$^+$ CD11c$^+$ lung cells from 3 week-old mice.

606 (F) Relative abundance of cholesterol in F4/80$^+$ CD11c$^+$ cells from BAL of adult mice detected by

607 GC/MS analysis (n=3 merged from 13-30 mice).

608 (G) Cholesterol uptake (left) and relative efflux (right) after incubation of CD11c$^+$ cells from BALs

609 of adult mice with fluorescently-labelled cholesterol (n=3 merged from 5-10 mice).

610 (H and I) Quantification of total cholesterol levels (H) and flow cytometric quantification of

611 numbers of F4/80$^+$ CD11c$^+$ cells (I) in BAL of mice treated with Simvastatin or control DMSO

612 for 8 weeks (n=5-13).

613 Data are merged from at least 2 independent experiments and presented as mean ± SEM. Dots

614 represent individual data points. Statistical analysis by unpaired (A, F and H) or paired (C and G)

615 Student's t-test. *p≤0.05, **p≤0.01, ***p≤0.001. See also Figure S6.

616 **Figure 6. High cholesterol handling activity determines the vulnerability of tissue**

617 **macrophages to OXPHOS impairment.**

618 (A) Langerin$^+$ cell numbers (LCs) quantified by fluorescence microscopy of epidermal sheets (left,

619 n=4-9) and flow cytometric quantification of numbers of F4/80$^+$ CD11b^{low} MHCII^{low} cells (RPMs)

620 in the spleen (right, n=5-7) of adult *CD11c^{Cre} Ndufs4^{f/-}* relative to *Ndufs4^{f/-}* mice, *CD11c^{Cre} Sdhb^{f/f}*

621 relative to *Sdhb^{f/f}* mice, *CD11c^{Cre} Uqcrq^{f/f}* relative to *Uqcrq^{f/f}* mice or *CD11c^{Cre} Cox10^{f/f}* relative

622 to *Cox10^{f/f}* mice.

623 (B) ATP levels in FACS-sorted CD11b$^+$ MHCII^{low} F4/80^{high} cells from the peritoneal lavage

624 (LPMs) of adult *Tfam^{f/f}* and *LysMΔTfam* mice (left, n=4-5) and in FACS-sorted F4/80$^+$ CD11b^{low}

625 MHCII^{low} cells from the spleen (RPMs) of adult *Tfam^{f/f}* and *CD11cΔTfam* mice (right, n=4-6).

626 (C) Flow cytometric quantification of relative MFI of Nile-Red staining of F4/80^{high} cells from the

627 peritoneal lavage (LPMs) of adult *Tfam^{f/f}* and *LysMΔTfam* mice (left, n=6-8) and of magnetically

628 purified F4/80$^+$ cells from the spleen (RPMs) of adult *Tfam^{f/f}* and *CD11cΔTfam* mice (right, n=9-

629 10).

630 (D) Analysis of *Atf4* mRNA levels in FACS-sorted CD11b$^+$ MHCII^{low} F4/80^{high} cells from the

631 peritoneal lavage (LPMs) of adult *Tfam^{f/f}* and *LysMΔTfam* mice (left, n=7-8) and in FACS-sorted

632 F4/80$^+$ CD11b^{low} MHCII^{low} cells from the spleen (RPMs) of adult *Tfam^{f/f}* and *CD11cΔTfam* mice

633 (right, n=8-9).

634 (E) Flow cytometric quantification of frequencies (left) and numbers (right) of F4/80$^+$ CD11b^{low}

635 MHCII^{low} cells in the spleen (RPMs) of *Tfam^{f/f}* and *CD11cΔTfam* mice treated with Simvastatin

636 or control DMSO for 8 weeks (n=10-13).

637 (F) Pearson's correlation of expression of OXPHOS-related genes (KEGG pathway mmu00190)

638 and genes regulating cholesterol transport (GO process GO:0032376) or fatty acid catabolism (GO

639 process GO:0009062) in mouse TMF populations using RNAseq data (see Figure 1E).

640 (G) Pearson's correlation of expression of OXPHOS-related genes (KEGG pathway hsa00190)

and genes regulating cholesterol transport (GO process GO:0032367), cellular lipid catabolism (GO process GO:0044242) or fatty acid catabolism (GO process GO:0009062) in TMF clusters identified in the scRNAseq data of indicated human organs (see Figure 1A).

(H) Diagram of proposed mechanism. FAO, fatty acid oxidation.

Data are merged from at least 2 independent experiments and presented as mean ± SEM. Dots represent individual data points. Statistical analysis by unpaired (A-E) Student's t-test or Pearson's correlation test (F and G). *p≤0.05, **p≤0.01, ***p≤0.001.

Figure 7. OXPHOS dysfunction reduces pro-inflammatory eWAT-MF presence during obesity and prevents metabolic syndrome and hepatosteatosis.

$Tfam^{f/f}$ and $LysM\Delta Tfam$ or $Uqcrq^{f/f}$ and $CD11c\Delta Uqcrq$ mice were fed an HFD for 9 weeks and analyzed.

(A) Quantification and representative images of magnetic resonance imaging to determine body mass (n=17).

(B) *Atf4* mRNA levels after FACS-sorting (left, n=5-6) or flow cytometric analysis of Ki67$^+$ (right, n=11) in CD64$^+$ F4/80$^+$ and CD206$^+$ or CD9$^+$ cells from eWAT.

(C) Flow cytometric quantification of numbers and representative plots of gated CD45$^+$ CD64$^+$ F4/80$^+$ cells (that also express indicated markers) in eWAT (n=13-15).

(D) Quantification and representative image of F4/80 (brown) and hematoxylin (blue)-stained eWAT sections (scale bar: 500µm, n=12-13).

(E) *Tnfa* and *Ccl2* mRNA levels in eWAT (n=10-16).

(F) White blood cell (WBC) counts in blood (n=13-15).

(G and H) mRNA levels of selected lipid metabolism (G) or mitochondrial respiration-related genes (H) in eWAT (n=6-13).

(I) Glucose levels measured over time in blood after intraperitoneal injection (time = 0) of insulin (n=13-16).

(J) Quantification and representative image of Oil-Red O (red) and hematoxylin (blue)-stained liver sections (scale bar: 100µm, n=8-9).

(K) Flow cytometric quantification of numbers of CD45$^+$ CD64$^+$ F4/80$^+$ cells (that also express indicated markers) in eWAT (n=18).

(L) Quantification of maximal and average temperature and representative infrared thermal images of the BAT area of obese mice (n=6-8).

Data are merged from at least 2 independent experiments and presented as mean ± SEM. Dots represent individual data points. Statistical analysis by unpaired Student's t-test (A-H and J-L) or two-way ANOVA with Geisser-Greenhouse correction (I). *p≤0.05, **p≤0.01, ***p≤0.001. See also Figure S7.

STAR Methods

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.

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 supplementary materials 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. *Tfam$^{f/f}$* (Larsson et al., 1998), *Ndufs4$^{f/f}$* (Kruse et al., 2008) and *Sdhb$^{f/f}$* (Cardaci et al., 2015) mice were kindly provided by Nils-Göran 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. *Ndufs4$^{f/-}$* mice were created by crossing floxed males with *Zp3^{Cre}* females (Lewandoski et al., 1997). All floxed mouse lines, as well as *Uqcrq$^{f/f}$* (Weinberg et al., 2019) and *Cox10$^{f/f}$* (Diaz et al., 2005) mice, were crossed with *CD11c^{Cre}* (Caton et al., 2007) or *LysM^{Cre}* mice (Clausen et al., 1999). 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, expect 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 Methods Details section. Cells were cultured at 37°C with 5% CO_2 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 Methods 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 levels were measured by bleeding from the tail vein using a handheld glucose meter (Contour Next, Bayer) at indicated times.

Food and water intake, consumed O_2 volume, expired CO_2 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) using a small animal magnetic resonance scanner 7 Tesla (Varian) and analyzed by ImageJ/Fiji 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 DNaseI 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 minced livers were digested for 20 min in HBSS with 0.5% BSA, 1 mg/ml Collagenase A and 50 μg/ml DNaseI with shaking at 37°C. To obtain single cell suspensions, ear skin was passed 5 times through a 18g syringe, adipose tissue was triturated 5 times with a 10 ml pipette and all tissues were squeezed through a 70 or 100 μm cell strainer (Corning). Peritoneal

763 cells were collected by lavage of the peritoneum with 8-10 ml PBS. Bronchoalveolar lavage (BAL)
764 was performed by inserting a venal catheter (BD) into the trachea and 3-10 washes with 0.3-1 ml
765 FACS buffer to harvest BAL cells. Blood was harvested by bleeding from the submaxillary vein
766 or cardiac puncture. All cell suspensions were subjected for 1-5 min (blood 3 times) to Red Blood
767 Cell Lysing Buffer, washed with FACS buffer and re-filtered through a 40 μm cell strainer and
768 either directly analyzed or further processed.

Flow cytometry and cell sorting

770 Single cell suspensions were incubated for 20 min at 4°C in PBS with 2% heat inactivated-FBS
771 and 0.5 mM EDTA (Sigma) with FcR block anti-mouse CD16/CD32 antibody and a mix of
772 fluorochrome-conjugated antibodies as indicated in the key resources table. DAPI was used to
773 exclude dead cells. Autofluorescence was determined using the UV or blue laser.
774 After antibody incubation, cells were stained with Nile-Red (1 μg/ml in PBS) for 10 min at room
775 temperature (RT) or with Dihydroethidium (DHE, 20 μM in R10 without Phenol Red or FBS) for
776 1 hour at 37°C, for ROS determination. For intracellular staining related to apoptosis, the Active
777 Caspase-3 Apoptosis Kit was used according to manufacturers' instructions in combination with
778 anti-active Caspase-3 (clone C92-605) and/or Ki67 (clone SolA15) antibodies. The LSRFortessa
779 or FACSymphony cell analyzers running FACSDiva software and FlowJo Version 10 software
780 were used to record and analyze data.
781 For FACS cell sorting: lung or spleen single cell suspensions were incubated with mouse CD11c
782 MicroBeads UltraPure or Anti-F4/80 MicroBeads UltraPure, respectively, loaded onto LS
783 columns and the positive fraction collected according to manufacturers' instructions before
784 fluorescent staining. Immune cells from kidney cell suspensions were enriched using a density
785 gradient centrifugation (Biocoll cell separation solution, 700 xg centrifugation for 30 min at RT).
786 Cells were flow-sorted using the FACSAria II cell sorter running FACSDiva software.
787 TMF populations were identified as follows: $F4/80^+$ $CD64^+$ $CD206^+$ or $CD9^+$ cells in iWAT and
788 eWAT (iWAT-MFs, eWAT-MFs); $CD45^+$ $F4/80^+$ $CD64^+$ cells in kidney (KMs); $F4/80^+$ $CD11b^{low}$
789 $MHCII^{low}$ cells in spleen (RPMs); $CD45^+$ Siglec F^- $Ly6G^-$ $Ly6C^-$ $F4/80^+$ cells in liver (KCs); $CD64^-$
790 $MHCII^+$ $CD11b^+$ $CD24^+$ cells in $CD45^+$ cells in skin (LCs); $CD11b^+$ $MHCII^{low}$ $F4/80^{high}$ cells in
791 peritoneal lavage (LPMs) and $CD45^+$ $F4/80^+$ $CD11c^+$ cells in lung (AMs).
792 For magnetic MACS cell sorting of BAL: AMs were purified using mouse CD11c MicroBeads
793 UltraPure and MS columns according to manufacturers' instructions.

Analysis of single cell RNA sequencing

795 All RNA-seq-related analyses were performed in R (version 4.2.1). Single-cell RNA-seq raw
796 counts data from the Human Cell Landscape project were downloaded from
797 https://figshare.com/articles/HCL_DGE_Data/7235471. After selecting datasets based on the
798 health status of the donors and absence of inflammatory cell infiltrates to ensure homeostatic
799 samples (Figure S1A), cells annotated as TMFs were independently analyzed using Bioconductor
800 packages (Huber et al., 2015) and Seurat (Butler et al., 2018). After ruling out cells with poor
801 quality metrics (counts ≥ 400 and ≤ 3000, detected genes ≥ 200 and ≤ 1250, and % of mitochondrial
802 genes ≤ 15), further analysis of resulting TMFs were performed at the pseudo-bulk level by
803 aggregating cells according to the tissue of origin. Single-cell profiles were summarized by
804 averaging counts per million (CPM) in order to force all cells to contribute with the same extent.
805 The number of pseudo-replicates for every tissue was determined by keeping a similar number of
806 detected genes per pseudo-bulk sample. Finally, pseudo-bulk samples were transformed with
807 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) (www.genome.jp/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 (Luo et al., 2009). 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 *Tfam$^{f/f}$* and *CD11cΔTfam* 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 (Mastrangelo et al., 2016). Briefly, 1mL 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 *Tfam$^{f/f}$* and *CD11cΔTfam* mice. Samples were subjected to two freeze–thaw cycles for metabolism quenching and complete metabolite extraction, specifically by placing the samples at -80ºC for 15 min and thawing them on ice for 10 min with brief vortex-mixing. The samples were then centrifuged at 20,000 xg 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 (15mg/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), integrated with a DB5-MS column (30 m x 0.25 mm, 0.25

854 μm film, Agilent Technologies). Metabolite deconvolution and identification were carried out
855 using Agilent MassHunter Unknowns Analysis version B.07.00, then, data was aligned in Agilent
856 Mass Profiler Professional version B.12.1 and exported to Agilent MassHunter Quantitative
857 Analysis version B.07.00. Metabolites were identified by comparing their retention time, retention
858 index and mass fragmentation patterns with those available in an in-house library including both
859 the NIST mass spectral database (version 2017) and Fiehn RTL library (version 2008). Samples
860 were normalized by IS as reference feature. The result was a matrix with the compounds in the
861 samples sorted by their characteristic retention time and target ion, and the relative abundance of
862 each compound for each sample.
863 Quality Control (QC) samples (n=4) were prepared by pooling equal volumes of cell extracts from
864 each sample by following protocols mentioned above. QC samples were injected at the beginning,
865 at the end and every six samples in order to assess the reproducibility of both sample preparation
866 and data acquisition. Raw data from all samples were processed as described above. Data quality
867 was assured by using the QC samples as reference by filtering the data matrix. Specifically,
868 metabolites present in 50% of the QC samples with a coefficient of variation below 50% were
869 retained and the resulting data matrix underwent principal component analysis (PCA) on
870 Metaboanalyst website (http://www.metaboanalyst.ca/) to assess the analytical reproducibility.
871 Cube root transformed metabolites' levels from knockout and wild type mice were compared using
872 Student's t test and the p-values adjusted using the Benjamini–Hochberg method correcting for
873 false discovery rate (FDR, q=0.05). Differences were considered statistically significant when
874 p≤0.05. Individual samples (n=3 per each genotype) were generated by merging FACs-sorted AMs
875 to 10^6 cells/sample from >10 independent experiments of more than 13-30 animals/genotype.

876 ***Nucleic acid analysis and quantitative PCR***
877 From FACS-sorted TMF cells, total DNA was isolated using the QIAamp DNA Mini Kit. Tissue
878 was homogenized using an IKA Ultra-Turrax T-10 disperser and total RNA of tissue and TMFs
879 was extracted with the RNeasy Micro or Mini Kit and reverse transcribed using the High Capacity
880 cDNA Reverse Transcription Kit with random hexamers following manufacturer's instructions.
881 Quantitative PCR was performed using the GoTaq® qPCR Master Mix in a 7900HT Fast Real-
882 Time PCR System (Applied Byosystem®). Please find all sequences of primers used in Table S1
883 in the supplementary information. 2-ΔCt mRNA expression values of mouse genes were calculated
884 relative to expression of *18S rRNA* and *mt-Co2* (mt-DNA) levels were calculated relative to *Sdh*
885 (nuclear DNA). For detection of *Xbp1* splice isoforms, the PCR product was purified using the
886 MinElute PCR Purification Kit and digested with the PstI-High-Fidelity restriction enzyme for 60
887 min at 37°C. Then, DNA bands were separated by agarose gel electrophoresis and imaged
888 following standard procedures and quantified using ImageJ/Fiji (Han et al., 2009).

889 ***In vitro treatments and lactate measurement***
890 10^5 MACS-purified BAL-AMs from pools of 3 week-old mice were analyzed directly or cultured
891 for 48 hours before staining and mRNA extraction. 0.2-1x10^5 MACS-purified BAL-AMs from
892 pools of adult mice were cultured for 6 hours or 24 hours, respectively, under presence or not of
893 PERK inhibitor GSK2606414, LXR agonists 1μM T0901317 or 0.1μM GW3965 before extraction
894 of mRNA. 3x10^5 MACS-purified BAL-AMs from pools of adult mice were cultured for 3 days to
895 collect supernatant for lactate measurement using the colorimetric Lactate Assay Kit following
896 manufacturers' instructions.

897 ***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 xg and the supernatant used for measurement of optical density at 600 nm using a spectral photometer (after a 1:3 dilution). Protein levels were determined with the Pierce™ BCA Protein Assay Kit and total cholesterol levels using the Cholesterol Quantitation Kit according to manufacturers' instructions. The cell pellet was merged with the rest of the BAL washes for determination of cell debris and cell analysis by flow cytometry.

$[1-^{14}C]$-Palmitate oxidation

Measurement of FA oxidation rates was performed as previously described (Huynh et al., 2014). $1.5x10^5$ 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 ^{14}C-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 $^{14}CO_2$ trapped in a filter paper disk with 20 μl of 1 M NaOH on the top of sealed vials. ^{14}C products were counted in an LS6500 liquid scintillation counter (Beckman Coulter). Scintillation values were converted to nmol $^{14}CO_2$ 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), $1.25x10^5$ 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 CO_2 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 (Mito-stress 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

$1-5x10^4$ 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

10^5 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 from 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 floating on PBS for 30 min at RT. Epidermal sheets were blocked with 2% BSA in PBS for 2 h at RT, incubated with Langerin-Alexa Fluor 488 antibody (clone 929F3.01) at 4°C 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$^+$ F4/80$^+$ CD11c$^+$ FACS-sorted AMs from the BAL of adult *Tfam$^{f/f}$* and *CD11cΔTfam* 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 Reynold'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 Cellpose (Stringer et al., 2021) 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 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/over-grooming. All n values represent biological replicates (different mice, primary cell preparations or in vitro experiments). Differences were considered significant when $P \leq 0.05$ and are indicated as ns, not significant, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

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A Workflow for scRNA-seq data analysis
Human Cell Landscape data

Selection of healthy participants and analysis of TMFs

Filtering tissues by number of TMFs

Building of pseudo-bulk samples

PC1

B PCA human macrophages

PC2 (8.6%)

PC1 (11%)

- Adipose Tissue
- Esophagus
- Gallbladder
- Heart
- Kidney
- Liver
- Lung
- Omentum
- Pleura
- Spleen

C

Pathway (human)	Adj. p-value	Absolute NES
ATP synthesis coupled electron transport	1.7E-19	3.54
Mitochondrial ATP synthesis coupled electron transport	1.7E-19	3.54
Aerobic electron transport chain	6.1E-18	3.44
NADH dehydrogenase complex assembly	2.0E-15	3.40
Mitochondrial respiratory chain complex I assembly	2.0E-15	3.40
Oxidative phosphorylation	8.8E-18	3.36
Respiratory electron transport chain	2.4E-16	3.26
Mitochondrial respiratory chain complex assembly	1.3E-16	3.20
Mitochondrial electron transport, NADH to ubiquinone	3.3E-11	3.15
Electron transport chain	1.7E-18	3.11

D OXPHOS genes

Enrichment score

Rank in PC1 scores (human)

E PCA mouse macrophages

PC2 (11.9%)

PC1 (27%)

- Brain Microglia
- Kidney MFs
- Peritoneal MFs
- Lean Inguinal Fat MFs
- Lean Mesenteric Fat MFs
- Liver Kupffer cells
- Lung Alveolar MFs
- Mesenteric Sheet MFs
- Spleen Red Pulp MFs
- Thymus MFs

F

Pathway (mouse)	Adj. p-value	Absolute NES
NADH dehydrogenase complex assembly	9.7E-04	2.34
Mitochondrial respiratory chain complex I assembly	9.7E-04	2.34
Histone demethylation	6.1E-03	2.23
Histone lysine demethylation	6.1E-03	2.23
Oxidative phosphorylation	9.0E-04	2.19
Mitochondrial respiratory chain complex assembly	1.1E-03	2.19
ATP synthesis coupled electron transport	3.6E-03	2.18
Mitochondrial ATP synthesis coupled electron transport	3.6E-03	2.18
Endoplasmic reticulum tubular network organization	2.7E-02	2.16
Embryonic camera-type eye morphogenesis	3.2E-02	2.12

G OXPHOS genes

Enrichment score

Rank in PC1 scores (mouse)

H Detected mitochondrial genes

	Mesenteric Sheet MFs	Lean Inguinal Fat MFs	Lean Mesenteric Fat MFs	Thymus MFs	Brain Microglia	Spleen Red Pulp MFs	Liver Kupffer cells	Kidney MFs	Peritoneal MFs	Lung Alveolar MFs	
-3.77*	-3.23*	-2.07*	0.41	0.69	1.03	2.17*	1.99*	2.54*	3.65*	Mt-nd2
-3.36*	-3.54*	-1.72*	0.89	0.33	1.21	1.83*	1.76*	2.19*	3.27*	Mt-nd4
-3.27*	-2.79*	-1.22*	0.01	0.57	1.05	1.64*	1.98*	1.96*	3.18*	Mt-nd5
-3.49*	-3.18*	-1.14*	0.43	0.54	1.42	1.59*	1.15	2.08*	3.51*	Mt-cytb
-3.46*	-2.73*	-1.64*	0.50	0.92	1.05	1.29*	1.13	2.46*	3.34*	Mt-nd1
-3.65*	-3.25*	-1.59	1.11	0.74	1.39	0.81	1.29	2.56*	3.38*	Mt-nd6
-1.94*	-1.45*	-1.63*	-1.11	0.90	1.68*	0.82	1.08	1.63*	3.37*	Mt-nd3
-3.16*	-1.21*	-0.91*	0.51	-0.42	0.79	0.99*	1.43*	1.56*	2.55*	Mt-co1
-3.26	**-2.67**	**-1.49**	**0.34**	**0.53**	**1.20**	**1.39**	**1.48**	**2.12**	**3.28**	**Average**

LogFC
-4 -2 0 2 4

A *Tfam* deletion in macrophages

B mt-DNA levels in macrophages

C Number of present macrophages

D Frequency of present macrophages

E

F Autofluorescence of macrophages

Legend (A):
- Tfam f/f
- CD11c ΔTfam
- LysM ΔTfam

Legend (E):
- Lean Inguinal Fat MFs
- Kidney MFs
- Spleen Red Pulp MFs
- Liver Kupffer cells
- Peritoneal MFs
- Lung Alveolar MFs

R=-0.896
p=0.016

A AM number

B Adult AM maturity

C Tfam f/f / CD11cΔTfam

D Adult BAL — BAL turbidy

E Proliferating 3wks AMs

F Apoptotic 3wks AMs

G Respiration of adult AMs

H Adult AM ΔΨm — TMRM / merged

I 3wks AM ATP content

J AM Lactate production

K ECAR

L TCA cycle metabolites

M TCA cycle-related genes

N AM number

O AM SiglecF / AM CD11b

A

Erp29
Casp9
Creb3
Eif2ak3
Ptpn1
Trp53
Casp3
Map3k5
Apaf1
Serinc3
Casp4
Creb3l1
Lrrk2
Bok
Txndc12
Itpr1
Ikbkg
Chac1
Trib3
Ero1l
Ddit3
Atf4
Bcl2
Ern1
Pmaip1
Selenok
Bbc3
Tmbim6
Bax
Spop
Grina
Bcl2l1

Tfam f/f   CD11c ΔTfam

Intrinsic apoptotic signaling pathway in response to ER stress
Adj. p-value=0.0046, NES=1.86

Z−score
−2 −1 0 1 2

B *Atf4*

Normalized transcript reads

***

Tfam f/f   CD11c ΔTfam

C *Xbp1* activation

% spliced *Xbp1* in total *Xbp1* mRNA

**

Tfam f/f   CD11c ΔTfam

spliced
unspliced A
unspliced B

Tfam f/f   CD11c ΔTfam

D *Atf4*

Relative mRNA levels/*18S rRNA*

*

Day 0   48h culture   Day 0   48h culture

E *Cdk1*

Relative mRNA levels/*18S rRNA*

***

Day 0   48h culture   Day 0   48h culture

Ccnb2

Relative mRNA levels/*18S rRNA*

● Tfam f/f
● CD11c ΔTfam

***

Day 0   48h culture   Day 0   48h culture

F

Tfam f/f   CD11cΔTfam

Autofluorescence

18.2   3.28   Day 0

25.0   14.7   48h culture

Ki67

Proliferation of cultured 3wks AMs

% Ki67+ cells

**

Day 0   48h culture   Day 0   48h culture

A Lipid content adult AMs

B

Lipid/Cholesterol whorls

C Palmitate oxidation

D

E

F Cholesterol

G Cholesterol uptake | Cholesterol efflux

H BAL cholesterol

I AM number

A Weight

Tfam f/f | LysMΔTfam

B *Atf4* expression — Proliferating eWAT-MFs

C Obese eWAT-MF number

Tfam f/f

CD11cΔTfam

D CLS quantification

Tfam f/f | LysMΔTfam

500μm

E eWAT cytokine levels

F Leukocyte count

G Lipid metabolism in eWAT

Pparg *Ppara* *Pnpla2* *Lipe* *Hmgcs2* *Hmgcl* *Cebpa* *Fasn*

Lipid catabolism | Lipolysis | Ketogenesis | Lipogenesis

H Mitochondrial respiration in eWAT

Ppargc1a *Uqcrq* *Cox10* *Atp5a1*

I Insulin tolerance test

J Liver lipid content

Tfam f/f | LysMΔTfam

100μm

K Obese eWAT-MF number

Total CD206+ MerTK+ CD11c+ CD9+

L Infrared thermal measurements

Uqcrq f/f | CD11cΔUqcrq

Maximal | Average

38.7

23.7

A

Langerhans cell number

Red pulp macrophage number

● Control (f/f or f/Δ)
● CD11c-Cre+ (f/f or f/Δ)

B

ATP content LPMs

ATP content RPMs

C

Lipid content LPMs

Lipid content RPMs

D

Atf4 LPMs

Atf4 RPMs

E

Splenic red pulp macrophages

F

Mouse macrophages

R=0.66, p=0.036
T-score using log–FC

R=0.66, p=0.039
T-score using log–FC

● Brain Microglia
● Kidney MFs
● Peritoneal MFs
● Lean Inguinal Fat MFs
● Lean Mesenteric Fat MFs
● Liver Kupffer cells
● Lung Alveolar MFs
● Mesenteric Sheet MFs
● Spleen Red Pulp MFs
● Thymus MFs

G

Human macrophages

R=0.76, p=0.01
T-score using log–FC

R=0.76, p=0.0099
T-score using log–FC

R=0.68, p=0.032
T-score using log–FC

● Adipose Tissue
● Esophagus
● Gallbladder
● Heart
● Kidney
● Liver
● Lung
● Omentum
● Pleura
● Spleen

H

Table S1. Related to STAR methods and key resources table.

REAGENT or RESOURCE	SOURCE
Oligonucleotides	
qPCR *Tfam* sense (5′)– CAGGAGGCAAAGGATGATTC–(3′), anti-sense (5′)– CCAAGACTTCATTTCATTGTCG–(3′)	(Desdín-Micó et al., 2020)
qPCR *Slc2a1* sense (5′)– GGGCATGTGCTTCCAGTATGT–(3′), anti-sense (5′)– ACGAGGAGCACCGTGAAGAT–(3′)	(Izquierdo et al., 2018)
qPCR *Atf4* sense (5′)– ATGGCCGGCTATGGATGAT–(3′), anti-sense (5′)– CGAAGTCAAACTCTTTCAGATCCATT–(3′)	This paper
qPCR *Bax* sense (5′)– CTACAGGGTTTCATCCAG–(3′), anti-sense (5′)– CCAGTTCATCTCCAATTCG–(3′)	This paper
qPCR *Cdk1* sense (5′)– AGAAGGTACTTACGGTGTGGT–(3′), anti-sense (5′)– GAGAGATTTCCCGAATTGCAGT–(3′)	(Izquierdo et al., 2018)
qPCR *Cnnb2* sense (5′)– GCCAAGAGCCATGTGACTATC –(3′), anti-sense (5′)– CAGAGCTGGTACTTTGGTGTTC –(3′)	(Izquierdo et al., 2018)
qPCR *Tnfa* sense (5′)– CCCTCACACTCAGATCATCTTCT –(3′), anti-sense (5′)– GCTACGACGTGGGCTACAG –(3′)	(Hernández-García et al., 2022)
qPCR *Ccl2* sense (5′)– GTCCCTGTCATGCTTCTGG –(3′), anti-sense (5′)– GCGTTAACTGCATCTGGCT –(3′)	This paper
qPCR *Abcg1* sense (5′)– TGACACATCTGCGAATCAC –(3′), anti-sense (5′)– AGGGGAAAGGTCAGAACG –(3′)	This paper
qPCR *Pparg* sense (5′)– GGAAGACCACTCGCATTCCTT –(3′), anti-sense (5′)– TCGCACTTTGGTATTCTTGGAG –(3′)	This paper
qPCR *Ppara* sense (5′)– ACAAGGCCTCAGGGTACCA –(3′), anti-sense (5′)– GCCGAAAGAAGCCCTTACA –(3′)	(Martínez-López et al., 2019)
qPCR *Pnpla2* sense (5′)– TTCACCATCCGCTTGTTGGAG –(3′), anti-sense (5′)– AGATGGTCACCCAATTTCCTC –(3′)	This paper
qPCR *Lipe* sense (5′)– TCCTCAGAGACCTCCGACTG –(3′), anti-sense (5′)– ACACACTCCTGCGCATAGAC –(3′)	This paper
qPCR *Hmgcs2* sense (5′)– ATACCACCAACGCCTGTTATGG –(3′), anti-sense (5′)– AATGTCACCACAGACCACCAG –(3′)	This paper
qPCR *Hmgcl* sense (5′)– ACTACCCAGTCCTGACTCCAA –(3′), anti-sense (5′)– TAGAGCAGTTCGCGTTCTTCC –(3′)	This paper
qPCR *Cebpa* sense (5′)– CAAGAACAGCAACGAGTACCG –(3′), anti-sense (5′)– GTCACTGGTCAACTCCAGCAC –(3′)	This paper
qPCR *Fasn* sense (5′)– AGAGATCCCGAGACGCTTCT –(3′), anti-sense (5′)– GCTTGGTCCTTTGAAGTCGAAGA –(3′)	This paper
qPCR *Ppargc1a* sense (5′)– TATGGAGTGACATAGAGTGTGCT –(3′), anti-sense (5′)– CCACTTCAATCCACCCAGAAAG –(3′)	(Matesanz et al., 2018)
qPCR *Uqcrq* sense (5′)– CCTACAGCTTGTCGCCCTTT –(3′), anti-sense (5′)– GATCAGGTAGACCACTACAAACG –(3′)	This paper
qPCR *Cox10* sense (5′)– AGAAGAQGCTATACAGGGATTGCC –(3′), anti-sense (5′)– CTGTGTGACATACATGCGCTT –(3′)	This paper
qPCR *Atp5a1* sense (5′)– CACAGCTGAGATGTCCTCCA –(3′), anti-sense (5′)– CATTGTCGGGTTCCAAGTTC –(3′)	This paper
qPCR *Nos2* sense (5′)– AATCTTGGAGCGAGTTGTGG –(3′), anti-sense (5′)– CAGGAAGTAGGTGAGGGCTTG –(3′)	This paper
qPCR *Cd80* sense (5′)– CTGGGAAAAACCCCCAGAAG –(3′), anti-sense (5′)– TGACAACGATGACGACGACTG –(3′)	This paper
qPCR *Il10* sense (5′)– GCTCTTACTGACTGGCATGAG –(3′), anti-sense (5′)– CGCAGCTCTAGGAGCATGTG –(3′)	(Hernández-García et al., 2022)

qPCR *Stat6* sense (5′)– CTCTGTGGGGCCTAATTTCCA –(3′), anti-sense (5′)– CATCTGAACCGACCAGGAACT –(3′)	This paper
qPCR *Tgfb1* sense (5′)– GCAGTGGCTGAACCAAGGA –(3′), anti-sense (5′)– AAAGAGCAGTGAGCGCTGAATC –(3′)	(Martínez-López et al., 2019)
qPCR *Vegfa* sense (5′)– AGTCCCATGAAGTGATCAAGTTCA –(3′), anti-sense (5′)– ATCCGCATGATCTGCATGG –(3′)	This paper
qPCR *Ifng* sense (5′)– CTGCCACGGCACAGTCATTG –(3′), anti-sense (5′)– TGCATCCTTTTTCGCCTTGC –(3′)	(Hernández-García et al., 2022)
qPCR *Il1b* sense (5′)– CTGAACTCAACTGTGAAATGCCA –(3′), anti-sense (5′)– AAAGGTTTGGAAGCAGCCCT –(3′)	(Hernández-García et al., 2022)
qPCR *Arg1* sense (5′)– CTCCAAGCCAAAGTCCTTAGAG –(3′), anti-sense (5′)– AGGAGCTGTCATTAGGGACATC –(3′)	(Izquierdo et al., 2018)
qPCR *Mrc1* sense (5′)– TGATTACGAGCAGTGGAAGC –(3′), anti-sense (5′)– GTTCACCGTAAGCCCAATTT –(3′)	(Rao et al., 2014)
qPCR *Clec10a* sense (5′)– CTCTGGAGAGCACAGTGGAG –(3′), anti-sense (5′)– ACTTCCGAGCCGTTGTTCT –(3′)	(Rao et al., 2014)
qPCR *Retnla* sense (5′)– CCAATCCAGCTAACTATCCCTCC –(3′), anti-sense (5′)– ACCCAGTAGCAGTCATCCCA –(3′)	(Rao et al., 2014)
qPCR *Siglecf* sense (5′)– CTGGCTACGGACGGTTATTCG –(3′), anti-sense (5′)– GGAATTGGGGTACTGGACTTG –(3′)	(Rao et al., 2014)
qPCR *Il4* sense (5′)– GGTCTCAACCCCCAGCTAGT –(3′), anti-sense (5′)– GCCGATGATCTCTCTCAAGTGAT –(3′)	(Rao et al., 2014)
qPCR *Il13* sense (5′)– CCTGGCTCTTGCTTGCCTT –(3′), anti-sense (5′)– GGTCTTGTGTGATGTTGCTCA –(3′)	(Rao et al., 2014)
qPCR *Ucp1* sense (5′)– GTGAACCCGACAACTTCCGAA –(3′), anti-sense (5′)– TGCCAGGCAAGCTGAAACTC –(3′)	(Rao et al., 2014)
qPCR *Cidea* sense (5′)– TGCTCTTCTGTATCGCCCAGT –(3′), anti-sense (5′)– GCCGTGTTAAGGAATCTGCTG –(3′)	(Rao et al., 2014)
qPCR *Dio2* sense (5′)– CAGTGTGGTGCACGTCTCCAATC –(3′), anti-sense (5′)– TGAACCAAAGTTGACCACCAG –(3′)	(Rao et al., 2014)
qPCR *Prdm16* sense (5′)– CAGCACGGTGAAGCCATTC –(3′), anti-sense (5′)– GCGTGCATCCGCTTGTG –(3′)	(Rao et al., 2014)
qPCR *Esrra* sense (5′)– GCAGGGCAGTGGGAAGCTA –(3′), anti-sense (5′)– CCTCTTGAAGAAGGCTTTGCA –(3′)	(Rao et al., 2014)
qPCR *Xbp1* sense (5′)– AAACAGAGTAGCAGCTCAGACTGC –(3′), anti-sense (5′)– TCCTTCTGGGTAGACCTCTGGGA –(3′)	(Han et al., 2009)
qPCR *18S rRNA* sense (5′)– GTAACCCGTTGAACCCCATT–(3′), anti-sense (5′)– CCATCCAATCGGTAGTAGCG–(3′)	This paper
qPCR *mt-Co2* sense (5′)–CTACAAGACGCCACAT–(3′), anti-sense (5′)–GAGGGGGAGAGCAAT–(3′)	(Matesanz et al., 2018)
qPCR *Sdh* sense (5′)–TACTACAGCCCCAAGTCT–(3′), anti-sense (5′)–TGGACCCATCTTCTATGC–(3′)	(Matesanz et al., 2018)

A

Dataset name	Geo accession code	Donor age	Donor sex	Tissue
Adult-Adipose1	GSM3943046	36	Male	Adipose
Adult-Esophagus2	GSM3980134	56	Male	Intestine
Adult-Gall-Bladder2	GSM3980136	58	Male	Gall-Bladder
Adult-Gall-Bladder1	GSM3980137	45	Male	Gall-Bladder
Adult-Heart1	GSM3980138	52	Male	Heart
Adult-Heart2	GSM3980139	47	Female	Heart
Adult-Kidney2	GSM4008619	66	Male	Kidney
Adult-Liver2	GSM4008625	52	Male	Liver
Adult-Liver4-1	GSM4008626	23	Female	Liver
Adult-Lung1	GSM4008628	21	Male	Lung
Adult-Lung2	GSM4008629	21	Female	Lung
Adult-Omentum2	GSM4008635	41	Male	Omentum
Adult-Pleura1	GSM4008645	46	Male	Pleura
Adult-Spleen1-1	GSM4008649	51	Female	Spleen

E Enriched gene sets PC1 (KEGG database)

Pathway (human)	Adj. p-value	Absolute NES
Oxidative phosphorylation	3.4E-33	3.84
Proteasome	9.4E-08	2.69
Antigen processing and presentation	5.7E-07	2.50
Ribosome	4.3E-13	2.49
Intestinal immune network for IgA production	5.2E-05	2.41
Porphyrin and chlorophyll metabolism	2.8E-04	2.40
Cardiac muscle contraction	3.6E-05	2.30
MAPK signaling pathway	7.4E-13	2.20
Cell adhesion molecules (CAMs)	1.0E-05	2.15
Focal adhesion	8.2E-09	2.09

F Enriched gene sets PC2 (GO database)

Pathway (human)	Adj. p-value	Absolute NES
Oxidative phosphorylation	7.9E-09	2.53
Cytoplasmic translation	6.7E-10	2.53
ATP synthesis coupled electron transport	4.3E-07	2.45
Mitochondrial ATP synthesis coupled electron transport	4.3E-07	2.45
ATP metabolic process	5.3E-10	2.43
Embryo implantation	2.7E-05	2.40
Aerobic electron transport chain	1.3E-05	2.37
Respiratory electron transport chain	1.2E-06	2.35
Ribosome assembly	7.8E-05	2.27
Ribosomal large subunit biogenesis	2.8E-04	2.17

G Enriched gene sets PC2 (KEGG database)

Pathway (human)	Adj. p-value	Absolute NES
Ribosome	4.8E-18	3.03
Oxidative phosphorylation	3.7E-16	2.83
Proteasome	2.8E-07	2.54
Cardiac muscle contraction	6.5E-06	2.38
Collecting duct acid secretion	2.9E-03	2.07
ECM-receptor interaction	9.6E-04	2.06
Protein export	5.6E-03	1.99
Glycolysis / Gluconeogenesis	3.8E-03	1.93
Hematopoietic cell lineage	4.2E-03	1.92
T cell receptor signaling pathway	3.8E-03	1.81

Figure S1. Related to Figure 1.

(A) List of publicly available scRNAseq datasets with donor information from the Human Cell Landscape (https://db.cngb.org/HCL/) that were used for the analysis of human TMF clusters shown in Figure 1A.
(B) Number of human TMF cells from each tissue used to build pseudo-bulk samples.
(C) Heatmap of samples clustered using hierarchical clustering based on Euclidean distances transformed into similarities and the complete agglomeration method of each pseudo-bulk sample of human TMF cells.

(D) Boxplots of number of detected genes per pseudo-bulk sample. Human TMF pseudo-bulk samples were built controlling for the number of detected genes per sample in order to avoid possible biases in further analysis. Ranges are within 7734 and 9733 genes.

(E to G) Pre-ranked enrichment analysis of the genes defining PC1 (E) or PC2 (F and G) of human TMFs in the reference pathways of the KEGG database (https://www.genome.jp/kegg/pathway.html) (E and G) or in the biological processes of the GO resource (http://geneontology.org/) (F). NES, normalized enrichment score; Adj., adjusted with Benjamini–Hochberg correction.

(H) Heat map of log-Fold change (FC) of all genes included in the KEGG reference pathway "oxidative phosphorylation" (hsa00190) in the TMF clusters identified in the human scRNAseq data analysis calculated comparing each individual tissue with the mean of all tissues. Genes (rows) and samples (columns) are ordered using hierarchical clustering of log-Fold changes and the complete agglomeration method.

A

GEO accession	Cell type	Sorting markers	Strain	Name	Tissue
GSM2932557	Macrophage	CD45+ CD11c+ SiglecF+	C57BL/6J	MF.Alv.Lu#1	Lung
GSM2932558	Macrophage	CD45+ CD11c+ SiglecF+	C57BL/6J	MF.Alv.Lu#2	Lung
GSM2932561	Macrophage	F4/80+ICAM2+CD5-CD19-CD43-	C57BL/6J	MF.PC#3	Peritoneal Cavity
GSM2932562	Macrophage	F4/80+ICAM2+CD5-CD19-CD43-	C57BL/6J	MF.PC#4	Peritoneal Cavity
GSM4057887	Macrophage	CD115+CD11b+F4/80loCD102loMHCII+CD226+	C57BL/6J	MF.226+II+480lo.PC#1	Peritoneal Cavity
GSM4057888	Macrophage	CD115+CD11b+F4/80loCD102loMHCII+CD226+	C57BL/6J	MF.226+II+480lo.PC#2	Peritoneal Cavity
GSM3455075	Macrophage	Lin-F4/80+CD64+	C57BL/6J	MF.64p.Th.1	Thymus
GSM3455076	Macrophage	Lin-F4/80+CD64+	C57BL/6J	MF.64p.Th.2	Thymus
GSM3455077	Macrophage	Lin-F4/80+CD64+	C57BL/6J	MF.64p.Th.3	Thymus
GSM3455078	Macrophage	Lin-F4/80+CD64+	C57BL/6J	MF.64p.Th.4	Thymus
GSM3455195	Macrophage	Clec4F+ Tim4+ CD45+ F4/80+ CD64+	C57BL/6J	MF.KC.Clec4FpTim4p64p.Lv.2	Liver
GSM3455196	Macrophage	Clec4F+ Tim4+ CD45+ F4/80+ CD64+	C57BL/6J	MF.KC.Clec4FpTim4p64p.Lv.3	Liver
GSM3455197	Macrophage	Clec4F+ Tim4+ CD45+ F4/80+ CD64+	C57BL/6J	MF.KC.Clec4FpTim4p64p.Lv.4	Liver
GSM3455299	Macrophage	CD45+ CD11b+ MHCII+ F4/80hi Lin- Ly6G-	C57BL/6J	MF.6Gn480hi.Kd.1	Kidney
GSM3455300	Macrophage	CD45+ CD11b+ MHCII+ F4/80hi Lin- Ly6G-	C57BL/6J	MF.6Gn480hi.Kd.2	Kidney
GSM3455386	Macrophage	CD45+ CD11b+ MerTK+ CD64+ F4/80+	C57BL/6J	MF.480p64pMerTKp.WAT.1	Inguinal Fat
GSM3455387	Macrophage	CD45+ CD11b+ MerTK+ CD64+ F4/80+	C57BL/6J	MF.480p64pMerTKp.WAT.2	Inguinal Fat
GSM3455388	Macrophage	CD45+ CD11b+ MerTK+ CD64+ F4/80+	C57BL/6J	MF.480p64pMerTKp.WAT.3	Inguinal Fat
GSM3455389	Macrophage	CD45+ CD11b+ CD64+ F4/80+ Lyve1+	C57BL/6J	MF.480p64pLyve1p.MS.1	Mesenteric Sheet
GSM3455390	Macrophage	CD45+ CD11b+ CD64+ F4/80+ Lyve1+	C57BL/6J	MF.480p64pLyve1p.MS.2	Mesenteric Sheet
GSM3455391	Macrophage	CD45+ CD11b+ CD64+ F4/80+ Lyve1+	C57BL/6J	MF.480p64pLyve1p.MS.3	Mesenteric Sheet
GSM3455392	Macrophage	CD45+ CD11b+ CD64+ F4/80+ Lyve1+	C57BL/6J	MF.480p64pLyve1p.MS.4	Mesenteric Sheet
GSM3455396	Macrophage	CD45+ CD11b+ MerTK+ CD64+ F4/80+	C57BL/6J	MF.480p64pMerTKp.MAT.1	Mesenteric Fat
GSM3455397	Macrophage	CD45+ CD11b+ MerTK+ CD64+ F4/80+	C57BL/6J	MF.480p64pMerTKp.MAT.2	Mesenteric Fat
GSM3455398	Macrophage	CD45+ CD11b+ MerTK+ CD64+ F4/80+	C57BL/6J	MF.480p64pMerTKp.MAT.3	Mesenteric Fat
GSM3455399	Macrophage	CD45+ CD11b+ MerTK+ CD64+ F4/80+	C57BL/6J	MF.480p64pMerTKp.MAT.4	Mesenteric Fat
GSM4057889	Macrophage	CD19-CD3-Ly6G-CD45loCD11b+ CD64+ F4/80loCD206-	C57BL/6J	MF.microglia.CNS#1	Brain
GSM4057890	Macrophage	CD19-CD3-Ly6G-CD45loCD11b+ CD64+ F4/80loCD206-	C57BL/6J	MF.microglia.CNS#2	Brain
GSM4057891	Macrophage	Mertk+ CD64+ CD11blo F4/80+	C57BL/6J	MF.RP.Sp#1	Spleen
GSM4057892	Macrophage	Mertk+ CD64+ CD11blo F4/80+	C57BL/6J	MF.RP.Sp#2	Spleen

B Enriched gene sets PC1 (KEGG database)

Pathway (mouse)	Adj. p-value	Absolute NES
Ribosome	6.4E-05	2.30
NOD-like receptor signaling pathway	1.3E-02	1.93
TGF-beta signaling pathway	1.3E-02	1.92
Cardiac muscle contraction	3.1E-02	1.80
Oxidative phosphorylation	1.3E-02	1.80
mRNA surveillance pathway	2.1E-02	1.79
Huntington's disease	1.3E-02	1.76
Natural killer cell mediated cytotoxicity	1.9E-02	1.74
RIG-I-like receptor signaling pathway	4.2E-02	1.72
Ubiquitin mediated proteolysis	1.3E-02	1.70

C Enriched gene sets PC2 (GO database)

Pathway (mouse)	Adj. p-value	Absolute NES
Aerobic respiration	2.5E-15	2.80
Oxidative phosphorylation	2.8E-11	2.71
ATP synthesis coupled electron transport	2.5E-09	2.67
Mitochondrial ATP synthesis coupled electron transport	2.5E-09	2.67
Cytoplasmic translation	1.4E-09	2.63
NADH dehydrogenase complex assembly	7.6E-08	2.57
Mitochondrial respiratory chain complex I assembly	7.6E-08	2.57
Cellular respiration	2.1E-12	2.55
Mitochondrial respiratory chain complex assembly	1.1E-08	2.55
Aerobic electron transport chain	6.9E-07	2.53

D Enriched gene sets PC2 (KEGG database)

Pathway (mouse)	Adj. p-value	Absolute NES
Ribosome	1.8E-28	3.40
Oxidative phosphorylation	2.5E-22	3.11
Parkinson's disease	5.8E-20	3.00
Alzheimer's disease	4.3E-22	2.95
Huntington's disease	1.1E-20	2.89
Proteasome	2.6E-10	2.66
Citrate cycle (TCA cycle)	6.7E-05	2.29
DNA replication	6.7E-05	2.18
Protein export	5.2E-04	2.15
Fatty acid metabolism	5.2E-04	2.03

E Genes included in the KEGG reference pathway "oxidative phosphorylation" (mmu00190)

F

Figure S2. Related to Figure 1.

(A) List of publicly available RNAseq data from the Immunological Genome Project (https://www.immgen.org/ImmGenpubs.html) that were used for the analysis of mouse TMF populations shown in Figure 1C-1E.

5

(B to D) Pre-ranked enrichment analysis of the genes defining PC1 (B) or PC2 (C and D) of mouse TMFs in the reference pathways of the KEGG database (B and D) or in the biological processes of the GO resource (C). NES, normalized enrichment score; Adj., adjusted with Benjamini–Hochberg correction.

(E and F) Heat map of log-Fold change (FC) of all genes included in the KEGG reference pathway "oxidative phosphorylation" (mmu00190) (E) and heatmap of Z score of *Tfam* (F) in the mouse TMF population RNAseq data analysis calculated comparing each individual tissue with the mean of all tissues. Genes (rows) and samples (columns) are ordered using hierarchical clustering of log-Fold changes and the complete agglomeration method.
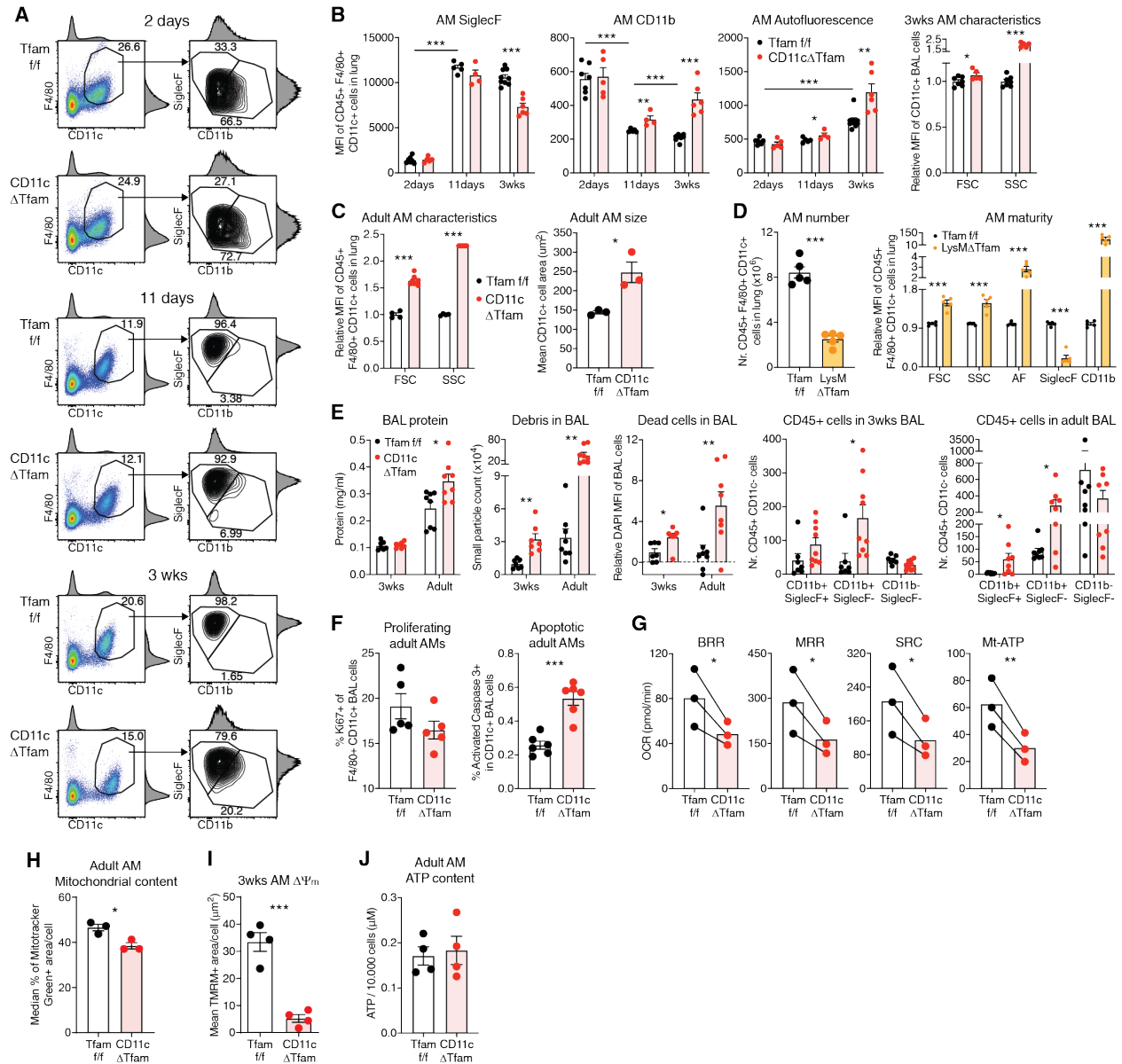
Figure S3. Related to Figure 3.

2 day-old, 11 day-old, 3 week (wks)-old or adult (6-8 week-old) mice or their AMs in the lung were analyzed.

(A and B) Representative plots (A, gated on CD45^{+} lung cells) and (relative) median fluorescence intensity (MFI) of autofluorescence, Siglec F, CD11b, FSC or SSC (B) of CD45^{+} F4/80^{+} CD11c^{+} cells in the lung or CD11c^{+} cells in the BAL of *Tfam^{f/f}* and *CD11cΔTfam* mice at various ages (n=4-9).

(C) Relative MFI of FSC or SSC of CD45^{+} F4/80^{+} CD11c^{+} cells in the lung (left, n=4-8) and cell size determined by fluorescence microscopy of CD11c^{+} cells from BAL of adult *Tfam^{f/f}* and *CD11cΔTfam* mice (right, n=3 [mean of 54-231 cells/mouse]).

(D) Flow cytometric quantification of numbers (left) and relative MFI of FSC, SSC, autofluorescence (AF), Siglec F or CD11b (right) of CD45^{+} F4/80^{+} CD11c^{+} cells in the lung of adult *Tfam^{f/f}* and *LysMΔTfam* mice (n=5).

(E) Analysis of BAL of 3 week-old or adult *Tfam^{f/f}* and *CD11cΔTfam* mice for protein levels, amount of debris, levels of dead DAPI^{+} cells and number of CD45^{+} CD11c^{-} CD11b[+ or −] Siglec F[+ or -] cells (n=7-9).

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(F) Flow cytometric quantification and representative plots of frequency of Ki67$^+$ (n=5, left) or activated caspase 3$^+$ cells (n=6, right) in (F4/80$^+$) CD11c$^+$ cells in BAL of adult *Tfam$^{f/f}$* and *CD11cΔTfam* mice.

(G) Basal respiratory rate (BRR), maximal respiratory rate (MRR), spare respiratory capacity (SRC) and respiration linked to mitochondrial ATP (mt-ATP) production of CD11c$^+$ cells from BAL of adult *Tfam$^{f/f}$* and *CD11cΔTfam* mice (n=3 merged from 5-10 mice).

(H) Quantification of mitochondrial content by fluorescence microscopy of MitoTracker Green staining of CD11c+ cells from BAL of adult *Tfam$^{f/f}$* and *CD11cΔTfam* mice (n=3 [mean of 54-231 cells/mouse]).

(I) Quantification of mitochondrial membrane potential (ΔΨm) by fluorescence microscopy of Tetramethylrhodamine methyl ester (TMRM) staining of CD11c$^+$ cells from BAL of 3 week-old *Tfam$^{f/f}$* and *CD11cΔTfam* mice (n=4 [mean of 9-85 cells/mouse]).

(J) ATP levels in F4/80$^+$ CD11c$^+$ cells from the BALs of adult mice (n=4).

Data are merged from at least 2 independent experiments and presented as mean ± SEM. Dots represent individual data points. Statistical analysis by unpaired (B-F, H-J) or paired (G) Student's t-test. *p≤0.05, **p≤0.01, ***p≤0.001.

A Differential expression

B

Upregulated Pathways (KEGG)	Adj. p-value	NES
Ribosome	0.0E+00	2.68
Lysosome	6.6E-05	2.00
Synaptic vesicle cycle	6.3E-03	1.93
Glutamatergic synapse	3.0E-03	1.91
Complement and coagulation cascades	9.1E-03	1.90
Cysteine and methionine metabolism	2.6E-02	1.82
Alanine, aspartate and glutamate metabolism	2.8E-02	1.79
Pantothenate and CoA biosynthesis	4.4E-02	1.79
Collecting duct acid secretion	5.3E-02	1.75
Biosynthesis of amino acids	4.1E-02	1.75

Downregulated Pathways (KEGG)	Adj. p-value	NES
Fanconi anemia pathway	3.8E-06	-2.27
Oxidative phosphorylation	0.0E+00	-2.26
DNA replication	3.8E-06	-2.25
Cell cycle	2.0E-07	-2.20
Homologous recombination	2.5E-04	-2.07
Thermogenesis	3.8E-06	-1.99
Mismatch repair	1.9E-03	-1.98
Cardiac muscle contraction	2.8E-02	-1.70
Base excision repair	6.1E-02	-1.63
Nucleotide excision repair	6.4E-02	-1.57

Upregulated Pathways (GO)	Adj. p-value	NES
Cytoplasmic translation	1.5E-06	2.19
Vascular transport	1.4E-03	2.01
Transport across blood-brain barrier	1.4E-03	2.01
Amino acid import across plasma membrane	2.9E-03	1.94
Anion transmembrane transport	4.5E-04	1.94
Lung alveolus development	3.4E-03	1.93
Modified amino acid transport	5.2E-03	1.93
L-amino acid transport	7.7E-03	1.93
Amino acid transmembrane transport	3.4E-03	1.93
Monocyte chemotaxis	9.4E-03	1.91
Response to interferon-gamma	8.5E-04	1.90
Amino acid import	7.5E-03	1.90
Cellular modified amino acid metabolic process	8.1E-04	1.90
Pigment cell differentiation	1.1E-02	1.89
Organic acid transmembrane transport	1.8E-03	1.89
Carboxylic acid transmembrane transport	1.8E-03	1.89
Intrinsic apoptotic signaling pathway in response to endoplasmic reticulum	4.6E-03	1.86
Cell junction disassembly	2.4E-02	1.86
L-alpha-amino acid transmembrane transport	1.4E-02	1.85
Positive regulation of endothelial cell migration	4.8E-03	1.85

Downregulated Pathways (GO)	Adj. p-value	NES
Mitotic sister chromatid segregation	0.0E+00	-2.74
Chromosome segregation	0.0E+00	-2.72
Nuclear chromosome segregation	0.0E+00	-2.70
Sister chromatid segregation	0.0E+00	-2.62
Meiotic cell cycle process	0.0E+00	-2.60
Meiotic nuclear division	0.0E+00	-2.59
DNA-dependent DNA replication	0.0E+00	-2.58
Meiotic cell cycle	0.0E+00	-2.50
Chromosome separation	0.0E+00	-2.47
DNA replication initiation	1.0E-07	-2.46
Nuclear division	0.0E+00	-2.44
DNA replication	0.0E+00	-2.44
Mitochondrial electron transport, NADH to ubiquinone	3.0E-07	-2.44
Cell division	0.0E+00	-2.44
Recombinational repair	0.0E+00	-2.43
Regulation of chromosome segregation	0.0E+00	-2.43
ATP synthesis coupled electron transport	1.0E-07	-2.43
DNA recombination	0.0E+00	-2.42
Double-strand break repair via homologous recombination	0.0E+00	-2.41
Electron transport chain	0.0E+00	-2.40

C

Metabolite	p-value	Adj. p-value	FC
Isoleucine	4.2E-04	1.6E-02	1.81
Cholesterol	9.7E-04	1.7E-02	1.71
Valine	1.7E-03	1.7E-02	1.86
Myo-inositol 6-phosphate	2.1E-03	1.7E-02	1.55
Fumaric acid	2.3E-03	1.7E-02	1.57
Glutamic acid	6.7E-03	3.7E-02	1.79
Cysteine	7.1E-03	3.7E-02	2.14
Glycerol 1-phosphate	8.8E-03	4.1E-02	1.68
Threonine	1.0E-02	4.1E-02	1.68
Isocitric acid	1.2E-02	4.3E-02	1.94
Proline	1.3E-02	4.3E-02	1.42
Aspartic acid	1.6E-02	4.8E-02	1.86
Myo-inositol	1.7E-02	4.8E-02	1.90
Malic acid	1.8E-02	4.8E-02	1.83
Glycine	2.3E-02	5.7E-02	1.40
Alanine	3.0E-02	6.3E-02	1.38
Glucose	3.1E-02	6.3E-02	1.82
6-Deoxyglucose	3.1E-02	6.3E-02	1.74
Serine	4.3E-02	8.4E-02	1.30

Metabolite	p-value	Adj. p-value	FC
Palmitelaidic acid	6.0E-02	1.1E-01	1.17
Arachidonic acid	6.9E-02	1.2E-01	1.27
Threose	7.5E-02	1.3E-01	2.12
Monoglyceride (16:0)	1.5E-01	2.4E-01	1.49
Oleic Acid	1.6E-01	2.4E-01	1.26
Glycerol	1.7E-01	2.4E-01	1.25
Lactic acid	1.7E-01	2.4E-01	1.18
Citric acid	1.8E-01	2.4E-01	1.23
Eicosapentaenoic acid	1.8E-01	2.4E-01	3.86
Succinic acid	2.5E-01	3.1E-01	1.13
Palmitoleic acid	3.0E-01	3.6E-01	1.25
Arachidic acid	3.1E-01	3.6E-01	1.21
Leucine	4.3E-01	4.9E-01	1.20
Myristic acid	4.7E-01	5.2E-01	1.09
Palmitic acid	5.8E-01	6.3E-01	1.09
Stearic acid	6.3E-01	6.6E-01	1.15
Decanoic acid	6.6E-01	6.7E-01	1.06
Pyruvic acid	6.7E-01	6.7E-01	1.06

D OXPHOS-related genes

E Cell cycle-related genes

F Apoptosis-related genes

J Amino acid metabolism-related genes

G *Slc2a1* expression adult AMs

H Glucose metabolism

I Glycolysis-related genes

K Amino acids
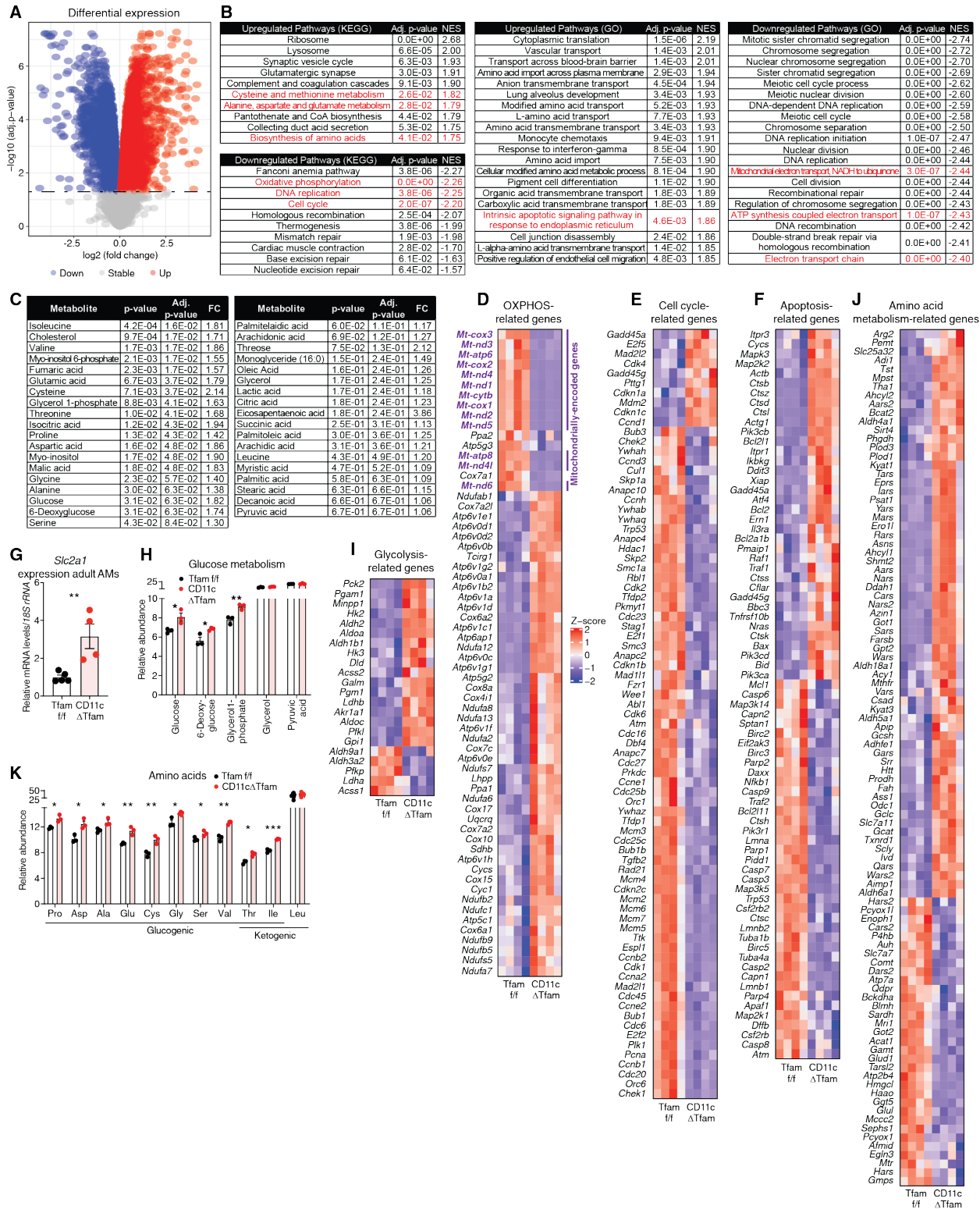
Z-score scale: 2, 1, 0, -1, -2

Figure S4. Related to Figure 3.

AMs from *Tfam^{f/f}* and *CD11cΔTfam* mice were analyzed.

(A) Volcano plot of deregulated genes detected in RNAseq of FACS-sorted CD45$^+$ F4/80$^+$ CD11c$^+$ lung cells from 3 week-old mice (n=4).

(B) Pre-ranked enrichment analysis of deregulated genes in the analysis shown in (A) in pathways of the KEGG database (left) or the GO resource (center and right). NES, normalized enrichment score *CD11cΔTfam* mice vs *Tfam$^{f/f}$* AMs; Adj., adjusted with Benjamini–Hochberg correction.

(C) Overview of metabolites detected by untargeted GC/MS analysis of FACS-sorted F4/80$^+$ CD11c$^+$ cells from BAL of adult mice (n=3 merged from 13-30 mice). FC, fold-change *CD11cΔTfam* mice vs *Tfam$^{f/f}$* AMs.

(D to F) Heat map of expression levels of deregulated (Adj. p-value <0.05) OXPHOS-related (KEGG reference pathway mmu00190, D), cell cycle-related (KEGG mmu04110, E) and apoptosis-related (KEGG mmu04210, F) genes detected in the RNAseq of FACS-sorted CD45$^+$ F4/80$^+$ CD11c$^+$ lung cells from 3 week-old mice shown in (A).

(G) *Slc2a1* mRNA levels in FACS-sorted F4/80$^+$ CD11c$^+$ lung cells from adult mice (n=4-5).

(H) Relative abundance of the indicated glucose metabolism intermediates detected by the GC/MS analysis of FACS-sorted F4/80$^+$ CD11c$^+$ cells from BAL of adult mice (n=3 merged from 13-30 mice) shown in (C).

(I and J) Heat map of expression levels of deregulated (Adj. p-value <0.05) glycolysis-related (KEGG mmu00010, I) and amino acid metabolism-related (GO reference pathway 0006520, J) genes detected in the RNAseq of FACS-sorted CD45$^+$ F4/80$^+$ CD11c$^+$ lung cells from 3 week-old mice shown in (A).

(K) Relative abundance of the indicated amino acids detected by the GC/MS analysis of FACS-sorted F4/80$^+$ CD11c$^+$ cells from BAL of adult mice (n=3 merged from 13-30 mice) shown in (C).

Data are merged from at least 2 independent experiments and presented as mean ± SEM. Dots represent individual data points. Statistical analysis by unpaired (G-K) with Benjamini–Hochberg correction (C) Student's t-test. *p≤0.05, **p≤0.01, ***p≤0.001.
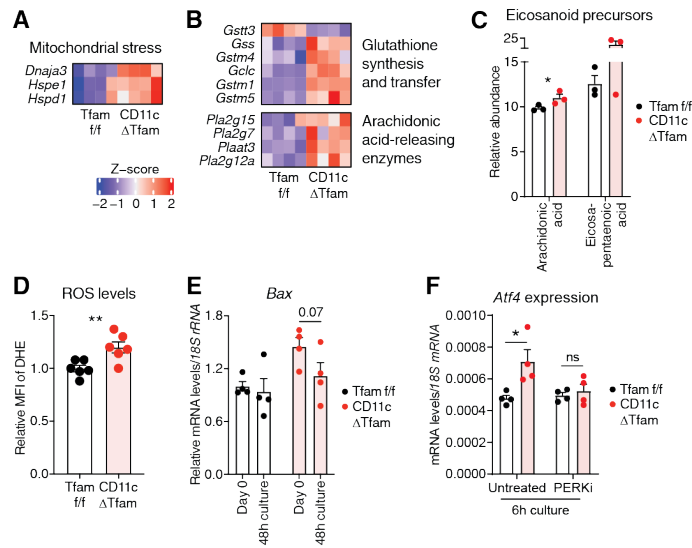
Figure S5. Related to Figure 4.

Tfam^{f/f} and *CD11cΔTfam* mice or their AMs in the lung were analyzed.

(A and B) Heat map of expression levels of selected mitochondrial stress-related genes (A) and glutathione synthesis and transfer-related genes (B, top) and genes encoding arachidonic acid-releasing enzymes (B, bottom) detected in the RNAseq of FACS-sorted CD45$^+$ F4/80$^+$ CD11c$^+$ lung cells from 3 week-old mice shown in Figure S4A.

(C) Relative abundance of eicosanoid precursors detected in the GC/MS analysis of FACS-sorted F4/80$^+$ CD11c$^+$ cells from BALs of adult mice (n=3 merged from 13-30 mice) shown in Figure S4C.

(D) Flow cytometric quantification of relative MFI of dihydroethidium (DHE, n=6) of F4/80$^+$ CD11c$^+$ lung cells from 3 week-old mice.

(E) CD11c$^+$ cells were magnetically purified from BALs of 3 week-old mice and either directly analyzed (Day 0) or cultured for 48h before analysis of *Bax* mRNA levels (n=4 merged from 2-4 mice).

(F) CD11c$^+$ cells were magnetically purified from BALs of adult mice and cultured for 6h under presence or not of 1μM PERK inhibitor GSK2606414 before analysis of *Atf4* mRNA levels (n=4 merged from 3-4 mice).

Data are merged from at least 2 independent experiments and presented as mean ± SEM. Dots represent individual data points. Statistical analysis by unpaired Student's t-test. *p≤0.05, ns not significant.
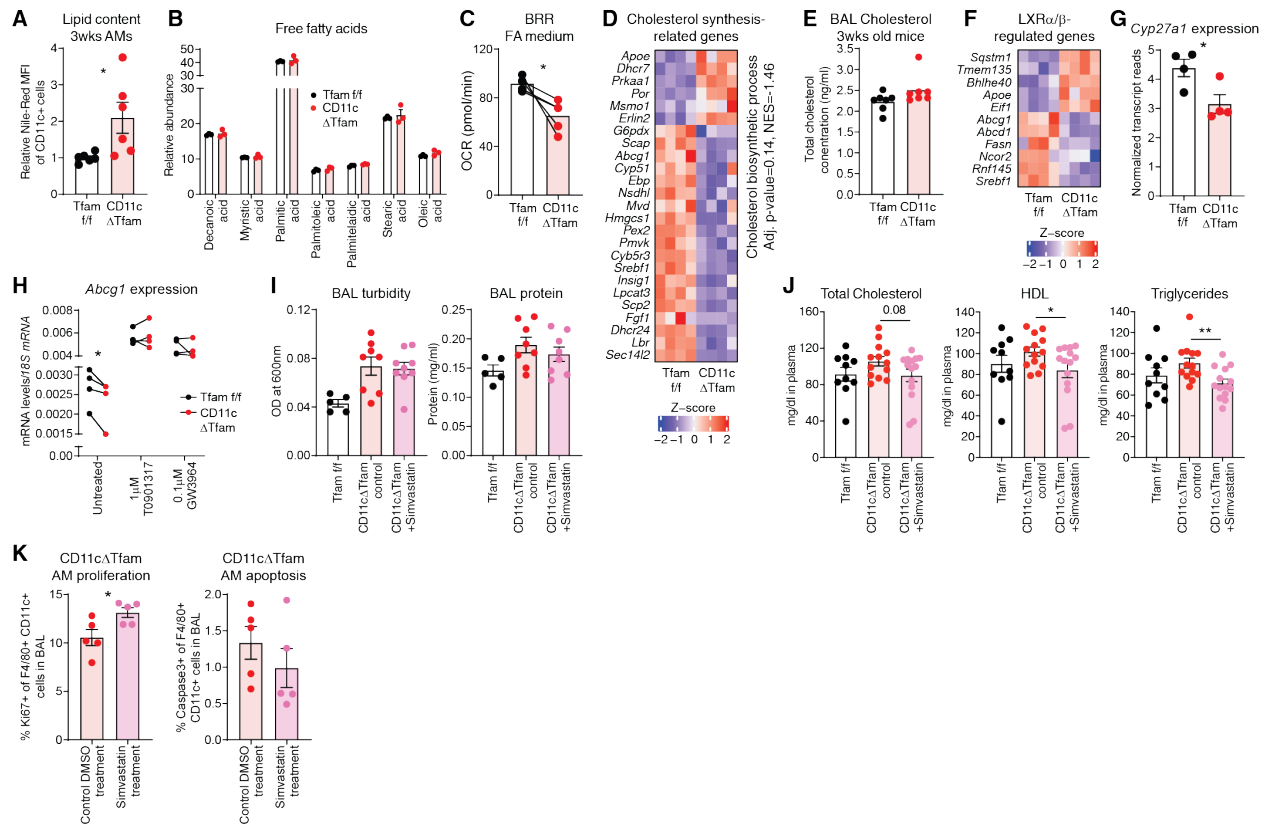
Figure S6. Related to Figure 5.

Tfam^{f/f} and *CD11cΔTfam* mice or their AMs in the lung were analyzed.

(A) Flow cytometric quantification of relative MFI of Nile-Red staining of CD11c$^+$ cells from BAL of 3 week-old mice (n=6).

(B) Relative abundance of free FAs detected in the GC/MS analysis of FACS-sorted F4/80$^+$ CD11c$^+$ cells from BALs of adult mice (n=3 merged from 13-30 mice) shown in Figure S4C.

(C) Basal respiratory rate (BRR) of CD11c$^+$ cells from BAL of adult *Tfam^{f/f}* and *CD11cΔTfam* mice in media containing 5mM L-carnitine and 50μM palmitoyl-CoA (n=4 merged from 5-10 mice).

(D) Heat map of expression levels of deregulated genes included in the "cholesterol biosynthetic process" (GO process GO:0006695) detected in the RNAseq of CD45$^+$ F4/80$^+$ CD11c$^+$ lung cells from 3 week-old mice.

(E) Quantification of total cholesterol levels in BAL of 3 week-old mice (n=7).

(F and G) Heat map of expression levels of deregulated genes controlled by LXRα/β (as determined on the DoRothEA database [https://dorothea.opentargets.io], F) and expression of *Cyp27a1* (G) detected in the RNAseq of CD45$^+$ F4/80$^+$ CD11c$^+$ lung cells from 3 week-old mice.

(H) CD11c$^+$ cells were magnetically purified from BALs of adult mice and cultured for 24h under presence or not of LXR agonists 1μM T0901317 or 0.1μM GW3965 before analysis of *Abcg1* mRNA levels (n=4 merged from 4-5 mice).

(I and J) Turbidity and protein levels in BAL (I) and well as levels of total cholesterol, HDL and triglycerides in plasma (J) of mice treated with Simvastatin or control DMSO for 8 weeks (n=5-13).

(K) Flow cytometric quantification of frequency of Ki67$^+$ (left) or activated caspase 3$^+$ cells (right) in F4/80$^+$ CD11c$^+$ cells in BAL of mice treated with Simvastatin or control DMSO for 3 weeks (n=5).

Data are merged from at least 2 independent experiments and presented as mean ± SEM. Dots represent individual data points. Statistical analysis by unpaired (A, G, J and K) or paired (C and H) Student's t-test. *p≤0.05, **p≤0.01.
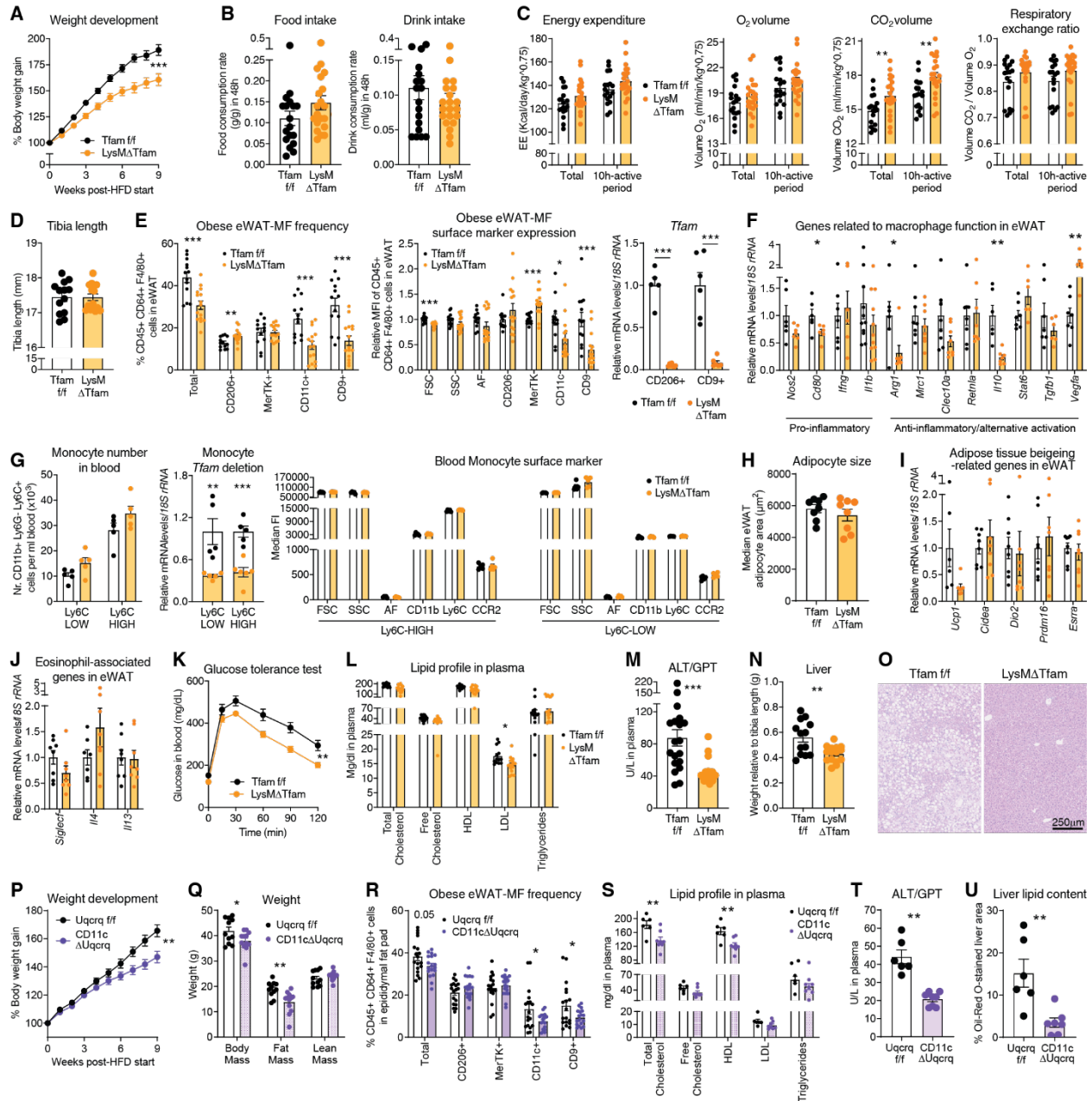
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Figure S7. Related to Figure 7.

Tfam^{f/f} and *LysMΔTfam* or *Uqcrq^{f/f}* and *CD11cΔUqcrq* mice were fed an HFD for 9 weeks and analyzed.

(A) Weight development over time on HFD diet start at week 0 (n=13-15).

(B) Food and drink intake of obese mice measured in metabolic cages (n=18-21).

(C) Energy expenditure, consumed O_2 volume, released CO_2 volume and calculated respiratory exchange ratio of obese mice measured in metabolic cages (n=18-22).

(D) Tibia length (n=13-15).

(E) Flow cytometric quantification of frequencies of $CD45^+$ $CD64^+$ $F4/80^+$ cells or cells that also express the indicated surface markers in alive cells from eWAT of obese mice (left, n=13-15). Relative median fluorescence intensity (MFI) of indicated parameters or markers on $CD45^+$ $CD64^+$ $F4/80^+$ cells from eWAT

of obese mice (center, n=13-15). *Tfam* mRNA levels in FACS-sorted CD45$^+$ CD64$^+$ F4/80$^+$ and CD206$^+$ or CD9$^+$ cells from eWAT of obese mice (right, n=5-7).

(F) mRNA levels of selected genes associated with macrophage functions in eWAT (n=6-11).

(G) Flow cytometric quantification of numbers (top left) and MFI of indicated parameters or markers (bottom) on CD11b$^+$ Ly6G$^-$ and Ly6C^{low} or Ly6C^{high} cells in blood of lean mice before start of HFD treatment (n=5). *Tfam* mRNA levels in FACS-sorted CD11b$^+$ Ly6G$^-$ and Ly6C^{low} or Ly6C^{high} cells from blood of lean mice (top right, n=5-6).

(H) Adipocyte size in obese eWAT measured by microscopy (n=9).

(I and J) mRNA levels of selected genes associated with adipose tissue beigeing (I) and eosinophil presence or function (J) in eWAT (n=6-8).

(K) Glucose levels over time in blood after intraperitoneal injection (time = 0) of glucose (n=13-16).

(L) Levels of total cholesterol, free cholesterol, HDL, LDL and triglycerides in plasma of HFD-fed mice (n=13-15).

(M) Alanine transaminase (ALT/GPT) levels in plasma (n=19-22).

(N) Weight of median lobe of livers from obese mice relative to tibia length (n=13-15).

(O) Representative image of eosin (pink) and hematoxylin (blue)-stained liver sections (scale bar: 250µm).

(P) Weight development over time on HFD diet start at week 0 (n=18-19).

(Q) Quantification of magnetic resonance imaging to determine body mass (n=11).

(R) Flow cytometric quantification of frequencies of CD45$^+$ CD64$^+$ F4/80$^+$ cells or cells that also express the indicated surface markers in alive cells from eWAT of obese mice (n=18).

(S) Levels of total cholesterol, free cholesterol, HDL, LDL and triglycerides in plasma of HFD-fed mice (n=6-8).

(T) Alanine transaminase (ALT/GPT) levels in plasma (n=6-7).

(U) Quantification of Oil-Red O-stained liver sections (n=6-7).

Data are merged from at least 2 independent experiments and presented as mean ± SEM. Dots represent individual data points. Statistical analysis by two-way ANOVA with Geisser-Greenhouse correction (A, K and P) or unpaired Student's t-test (B-J, L-N and Q-U). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.