Tetracycline Antibiotics Induce Host-Dependent Disease Tolerance to Infection

Graphical Abstract

Highlights

- Doxycycline protects from sepsis beyond its direct antibacterial activity
- Doxycycline protection from infection is microbiome-independent
- Inhibition of mitochondrial protein synthesis induces disease tolerance
- Mild and transient perturbations of the mitochondrial ETC induce disease tolerance

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In Brief
Several classes of antibiotics have beneficial effects on the outcome of infections that cannot be explained by their direct antibacterial activities alone. Colaço et al. show that inhibition of mitochondrial protein synthesis by ribosomal-targeting antibiotics perturbs the mitochondrial electron transport chain and induces disease tolerance to infection.
Tetracycline Antibiotics Induce Host-Dependent Disease Tolerance to Infection

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SUMMARY

Several classes of antibiotics have long been known to have beneficial effects that cannot be explained strictly on the basis of their capacity to control the infectious agent. Here, we report that tetracycline antibiotics, which target the mitoribosome, protected against sepsis without affecting the pathogen load. Mechanistically, we found that mitochondrial inhibition of protein synthesis perturbed the electron transport chain (ETC) decreasing tissue damage in the lung and increasing fatty acid oxidation and glucocorticoid sensitivity in the liver. Using a liver-specific partial and acute deletion of Crif1, a critical mitoribosomal component for protein synthesis, we found that mice were protected against sepsis, an observation that was phenocopied by the transient inhibition of complex I of the ETC by phenformin. Together, we demonstrate that mitoribosome-targeting antibiotics are beneficial beyond their antibacterial activity and that mitochondrial protein synthesis inhibition leading to ETC perturbation is a mechanism for the induction of disease tolerance.

INTRODUCTION

Optimal organismal function and survival in adverse conditions require robust homeostatic responses to variable and challenging environmental conditions (Rajan and Perrimon, 2011). Understanding core physiological principles in response to infection and their genetic circuitry are current central quests in biology (Chovatiya and Medzhitov, 2014). Sepsis is a prime example of extreme homeostasis disruption caused by infection and therefore constitutes an excellent model to study homeostatic circuits and inter-organ communication principles (Colaço and Moita, 2016). Sepsis is a complex disorder caused by a non-adaptive host response to an infection (Singer et al., 2016), leading to acute organ dysfunction and consequent high risk of death (Cecconi et al., 2018). The pathophysiology of sepsis includes an acute burst in pro-inflammatory cytokine production (Wiersinga et al., 2014) and metabolic failure (Van Wuytenghe et al., 2018), yet its molecular bases remain poorly understood. Current management of sepsis patients is limited to control of infection with antibiotics and organ support measures, with most attempts to modulate immune response resulting in failure (Cohen et al., 2015).

Surviving a severe infection requires the synergy between two evolutionarily conserved defense strategies that can limit host disease severity. The extensively explored concept of resistance relies on pathogen recognition, signaling transduction pathways, and effector mechanisms to reduce pathogen load, whereas disease tolerance provides host tissue damage control and limits disease severity irrespective of pathogen load. Yet resistance mechanisms are not enough to guarantee recovery from infection. In fact, many sepsis patients die (currently ∼25%) despite effective eradication of the inciting pathogen. Therefore, disease tolerance-based therapies are likely to be critical adjuvant components of sepsis treatment (Figueiredo et al., 2013; Ganeshan et al., 2019; Weis et al., 2017).

All eukaryotic organisms are equipped with surveillance mechanisms to detect and correct perturbations in homeostasis. Organellar dysfunction caused by pathogens, toxins, drugs, physical insults, or nutritional changes rapidly trigger a compensatory response (Sawa et al., 2016). Such stress responses are critical for the initiation of an effective immune response (Colaço and Moita, 2016) and cytoprotective-based lifespan extension programs (Shore and Ruvkun, 2013). Mitochondria, in particular, have evolved surveillance programs to monitor physiological
Figure 1. Doxycycline Confers Protection in a Mouse Model of Bacterial Sepsis

(A–C) Survival (A), rectal temperature (B), and % initial body weight (C) after i.p. infection of mice with TetR CamR E. coli and i.p. treatment with 1.75 mg/g body weight doxycycline at 0, 24, and 48 h.

(D) Bacterial load in mouse blood, liver, lung, and kidney at the indicated time points after infection.

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perturbations and initiate cytoprotection mechanisms (Yun and Finkel, 2014). Pioneer work in C. elegans reveals that genetic defects in the electron transport chain (ETC) (Dillin et al., 2002) or inhibition of mitochondrial protein synthesis (Houtkooper et al., 2013) results in extended lifespan. In mice, metabolic benefits arising from inhibition of ETC activity have been reported in the context of obesity and insulin resistance (Chung et al., 2017; Masand et al., 2018; Pospisilik et al., 2007).

In this study, we set out to identify protective mechanisms against infection activated by mild disruption of core cellular functions. We found that mitochondrial protein synthesis inhibition by mitoribosome-targeting antibiotics like doxycycline increased survival independently of its antibiotic effects. These results were associated with changes in mitochondrial function, fatty acid metabolism, and response to glucocorticoids, which could be phenocopied by mild and transient chemical and genetically induced perturbations of mitochondrial ETC.

RESULTS

Doxycycline Confers Protection in a Mouse Model of Bacterial Sepsis

To identify disease tolerance mechanisms that resolve perturbation of core cellular functions, we selected a panel of clinically approved drugs—including menadione, bortezomib, trifluoperazine, and mitoribosome-targeting antibiotics—known to interfere with basic cellular functions and pathways. These drugs were intraperitoneally (i.p.) injected into C57BL/6J mice at the time of i.p. infection with *E. coli* strain carrying resistance to tetracyclines (TetR) and chloramphenicol (CamR) to control for the effects of ribosomal-targeting antibiotics directly on bacterial viability. Treatment with low-dose doxycycline, a tetracycline antibiotic known to bind to the mitochondrial ribosome and block translation of mitochondrial-encoded mRNA (Lin et al., 2018), revealed a robust and reproducible increase in survival (Figure 1A). Doxycycline-treated mice had less severe hypothermia than PBS-treated controls (Figure 1B) and showed slightly lower body weight at 48 h (Figure 1C). Treatment with chloramphenicol, a structurally unrelated ribosome-targeting antibiotic, also conferred protection in this sepsis model (Figures S1A–S1C).

We found no differences in the bacterial loads of doxycycline-treated mice in either blood (Figure 1D, left panel) or tissue samples (Figure 1D, right panel) collected at 6, 12, and 30 h after infection, except for a modest reduction in kidney colony-forming units (CFUs) only at 30 h (Figure 1D, right panel). We observed no differences in the growth curves of the TetR *E. coli* over time *in vitro* in the presence or absence of doxycycline (Figure S1D). These results suggest that doxycycline induces disease tolerance by limiting disease severity without affecting pathogen load. Despite similar bacterial burden between groups, doxycycline-treated mice showed reduced tissue damage (Figure 1E–1G). Serum concentrations of the liver damage markers aspartate transaminase (AST) and alanine transaminase (ALT) revealed significantly reduced AST concentrations at 12 h (Figure 1E). The kidney damage marker creatinine was markedly reduced at 30 h, whereas the muscle damage marker creatine kinase (CPK) showed modest differences (Figure 1E). Lactate dehydrogenase (LDH), an unspecific cell damage marker, showed reduced concentrations at 12 h (Figure 1E). Reduced tissue damage was also documented in a blind histopathology analysis of liver, lung, and kidney, in which tissues were scored for the presence and dimension of necrotic areas as well as leukocyte infiltration (Figures 1F and 1G). In all analyzed tissues, doxycycline-treated mice globally scored lower for damage (Figure 1G). These changes were more pronounced in the liver, where necrotic areas of doxycycline-treated mice were markedly reduced, and in the lung, where we observed reduced neutrophil infiltration, hemorrhage, and thickening of the alveolar wall upon doxycycline treatment (Figure 1F). In infected mice, tumor necrosis factor α (TNFα) concentrations were significantly reduced at 30 h (Figure 1H). However, in mouse bone marrow-derived macrophages (BMDMs) pre-treated with doxycycline, no differences were found in cytokine concentrations (Figure S1E), suggesting that doxycycline induces disease tolerance without directly affecting the inflammatory response.

We then tested the protective effects of doxycycline across different groups of pathogens. In a model of influenza infection, mice were intranasally challenged with a sublethal dose (100 pfu/mouse) of Influenza PR/8, and doxycycline was injected i.p. at days 4, 5, and 6 post-infection, the period when viral loads are highest and lung lesions become apparent. Doxycycline-treated mice lost body weight at a similar pace but recovered faster after day 5, reaching a significant difference in body weight at day 10 post-infection (Figure S1F). These results suggest that doxycycline may limit lung tissue damage, which results in faster recovery. We found no differences in survival rates of treated mice in a model of systemic fungal infection induced by intravenous injection of *Candida albicans* (Figure S1G) or in a model of cerebral malaria induced by *Plasmodium berghei* ANKA (Figure S1H) despite reduced percentage of infected red blood cells upon doxycycline treatment (Figure S1I). These results suggest that doxycycline-induced mechanisms of disease tolerance confer protection in bacterial and viral but not in fungal or parasitic infections.

Doxycycline Improves Lung Pathology

To investigate the improvement of lung pathology upon doxycycline treatment, we tested the effect of local administration of the drug to the lung. A single intratracheal administration of doxycycline 2 h before *E. coli* i.p. infection resulted in increased survival

(8) Conclusions of the organ damage markers aspartate transaminase (AST), alanine transaminase (ALT), creatinine (CREA), creatine kinase (CPK), and lactate dehydrogenase (LDH).

(F) Hematoxylin-eosin-stained liver, lung, and kidney 30 h after infection.

(G) Organ damage score in Hematoxylin-eosin-stained tissues 30 h after infection. Score 0 = no lesions; 1 = very mild; 2 = mild; 3 = moderate; 4 = severe lesions.

(H) TNFα and interleukin (IL)-6 concentrations in mouse serum.

(A) Represents pooled data from 12 independent experiments. (B) and (C) represent mean ± SD pooled from six independent experiments. (D), (E), (G), and (H) represent pooled data from at least two independent experiments; squares represent individual mice and bars indicate the mean. (F) Shows representative images of two independent experiments. Scale bars indicate 500 μm (liver), 200 μm (lung), and 100 μm (kidney). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. See also Figure S1.
To explore the molecular mechanisms induced by doxycycline in the lung, we performed bulk RNA sequencing (RNA-seq) in mouse lungs 12 h after i.p. infection with *E. coli* and i.p. doxycycline treatment (Table S1). In the absence of doxycycline, we found a large number of expected genes upregulated upon infection (Figure 2B), mostly related to an acute inflammatory response (Figure S2A). Doxycycline treatment did not change the expression of the majority of these genes but instead resulted in the strongly reduced expression of a small group of genes in both non-infected and infected groups (Figure 2C; Figures S2B and S2C), suggesting specific drug-induced changes. Functional clustering analysis in non-infected, doxycycline-treated mice showed consistency in the function of downregulated genes, with 60% of the genes clustering in pathways related to keratinization and epithelium differentiation (Figure 2D; Figure S2D). In particular, the basal cell markers *Krt6a* and *Krt6b* were strongly downregulated (Figure 2D), suggesting that doxycycline might induce differentiation of lung progenitor cells (Hackett et al., 2011), leading to a more effective repair of the lung epithelium, without the cornification and pathologic fibrosis in response to infection or other forms of stress, including cigarette smoke (Hu et al., 2018). To directly test this possibility, we used diphteria toxin to deplete basal cells from mice expressing the diphteria toxin receptor under a *Krt6a* promoter (Krt6a-DTR mice) (Zuo et al., 2015). Krt6a-DTR mice infected with *E. coli* were still protected by doxycycline treatment (Figure S2E), suggesting that basal cells were unlikely to be the single target of the drug and that the enhanced lung repair capacity induced by doxycycline depends on the combination of its effect on multiple cell lineages.

**Doxycycline Improves Liver Pathology**

To begin exploring the protective effect of doxycycline on the liver, we performed bulk RNA-seq in mouse livers 8 h after infection.
Figure 3. Fatty Acid Oxidation and Response to Glucocorticoids Are Essential for Sepsis Outcomes

(A) Volcano plot with differential expression of liver genes affected by i.p. E. coli infection. Numbers indicate genes with log2 fold change < -5 or > 5 and p < 0.05.

(B) Scatterplot of genes affected by i.p. doxycycline treatment in infected versus non-infected groups. Yellow dots indicate genes differentially expressed in infected mice; gray dots indicate non-statistically significant genes (p > 0.05).

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E. coli infection (Table S2). Similarly to the lung, infection resulted in upregulation of a large number of genes associated with an inflammatory response in the liver (Figure 3A; Figure S3A). However, when comparing PBS and doxycycline-treated mice in the absence of infection, we found no statistically significant differential gene expression (Figure 3B), suggesting that the transcriptional signature of tissue repair found in the lung (Figures 2C and 2D) is not present in the liver. Functional analysis of E. coli-infected, doxycycline-treated mice revealed a discrete number of up- and downregulated genes compared to PBS-treated controls (Figure 3B), with a single significant cluster that was related to decreased production of collagen in infected, doxycycline-treated mice (Figure S3B). Given that collagen is a marker of liver fibrosis associated with disease severity in sepsis (Gadd纳斯 et al., 2009), these findings further support a role for doxycycline in limiting liver damage from early stages of infection.

To further dissect the protective effects of the drug in the liver, we investigated metabolic profiles in this organ in the presence of doxycycline and infection, given the centrality of liver function and metabolic changes during sepsis (Fleury et al., 2019; Gane-Shan et al., 2019). We used an untargeted metabolomics approach to study metabolic changes in the liver 8 h after infection. Analysis of the top up- and downregulated metabolites identified two main signatures reflecting a pronounced accumulation of acylcarnitines (highlighted in yellow) and glucocorticoids (highlighted in gray) in the liver of infected mice (Figure 3C; Table S3). To begin exploring these signatures, we measured mRNA of Ppara, a known master regulator of fatty acid oxidation (FAO), and several of its transcriptional targets, including Cpt1, Cpt2, and SLC25A20, responsible for long-chain fatty acid (LCFA) import into the mitochondria, at 8 and 24 h after infection. We consistently found a decreased expression of all of the analyzed targets upon infection, which was maintained for at least 24 h (Figure 3D), highlighting lipid metabolic dysfunction as a hallmark of sepsis pathophysiology. To functionally test the importance of LCFA import into the mitochondria for sepsis pathophysiology, we treated infected mice with etomoxir (Divakaruni et al., 2018), a frequently used inhibitor of CPT1—the enzyme that catalyzes the conversion of free fatty acids (FFAs) to acylcarnitines before their import into the mitochondrial matrix. We observed a significant increase in mortality as compared to infected non-treated controls (Figure 3E, upper panel), a result phenocopied by perhexiline, another CPT1 inhibitor (Figure S3C). Doxycycline did not improve survival in the presence of CPT1 inhibition by etomoxir (Figure 3E, lower panel). These results were substantiated by liver specific short hairpin RNA (shRNA)-mediated silencing of Cpt2 (Figures S3D and S3E). A similar effect was found for glucocorticoid response, as mice treated with mifepristone, a glucocorticoid receptor (GR) antagonist, had significantly worse infection outcomes (Figure 3F, upper panel), which prevented doxycycline-increased survival (Figure 3F, lower panel). Taken together, these results demonstrate that both LCFA metabolism and response to glucocorticoids are necessary for recovery from sepsis and that impairment of these pathways correlates with worse infection outcomes.

**Doxycycline Decreases Acylcarnitine Accumulation and Improves Response to Glucocorticoids**

We used HPLC-MS analysis to identify acylcarnitine and FFA species in mouse liver 8 h after infection and doxycycline treatment. Doxycycline partially decreased the accumulation of acylcarnitines and FFA upon infection (Figure 4A; Table S4), suggesting that it may improve the block in LCFA transport into the mitochondria caused by infection. Several attempts to correct LCFA transport in the liver by overexpressing several genes involved in this pathway, alone or in combination, using an adeno-associated virus (AAV) serotype 8 driven by liver thyroid hormone-binding globulin (TBG) promoter, conferred little to no survival advantage during infection (Figures S4A–S4C). An attempt to bypass the carnitine shuttle by orally supplementing mice with octanoic acid, a medium-chain FFA (C8:0) that does not require active transport into the mitochondria or conjugation with carnitine, was also not capable of consistently rescuing mice (Figure S4D). Likewise, treatment with the PPARy agonist CP868388 at the time of infection was not enough to significantly improve survival (Figure S4E).

Sepsis is characterized by resistance to glucocorticoids (Dendoncker and Libert, 2017). Accordingly, pre-treatment of mice with the synthetic glucocorticoid dexamethasone before infection with E. coli failed to significantly increase survival (Figure S4F). Doxycycline treatment did not significantly change glucocorticoid species in infected mice (Figure 4B; Table S3). To explore the effect of doxycycline in glucocorticoid signaling, we probed liver extracts collected 8 h after infection with and without doxycycline treatment for total GR and its phosphorylation on serine (S) residues S234 and S220 in mice (corresponding to S226 and S211 in humans, respectively), which are markers of GR activation (Bouazza et al., 2012). Infected, PBS-treated mice showed markedly reduced amounts of all analyzed forms at 8 h, supporting the hypothesis of glucocorticoid resistance in sepsis (Figure 4C). Doxycycline treatment increased phospho-S226 and to a lesser extent phospho-S211, both in the presence and absence of infection, while total GR amounts were also moderately increased in doxycycline-treated, E. coli infected mice (Figure 4C). To test the relevance of these findings, we used AAV8 to deliver a dominant-negative (DN) form of GR by mutating both S234 and S220 to alanine (A). Mice that had their livers transduced with the DN GR were significantly more susceptible to infection than mice that received AAV8-GFP (Figure 4D). These observations indicate that doxycycline substantially increases GR activation in response to glucocorticoids, which is normally blunted in sepsis (Dendoncker et al., 2019). Together, these results suggest that LCFA transport into the mitochondria and glucocorticoid signaling are perturbed and
are necessary for survival in sepsis. While independently rescuing these pathways is not sufficient to substantially improve the outcome of a severe infection, doxycycline treatment simultaneously restores both pathways.

**Low-Dose Doxycycline Affects Mitochondrial Function**

**In Vivo**

The role of microbiota in host physiology has been increasingly acknowledged in the context of sepsis (Haak et al., 2018). As an antibiotic, doxycycline induces changes in microbiome composition that may indirectly affect host fitness. To address the contribution of microbiota for doxycycline-induced protection against sepsis, we tested the effect of doxycycline treatment in the TetR *E. coli* sepsis model in germ-free mice. Both survival (Figure 5A) and body temperature (Figure S5A) were significantly improved in doxycycline-treated mice, whereas no differences were found in body weight (Figure S5B). These results largely phenocopy the protective effect observed in conventionally raised, specific pathogen-free mice, demonstrating a host-dependent disease tolerance mechanism.

Doxycycline is known to work by blocking the binding of aminoacyl-tRNA (aa-tRNA) to the A site of the ribosome,
decreasing the use of the elongation factor Tu (TUFM), which is part of the ternary complex (aa-tRNA, TUFM, and guanosine triphosphate [GTP]) that decodes the gene open reading frame (Lin et al., 2018). We therefore tested the possibility that doxycycline treatment could increase the availability of TUFM, which could then interact with NLRX1 and cause a reduction of type I interferon and enhancement of autophagy, as previously demonstrated (Lei et al., 2013). We did not observe a substantial improvement in survival (Figure S5C) after we delivered AAV8 to overexpress TUFM in the liver (Figure S5D) and therefore conclude that this mechanism does not play a critical role in the promotion of disease tolerance induced by doxycycline.

Doxycycline has been reported to block synthesis of mitochondrial-encoded proteins at relatively high doses across several model organisms, including mice (Michel et al., 2015; Moullan et al., 2015). We used mitochondrial fractions of liver extracts from mice that were treated with doxycycline for 16 h to examine the relative abundance of proteins of the ETC encoded by the nucleus (NDUFS1 and HSP60) and by the mitochondrial DNA (MT-ND1 and MT-ND6). While the nuclear encoded NDUFS1 and HSP60 remained unchanged, the mitochondria-encoded MT-ND1 and MT-ND6 decreased their abundance when compared to the PBS-treated control animals (Figure 5B). Functional changes in mitochondrial function could also be detected in vivo by measuring the enzymatic activity of ETC complexes in mouse liver collected 12 h after doxycycline treatment. We found strongly reduced activity of complexes III and IV (54% and 60% of the control, respectively) and a slight, possibly compensatory, activation of complexes I and II (115% and 118% of the control, respectively) (Figure 5C). These changes in function were not reflected in impaired mitochondrial integrity as judged by citrate synthase (CS) activity (Figure 5D) or morphology as analyzed by transmission electron microscopy up to 24 h after doxycycline treatment (Figure 5E). We did not find consistent transcriptional changes in the lung, liver, or kidney suggestive of induced mitochondrial unfolded protein response (UPR(mt)) in several independent experiments using reporters that included the measurement of mRNA by quantitative PCR (qPCR) of Hspdd1, Atf4, Atf5, ClpP, and Chop (data not shown). Inhibition of ETC function can lead to the generation of reactive oxygen species (ROS) (Zhao et al., 2019). We measured peroxide ROS generation in doxycycline-treated HepG2 cells using the MitoSOX fluorescent reporter and found a modest increase in mitochondrial ROS (Figure S5E), without affecting the mitochondrial mass as measured by Mitotracker green (Figure S5F). The ataxia telangiectasia mutated (ATM) kinase, which mediates disease tolerance in response to anthracycline treatment (Figueiredo et al., 2013), can be activated by ROS (Choy and Watters, 2018). To test the hypothesis that doxycycline could mediate activation of ATM by ROS, we used AAV8-Cre-mediated depletion of ATM in the liver and monitored survival in response to infection. We found no substantial effect on the protective phenotype of doxycycline (Figure S5G) and concluded that the doxycycline induction of disease tolerance was independent of ATM activation. Taken together, our data

Figure 5. Low-Dose Doxycycline Affects Mitochondrial Function In Vivo

(A) Survival of germ-free mice after infection with E. coli and treatment with doxycycline.

(B) Protein amounts of mitochondrial-encoded (MT-ND1, MT-ND6) and nuclear-encoded (NDUFS1, HSP60) proteins in the mitochondrial fraction of liver extracts 16 h after doxycycline treatment. Each lane represents one mouse.

(C) Enzymatic activity of ETC complexes in liver 12 h after doxycycline treatment. Activity is expressed as % of PBS-treated control, normalized for citrate synthase (CS) activity.

(D) CS activity (expressed as % of control) in liver 12 h after doxycycline treatment.

(E) Representative images of two independent experiments of transmission electron microscopy in mouse skeletal muscle after doxycycline treatment. Scale bar represents 500 nm.

(A) Represents pooled data from four independent experiments. Data in (B) are representative from two independent experiments. (C) and (D) represent mean ± SD of 12 mice/group from two independent experiments. (E) represents data from one experiment; scale bars represent 500 nm. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. See also Figure S5.
Figure 6. Mild, Transient Perturbations in Mitochondrial Function Are Associated with Increased Survival in Sepsis

(A and B) CRIF1 and Cre recombinase protein expression (A) and Crif1 mRNA expression (B) in Crif1lox/lox or Crif1lox/lox/C0 mice 7 days after injection of AAV8-TBG-expressing Cre recombinase (Ad. Cre) or GFP as a control (Ad. GFP).

(C) Survival of Crif1lox/lox or Crif1lox/lox/C0 mice, previously injected with AAV8-TBG-CRE, after infection with E. coli.

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indicate that doxycycline triggers changes in mitochondrial ETC function without compromising mitochondrial integrity or other major mitochondrial functional features.

**Mild, Transient Perturbations in Mitochondrial ETC Increase Survival in Sepsis**

To determine a causal link between mild perturbations in mitochondrial ETC function and disease tolerance, we depleted CR6-interacting factor 1 (CRIF1, also known as Gadd45 gip1), which is a mitoribosomal protein with an important role in the assembly and function of ETC complexes (Kim et al., 2012). Tissue-specific Crif1⁻/⁻ reduces ETC activity, resulting in systemic metabolic benefits (Chung et al., 2017). Prompted by the doxycycline-induced improvement in liver pathology and metabolism during sepsis, we tested the impact of targeted liver deletion of CRIF1 in sepsis. Intravenous injection of Cre-expressing AAV8 in homozygous Crif1lox/lox or heterozygous Crif1lox/⁻ mice led to high protein expression of Cre recombinease after 7 days, accompanied by reduced CRIF1 protein (Figure 6A) and mRNA (Figure 6B). Mice homozygous for the lox allele suffered almost full deletion in mRNA (98%) and protein, whereas heterozygous mice showed a 30% decrease in mRNA and only modest decrease in protein expression (Figure 6A). E. coli infection performed 7 days after AAV8-Cre injection resulted in increased survival of Crif1⁻/⁻ mice but not Crif1lox/lox littermates (Figure 6C), thus supporting the notion of a beneficial role for mild, transient mitochondrial perturbations but deleterious effect for severe perturbations of ETC mitochondrial function. Crif1⁻/⁻ mice also showed less severe hypothermia (Figure 6A), similar to doxycycline-treated mice (Figure 1B), but no differences in body weight when compared to controls (Figure 6B). Bacterial loads in blood of Crif1⁻/⁻ deficient mice were similar to the controls (Figure 6D), supporting the notion of disease tolerance induced by mild mitochondrial perturbations.

To address the question of whether the induction of disease tolerance by ETC required general or specific perturbations of ETC complexes, we used the biguanide antidiabetic drug phenformin, a known potent inhibitor of complex I of the ETC (Dykens et al., 2008). A single dose of phenformin resulted in a substantial survival advantage (Figure 6E) and improvement in body temperature control (Figure 6F), with modest effects in body weight (Figure 6D). Importantly, administration of two doses of phenformin, separated by 24 h, lead to increased mortality in contrast to a single administration (Figure 6E). Phenformin, similarly to doxycycline, conferred protection to sepsis by inducing disease tolerance mechanisms, as bacterial load in mouse blood collected 24 h after infection showed no difference between PBS and phenformin-treated mice (Figure 6F). Activity of ETC complexes in mouse liver collected 12 h after phenformin treatment revealed strongly decreased complex I activity (61% of the control) (Figure 6G), whereas complexes II, III, and IV, as well as CS showed little to no changes (Figure 6F). Additionally, phenformin-treated mice present lower concentration of serum inflammatory markers (Figure S6G), lower scores of histological damage (Figures S6H and S6I), and decreased accumulation of acylcarnitines (Figure 6H; Table S4), in good agreement with the doxycycline observations. In summary, we show that mild and transient perturbations in mitochondrial function, which may affect the activity of different complexes of the ETC, activate disease tolerance mechanisms in a mouse model of bacterial sepsis.

**ETC Inhibition Decreases Lipid Accumulation in the Liver**

To identify mechanisms of action downstream of ETC perturbation commonly induced by doxycycline and phenformin, we performed bulk RNA-seq in mouse livers but this time 20 h after E. coli infection and drug treatment to increase the probability of finding substantial changes in gene expression. We found 435 genes induced more than 2-fold (log2 fold change > 1) by both doxycycline and phenformin in E. coli-infected mice and 15 genes that were downregulated more than 2-fold (Figure 7A). Using diverse bioinformatics tools, we did not find a significant over-representation for any pathways. However, a manual curation of this list identified two genes involved in the conjugation of long-chain fatty acids, as well as three adrenergic receptors (Adrb3, Ada1b, and Adra2b) that were commonly induced by more than 4-fold (log2 fold > 2) (Figure 7A). qPCR analysis of independent samples confirmed substantial induction of Adrb3, Ada1b, and Adra2b (Figure 7B). We also observed induction of Adra1a and Adrb1 but not of Adrb2, which showed downregulation after doxycycline treatment (Figure 7B). To functionally identify which class of adrenergic receptors might contribute for the protection phenotype conferred by ETC inhibition, we co-treated infected mice with phenformin in combination with either an α (prazosin) or a β (propranolol) adrenergic blocker. While prazosin did not substantially affect the protection of phenformin, mice treated with propranolol were no longer protected by phenformin against infection (Figure 7C). Mice treated with doxycycline or phenformin had consistent and substantially lower lipid accumulation in the liver in response to infection as measured by oil red staining (Figures 7D and 7E). Acylcarnitine concentrations in the liver 20 h after infection show similar substantial decreased amounts in doxycycline- and phenformin-treated mice, as exemplified for stearoylcarnitine (Figure 7F). These results suggest that doxycycline or phenformin treatment induces sensitization to circulating adrenergic agonists, which causes decreased lipid accumulation in the liver.

(D) Bacterial loads in Crif1⁻/⁻ mouse blood 24 h after infection.

(E) Enzymatic activity of the ETC complex I in liver at 12 h. ETC activity is expressed as % of PBS-treated control, normalized for CS activity.

(F) HPLC-MS analysis of FAO metabolites in liver 8 h after infection and phenformin treatment. Each square represents one mouse. Data in (A) is from a single experiment. (B) represents mean ± SD of four mice per group assayed in triplicate. (C) represents pooled data from three independent experiments. (D) represents data from a single experiment. Bars indicate the mean. (E) represents pooled data from four independent experiments. (F) represents pooled data from two independent experiments. Bars indicate the mean. (G) represents mean ± SD of five mice per group from a single experiment. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. See also Figure S6.
Figure 7. ETC Inhibition Decreases Lipid Accumulation in the Liver

(A) Scatterplot of liver genes changed 20 h after infection in doxycycline- versus phenformin-treated groups. Differentially expressed genes are indicated in yellow (doxycycline-treated), cyan (phenformin-treated), or green (both conditions); gray dots indicate non-statistically significant genes ($p > 0.05$). Adrenergic receptors and genes involved in taurine conjugation to very long and long-chain fatty acids in the peroxisome are highlighted in dark blue and red, respectively.

(B) Expression of adrenergic receptors by qPCR in liver at 30 h post-infection. Each square represents one mouse; bars indicate the mean.
DISCUSSION

Both resistance and disease tolerance mechanisms are necessary to minimize loss of function and promote survival in response to infection (Soares et al., 2017). The requirement for synergy of both defense strategies is perhaps best illustrated in sepsis, where effective control and elimination of the inciting pathogen is not sufficient to ensure survival, especially in cases of pronounced tissue damage. In the current study, we asked whether perturbation of core cellular functions, in addition to DNA damage responses initiated by low-dose anthracyclines (Figueiredo et al., 2013), could also lead to protective responses against severe infection. We identified tetracycline antibiotics as a class of drugs capable of activating disease tolerance mechanisms. Our findings are in good agreement with the long-known effects of several classes of antibiotics on the course and outcome of infections that cannot be explained by their direct antibacterial activities alone (Tauber and Nau, 2008).

Our results have shown that disease tolerance induced by doxycycline is pathogen group specific given that we observed increased survival in bacterial sepsis, faster recovery in viral lung infection but no salutary effects in fungal infection or protection against cerebral malaria. These findings support the notion that tissue damage control mechanisms confer disease tolerance in a pathogen-specific manner because distinct forms of stress and damage require dedicated tissue repair responses (Soares et al., 2017; Wang et al., 2016).

Characterization of doxycycline effects in organs where tissue damage was most improved uncovered potential explanations for doxycycline protection. In the lung, we found evidence of decreased tissue damage and a doxycycline-induced gene expression signature that suggested increased repair. We did not observe the same transcriptional signature in the liver. Using untargeted metabolomics, we identified two clear molecular signatures induced by infection in the liver. The accumulation of acylcarnitines and FFA upon infection suggested that severe infection induced a block in LCFA transport into the mitochondria. A second signature pointed to a sharp increase in multiple glucocorticoid species, suggesting that the infection imposes a strong stress response in the liver but also to one of the known hallmarks of sepsis pathology: resistance to glucocorticoid treatment (Dendoncker et al., 2019; Van Wyngene et al., 2018).

We also found that infection decreased markers of GR activation including the phosphorylation of human S211 and S226. Both of these signatures were critical for survival given that our data demonstrated decreased survival of infected animals by etomoxir-mediated inhibition of CPT1 and GR inhibition by mifepristone or a DN form of GR. Modulation of each of these processes alone was not enough to substantially improve survival, but doxycycline positively impacted both of these processes, likely contributing to increased survival by reprogramming liver metabolism during infection. In fact, this shift to catabolic programs in parenchymal tissues has been associated with tissue protection, resistance to stress, and disease tolerance across several experimental models (Wang et al., 2019). In the specific case of the liver, transient inhibition of ETC by doxycycline or phenformin may depend on the sensitization to adrenergic agonists by upregulating adrenergic receptors, in good agreement with the recent observation that Adrb1/2-mediated sympathetic outflow is required for sepsis survival (Luan et al., 2019). Several adrenergic receptors are known GR targets (Sakaue and Hoffman, 1991; Aksoy et al., 2002), and our data show that doxycycline sensitizes GR for the highly increased corticoid species present in the liver in response to infection.

The positive effects induced by doxycycline treatment were likely to be initiated by the partial and transient perturbation of ETC activity because a stronger and maintained inhibition of ETC function—such as the repeated administration of the complex I inhibitor phenformin—was by contrast potentially deleterious for infection survival. Induction of disease tolerance by doxycycline resulted from mild perturbation of the activity of ETC complexes III and IV, which were likely due to the mito-nuclear imbalance caused by inhibition of protein synthesis in the mitochondria. Notably, gasotransmitters like carbon monoxide, which is known to inhibit complex IV activity (Zuckerbraun et al., 2007), have been reported to induce disease tolerance (Pamplona et al., 2007). Perturbation of other ETC complexes also induce disease tolerance to infection, since phenformin, a strong complex I inhibitor, similarly induces disease tolerance.

ETC perturbations triggered not only local protective responses but also systemic effects, as demonstrated by improved survival in response to direct administration of doxycycline to the lung and by liver-specific genetic deletion of CRIF1.

The requirement for mild transient perturbations of ETC function to achieve protection and the deleterious nature of stronger and maintained perturbations suggests an hormetic response induced by perturbations of core cellular functions that trigger compensatory cytoprotective responses. Most pathogens, unlike commensals, will cause mild disruption of homeostatic functions as a requirement for host invasion and completion of their life cycles. Sensing of homeostasis deviations can conceivably be used by the host as a sign of infection, in addition to direct recognition, to better evaluate the level of threat and to initiate the necessary defense programs (Colaço and Moita, 2016). As tissue damage is a universal consequence of infection, it is possible that sensing of homeostasis disruption resulting from infection could lead not only to the initiation of resistance immune mechanisms by the host but also to tissue repair programs (Colaço and Moita, 2016). In this context, a mild stress imposed by doxycycline may mimic an active infection and trigger a program to re-establish homeostasis based on tissue repair and metabolic reprogramming.

In addition to the protective role of ATM-dependent DNA damage responses, our current study adds an independent pathway
leading to disease tolerance that can be initiated by inhibition of mitochondrial protein synthesis leading to perturbations of ETC function. The elucidation of the signal generated by ETC function perturbation, the mechanisms that sense it, and its downstream transduction and molecular effectors are challenging but are critical goals to understanding cytoprotection and disease tolerance in vertebrates, which ultimately can be key for the development of therapies against infection, in particular sepsis.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead Contact
  - Materials Availability
  - Data and Code Availability
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Mice
  - Cell lines
  - Primary cell cultures
  - Bacterial cultures
  - Fungal cultures
- **METHOD DETAILS**
  - E. coli-induced sepsis model and drugs treatments
  - Other infection models
  - Liver-specific gene editing with Adeno-associated virus (AAV)
  - Ablation of KRT6+ cells
  - Colony Forming Units assay
  - Biochemical assays in mouse serum and supernatant from BMDMs
  - Histopathology
  - Transmission Electron microscopy
  - Immunoblotting
  - Gene expression analyses
  - Liver metabolomics
  - Electron transport chain (ETC) complex activity
  - Flow cytometry
- **QUANTIFICATION AND STATISTICAL ANALYSIS**

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.immuni.2020.09.011.

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AUTHOR CONTRIBUTIONS

Conceptualization, L.F.M. with input from H.G.C.; Methodology, H.G.C., A.B., V.B., and T.K.; Formal Analysis, H.G.C., A.B., V.B., and T.K.; Investigation, H.G.C., A.B., A.N.-C., E.S., D.P., T.V., K.L.W., G.G., P.F., V.B., S.W., and T.K.; Resources, H.-S.Y. and M.S.; Data Analysis and Curation, A.B.; Writing – Original Draft, H.G.C. and L.F.M.; Supervision, L.F.M.; Funding Acquisition, L.F.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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**STAR** METHODS

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| alpha/beta-Tubulin Antibody | Cell Signaling Technology | Cat# 2148; RRID:AB_2288042 |
| Cre Recombinase (D7L7L) XP® Rabbit mAb antibody | Cell Signaling Technology | Cat# 15036; RRID:AB_2798694 |
| Anti-rabbit IgG, HRP-linked Antibody | Cell Signaling Technology | Cat# 7074; RRID:AB_2099233 |
| Anti-CRIF1 antibody | Abcam | Cat# ab226244; RRID:AB_2798694 |
| MT-ND1 antibody | Abcam | Cat# ab181848; RRID:AB_2687504 |
| MT-ND6 antibody | ThermoFisher | Cat# PA5-75196; RRID:AB_2718924 |
| NDUFS1 antibody | Abcam | Cat# ab157221; RRID:AB_2857900 |
| HSP60 antibody | Cell Signaling Technology | Cat# 12165; RRID:AB_2636980 |
| Glucocorticoid Receptor (D6H2L) XP® Rabbit mAb antibody | Cell Signaling Technology | Cat# 12041; RRID:AB_2631286 |
| Phospho-Glucocorticoid Receptor (Ser211) Antibody | Cell Signaling Technology | Cat# 4161; RRID:AB_2155797 |
| Phospho-Glucocorticoid Receptor (Ser226) (D9D3V) Rabbit mAb antibody | Cell Signaling Technology | Cat# 97285; RRID:AB_2800276 |
| **Bacterial and Virus Strains** | | |
| Escherichia coli MG1655 TetR CamR | This paper | N/A |
| Influenza A/Puerto Rico/8/34 (H1N1) | Maria João Amorim (Instituto Gulbenkian de Ciência) | (de Wit et al., 2004) |
| AAV8-TBG-iCre | Vector Biolabs | Cat# VB1724 |
| AAV8-TBG-eGFP | Vector Biolabs | Cat# VB1743 |
| AAV8-GFP-U6-m-CPT2-shRNA | Vector Biolabs | Cat # shAAV-256065 |
| AAV8-GFP-U6-scrmb-shRNA | Vector Biolabs | Cat # 77777 |
| AAV8-TBG-m-Cpt1a | Vector Biolabs | Cat # AAV-250982 |
| AAV8-TBG-m-CPT2 | Vector Biolabs | Cat # AAV-256065 |
| AAV8-TBG-m-SLC25A20 | Vector Biolabs | Cat # AAV-272202 |
| AAV8-TBG-m-TUFM | Vector Biolabs | Cat # AAV-275348 |
| AAV8-TBG-m-NR3C1 (S220/234A) | Vector Biolabs | Cat # AAV-266053 |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| Doxycycline hyclate | Sigma-Aldrich | Cat# D9891 |
| Phenformin Hydrochloride | Sigma-Aldrich | Cat# P7045 |
| Chloramphenicol | Sigma-Aldrich | Cat# C0378 |
| Ubiquinone | Sigma-Aldrich | Cat# C7956 |
| Bovine Serum Albumin (fatty acid free) | Sigma-Aldrich | Cat# A6003 |
| Succinic acid | Sigma-Aldrich | Cat# S7501 |
| Decylubiquinone | Sigma-Aldrich | Cat# D7911 |
| Malonic acid | Sigma-Aldrich | Cat# M1296 |
| Rotenone | Santa Cruz Biotechnology | Cat# sc-203242 |
| Antimycin A | Sigma-Aldrich | Cat# A8674 |
| 5,5¢-Dithiobis(2-nitrobenzoic acid) (DTNB) | Sigma-Aldrich | Cat# D218200 |
| Acetyl coenzyme A trilithium salt | Santa Cruz Biotechnology | Cat# sc-214465B |
| Potassium cyanide | Sigma-Aldrich | Cat# 60178 |
| β-Nicotinamide adenine dinucleotide, reduced dipotassium salt | Sigma-Aldrich | Cat# N4505 |

(Continued on next page)
Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Dichloroindophenol sodium salt hydrate | Sigma-Aldrich | Cat# D1878 |
| Cytochrome c from bovine heart | Sigma-Aldrich | Cat# 30398 |
| Sodium hydrosulfite | Sigma-Aldrich | Cat# 71699 |
| Potassium borohydride | Sigma-Aldrich | Cat# 438472 |
| Oxaloacetic acid | Sigma-Aldrich | Cat# 04126 |
| SuperScript® II Reverse Transcriptase | Invitrogen | Cat# 18064014 |
| Oligo(dt)12-18 Primer | Invitrogen | Cat# 18418012 |
| RNaseOUT™ Recombinant Ribonuclease Inhibitor | Invitrogen | Cat# 10777019 |
| iTaq Universal SYBR Green Supermix | Bio-rad | Cat# 1725125 |
| RNaseasy Mini Kit | QIAGEN | Cat# 50974106 |
| RNase-Free DNase Set | QIAGEN | Cat# 50979254 |
| cOmplete, Mini, EDTA-free | Roche | Cat# 1183617001 |
| PHOSSTOP | Roche | Cat# 4906837001 |
| (+)-Etomoxir sodium salt hydrate | Sigma-Aldrich | Cat# E1905 |
| CP-868388 | Sigma-Aldrich | Cat# P20149 |
| Mifepristone | Sigma-Aldrich | Cat# M8046 |
| Dexamethasone | Merck Sharp & Dohme | Oradexon 5 mg/mL |
| Octanoic acid | Sigma-Aldrich | Cat# C2875 |
| Propranolol hydrochloride | Sigma-Aldrich | Cat# P0884 |
| Prazosin hydrochloride | Sigma-Aldrich | Cat# P7791 |
| Perhexiline maleate salt | Sigma-Aldrich | Cat# SML0120 |
| Trizol reagent | Ambion | Cat# 15596018 |
| Diphtheria Toxin, Unnicked, *Corynebacterium diphtheriae* | Calbiochem | Cat# 322326 |

Critical Commercial Assays

| Mouse TNF-α ELISA MAX™ Standard | BioLegend | Cat# 430902 |
| Mouse IL-6 ELISA MAX™ Standard | BioLegend | Cat# 431302 |
| QuantiChrom Creatinine | Bioassay Systems | Cat# DICT |
| QuantiChrom Lactate Dehydrogenase | Bioassay Systems | Cat# D2DH |
| EnzyChrom Creatine Kinase | Bioassay Systems | Cat# ECPK |
| EnzyChrom Alanine Transaminase | Bioassay Systems | Cat# EALT |
| EnzyChrom Aspartate Transaminase | Bioassay Systems | Cat# EASTR |
| Mouse IL-10 ELISA MAX™ Standard | BioLegend | Cat# 431411 |
| Mouse IL-12/IL-23 (p40) ELISA MAX™ Standard | BioLegend | Cat# 431601 |
| MitoSOX™ Red Mitochondrial Superoxide Indicator | Thermo Fisher | Cat# M36008 |
| MitoTracker™ Green FM | Thermo Fisher | Cat# M7514 |

Deposited Data

RNA-Seq data and code

This paper | https://bit.ly/3bzSlgM |

Experimental Models: Cell Lines

Bone marrow-derived macrophages

This paper | N/A |

Hep G2

ATCC | Cat# HB-8065 |

Experimental Models: Organisms/Strains

*Candida albicans* (Robin) Berkhour SC5314

Salomé LeibundGut-Landmann (University of Zurich, Switzerland) | (Gillum et al., 1984) |

C57BL/6J *Mus musculus*

Instituto Gulbenkian de Ciência | IMSR Cat# JAX:000664, RRID:IMSR_JAX:000664 |

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead Contact**
Further information and requests for resources and reagents may be directed to and will be fulfilled by the Lead Contact Luis F. Moita (lmoita@igc.gulbenkian.pt).

**Materials Availability**
This study did not generate new unique reagents, except for the TetR CamR E. coli strain, which will be made available upon request.

**Data and Code Availability**
The RNA-seq datasets and scripts generated during this study are available on GitHub: https://bit.ly/3bzSIgM.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Mice**
All animal studies were performed in accordance with Portuguese regulations and approved by the Instituto Gulbenkian de Ciência ethics committee and DGAV. C57BL/6J mice were obtained from Instituto Gulbenkian de Ciência or Charles River Laboratories (France). Male mice, 8 to 12 weeks old were used in all experiments. *Crif1lox/lox* mice (Kwon et al., 2008) were obtained from M. Shong (Chungnam National University School of Medicine, Daejeon, South Korea). KRT6-DTR mice (Zuo et al., 2015) were obtained from W. Xian and F. McKeon (Harvard Medical School, Boston, MA, USA). ATM−/− mice (Borghesani et al., 2000) were obtained from F. Alt (Harvard Medical School, Boston, MA, USA). Mice were maintained under specific pathogen-free (SPF) or germ-free (GF) conditions with 12 h light/12 h dark cycle, humidity 50%–60%, ambient temperature 22 ± 2°C and food and water *ad libitum*. For all experiments, age-matched mice were randomly assigned to experimental groups.

**Cell lines**
HepG2 (male) human hepatocellular carcinoma cells were cultured in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) Penicillin-Streptomycin at 37°C with 5% CO2. Three to five days before experiments, medium was changed to DMEM with 10% FBS with no addition of antibiotics.

**Primary cell cultures**
BMDMs were differentiated from adult (typically 8-week-old) C57BL/6J male mice. After euthanasia by CO2 inhalation, the mouse skin was sterilized with 70% ethanol and femurs and tibia of hind limbs were removed, stripped of muscle and rinsed in RPMI medium. Bone marrow cells were flushed from cut bones using an insulin syringe with a 30G needle into 10 mL of RPMI medium. Cells were then pelleted by centrifugation at 450 g for 5 min and the cell pellet resuspended in 10 mL of RPMI supplemented with 10% (v/v) FBS and 0.2% (v/v) penicillin/streptomycin. Cells were counted and plated at a density of 3 × 10⁶ cells (including red blood cells) per 10 mL of RPMI medium supplemented with 10% FBS and 0.2% penicillin/streptomycin, with 30% of L929-conditioned medium. After three days, an equal volume of fresh medium with 30% of L929-supernatant was added to the cells. After four additional incubation days, the medium was replaced by 10 mL of fresh medium with 30% of L929-supernatant. 24 h-48 h afterward, cells were scraped from plates, counted and seeded in C10 medium. L929-conditioned medium: L929 cells were cultured in T175 flasks, in 40 mL of DMEM medium with...
10% (v/v) FBS and 1% (v/v) Penicillin/Streptomycin and grown to confluency. The culture medium was left unchanged for 5 days, for good production of M-CSF. Cells were then centrifuged at 290 g for 5 min and the supernatant was collected and filter sterilized. C10: RPMI medium 1640 supplemented with: 10% (v/v) Fetal Bovine Serum (FBS), 1% (v/v) Penicillin-Streptomycin, 1% (v/v) L-Glutamine, 1% (v/v) Non-essential amino acids, 1% (v/v) HEPES buffer, 0.05 M 2-Mercaptoethanol.

**Bacterial cultures**

*Escherichia coli* K12 MG1655 carrying resistance to chloramphenicol was made resistant to tetracyclines by P1 phage transduction (P1 phage lysate was a gift of Roberto Balbontìn from the Evolutionary Biology group at the IGC). All bacterial cultures were carried out in Luria-Bertani broth supplemented with 10 μg/mL doxycycline (LB+doxy), except for survival studies in chloramphenicol-treated mice, in which bacterial cultures were made in LB + 50 μg/mL chloramphenicol.

**Fungal cultures**

*Candida albicans* (Robin) Berkhout (*Gillum et al., 1984*) were cultured in yeast culture medium (YPD) for 16-20 h at 30°C, 180 rpm.

**METHOD DETAILS**

*E. coli*-induced sepsis model and drugs treatments

A starter culture from a single *E. coli* colony was incubated overnight (12-16 h) at 37°C, 200 rpm. The next morning, the culture was diluted 1:50 in LB+doxy and incubated for 2.5 h until late exponential phase was reached (OD600nm = 0.8-1.0). The culture was then centrifuged at 4400 g for 5 min at room temperature, washed with PBS and resuspended in PBS to obtain an OD600nm = 4.5-5.0, corresponding to 1-2x10^9 CFU/mL. This bacterial suspension was immediately injected intraperitoneally (200 μL/mouse) in mice using a 27G-needle. Infections were always performed in the morning. The concentration of the inoculum was determined by plating 10^-6 and 10^-7 dilutions in LB+doxy agar plates and incubating overnight at 37°C. Doxycycline hyclate was dissolved in PBS and injected intraperitoneally (200 μL/mouse) at 1.75 μg/g body weight 0, 24 and 48 h after infection. The following drugs were dissolved in the indicated vehicles and injected intraperitoneally (200 μL/mouse, except mifepristone: 50 μL/mouse) at the time of infection (except if otherwise stated) and at the indicated concentrations: chloramphenicol (vehicle: 5% cyclodextrin, dose 50 μg/g); phenformin (vehicle: PBS, dose 100 μg/g); etomoxir (vehicle: PBS, dose 15 μg/g); mifepristone (vehicle: 100% DMSO, dose 30 μg/g); CP668388 (vehicle: 7% DMSO, dose 3 μg/g), dexamethasone (vehicle: PBS, dose 5 μg/g), perhexiline (vehicle DMSO, dose: 8 μg/g), propranolol (vehicle: 4.2% methanol, dose 2 μg/g, injected 30 min before infection and 2 and 10 h after infection), prazosin (vehicle: 100% methanol, dose 2 μg/g, injected 30 min before infection and 2 and 10 h after infection). Octanoic acid was dispersed in 0.5% methylcellulose and supplemented by oral gavage (200 μL/mouse) at 2, 8, 24, and 48 h post-infection. Intratracheal administration of doxycycline was performed as previously described (*DuPage et al., 2009*). Briefly, mice were anesthetized with an intraperitoneal injection of 450 μg/g avertin, placed on an intubation platform (*Labinventions.com*), and intubated using a 22G, 1-inch catheter. Doxycycline (1.75 μg/g body weight in 50 μL PBS) was then pipetted into the opening of the catheter and the catheter was removed after all volume was inhaled. Mice were allowed to recover from anesthesia and infection with *E. coli* was performed 2 h later. Body weight and rectal temperature were determined 0, 24 and 48 h after infection. For survival experiments, mice were closely monitored during one week for survival and health status. Moribund animals (i.e., shivering or unable to maintain upright position) were euthanized. For tissue analysis, mice were sacrificed at the indicated time-points by CO2 inhalation, blood was collected by cardiac puncture and organs were harvested, immediately frozen in liquid nitrogen and stored at −80°C. Blood was centrifuged at 1600 xg for 5 min and serum collected and stored at −80°C.

**Other infection models**

For influenza virus infection, mice were anesthetized by inhalation of isoflurane and intranasally inoculated with a sublethal (100 pfu/mouse) dose of Influenza A PR/8 (*de Wit et al., 2004*) in 30 μL PBS. Infected mice were treated with an intraperitoneal injection of 1.75 μg/g doxycycline at days 4, 5 and 6 post-infection. Infection with GFP-transgenic *Plasmodium berghei* ANKA was performed as described (*Pamplona et al., 2007*). Briefly, mice were given an intraperitoneal injection containing 1x10^5 infected red blood cells from a previously infected mouse. Doxycycline (1.75 μg/g body weight) was injected daily starting at the time of infection. Blood samples were taken from the tail vein and analyzed in FACSCalibur to determine parasitemia (expressed as % of GFP-positive red blood cells).

**Liver-specific gene editing with Adeno-associated virus (AAV)**

AAV serotype 8 constructs were diluted in sterile PBS and 5x10^11 gc/mouse were delivered by retro-orbital injection. All subsequent experiments were performed 7 days after AAV injection, except for AAV8-TBG-NR3C1, in which mice were infected 24 h later.

**Ablation of KRT6+ cells**

Mice hemizygous for the human diphtheria toxin receptor inserted in the Krt6a locus (Krt6a-DTR) and wild-type littermates were treated with 12 ng/g diphtheria toxin (DT) by intra-tracheal administration, as described above. Experiments were performed 4 days after DT administration.
Colony Forming Units assay
Freshly collected samples of liver, lung and kidney were homogenized in 1 mL sterile PBS using TissueLyser II (QIAGEN). Colony forming units (CFU) were determined in blood and organs by serially diluting in sterile PBS and plating in LB+doxy agar plates. At least three dilutions were plated per condition. CFU were counted after incubating plates at 37°C for 16 h.

Biochemical assays in mouse serum and supernatant from BMDMs
Cytokine concentrations were determined using the ELISA kits indicated in the KRT, according to the manufacturer’s instructions. Serological makers of organ damage were determined using the colorimetric kits indicated in the KRT according to the manufacturer’s instructions. All absorbance readings were performed in 96-well plates using an Infinite M200 plate reader (Tecan).

Histopathology
Mouse liver, lung, and kidney were collected 30 h after infection and immediately fixed in 10% buffered formalin. Samples were then embedded in paraffin, sectioned (3 μm) and stained for hematoxilin and eosin according to standard procedures. Slides were analyzed with a DMLB2 microscope (Leica), and images were acquired with the NanoZoomer-SQ Digital slide scanner (Hamamatsu Photonics). Blind histopathology analysis was performed by a trained pathologist at the Instituto Gulbenkian de Ciência Histopathology Unit. Tissues were scored for damage, namely necrosis and leukocyte infiltration. For lipid droplet quantification, livers collected 20 h after infection were fixed in 10% buffered formalin, embedded in 30% sucrose and cryo-sections were made at 8 μm. Samples were then stained with Oil Red A. A blind analysis was performed by taking a picture of liver at 10x and measuring the area of lipids using the color threshold plugin of ImageJ software and estimating the fraction within the liver area.

Transmission Electron microscopy
Mice were euthanized 24 h after doxycycline treatment, perfused with 10 mL cold PBS through the left ventricle, followed by perfusion with 10 mL 2% formaldehyde. The gastrocnemius muscle was excised, cut in small pieces and fixed for 1 h in 2% formaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4. Secondary fixation was performed with 1% osmium tetroxide for 30 min, followed by staining with 1% tannic acid for 20 min and 0.5% uranyl acetate for 1 h. Samples were then dehydrated in a graded series of ethanol and embedded in Embed-812 epoxy resin. Sections (70 nm) were made using a Leica UC7 ultramicrotome and picked on slot grids coated with 1% formvar in chloroform. Samples were then post-stained with 1% uranyl acetate for 7 min and Reynolds lead citrate for 5 min. Transmission electron microscopy images were acquired on a Hitachi H-7650 microscope operating at 100 KeV and equipped with a XR41M mid mount AMT digital camera.

Immunoblotting
For analysis of total cellular fractions, frozen tissue samples were homogenized in RIPA buffer containing protease and phosphatase inhibitor cocktails using TissueLyser II. Cell and tissue homogenates were centrifuged at 20000 x g for 10 min at 4°C, the supernatant was collected and proteins quantified by the Bradford method. For subcellular fraction analysis, mitochondria were isolated from liver according to (Elkholi et al., 2019). Briefly, liver cuttings were harvested in mitochondrial isolation buffer (MIB: 200 mM mannitol, 68 mM sucrose, 10 mM HEPES-KOH pH 7.4, 10 mM K 1 mM EDTA, 1 mM EGTA, 0.1% BSA, supplemented with protease inhibitors). Liver pieces were minced and incubated on ice for 20 min, then homogenized using a 2 mL Potter-Elvehjem Teflon dounce (20 strokes). The homogenate was centrifuged for 10 min at 800 x g at 4°C, the supernatant collected, and centrifuged again using the same conditions to ensure that no unlysed cells or nuclei were present. The resulting supernatant was centrifuged for 10 min at 8000 x g at 4°C. The supernatant was collected as cytosol; the pellet was washed once in MIB, lysed in RIPA buffer and collected as mitochondrial fraction.

SDS-PAGE was performed by loading 20 μg total protein onto 10 or 12% polyacrylamide gel. Proteins were then transferred onto nitrocellulose membranes, blocked with 5% low-fat milk and incubated with primary antibodies for 16 h at 4°C. HRP-conjugated secondary antibodies were incubated for 1 h at room temperature and developed with ECL Prime. Chemiluminescence was acquired with GE Amersham Imager 680.

Gene expression analyses
RNA extraction and qPCR
For lung RNA-Seq, both lungs were harvested, cleaned from fat and bronchi and homogenized in 1 mL Trizol using a TissueLyser II. Liver samples (~50 mg) were homogenized in 500 μL Trizol. Homogenates from both tissues were centrifuged at 20000 x g for 3 min at 4°C and 500 μL supernatant were used for RNA extraction. Extraction was performed with 100 μL chloroform and the aqueous layer was transferred to a RNeasy mini spin column. RNA purification was performed according to the manufacturer’s protocol including one step of in-column DNase treatment. RNA was quantified in Nanodrop and 1 μg total RNA was used to synthesize cDNA using SuperScript II and Oligo dT. Real-time quantitative PCR was performed using Sybr Green reagent and ABI QuantStudio 7 equipment. Relative gene expression is reported as 2ΔΔCt relative to a control gene (Actb or Gapdh). A list of the oligonucleotide sequences used can be found in Table S6.
RNA-Seq
Total RNA samples were checked for quality using AATI Fragment Analyzer. Only samples with RNA Quality Number (RQN) > 7 and clearly defined 28S and 18S peaks were considered for downstream analysis.

mRNA libraries were prepared, pooled and sequenced (75 bp, single end) using NextSeq500.

Quality Assessment and Alignment
Prior to alignment, quality of the sequences was assessed using FASTQC and MultiQC (Ewels et al., 2016). Sequences were then aligned against the Mus musculus genome version 97, with the annotation file for the genome version 97 (for analyses in Figures 2 and 3) or version 99 (for the analysis in Figure 7), both obtained through the website of Ensembl. The alignment was performed using STAR (Dobin et al., 2013), with default parameters and with the option of GeneCounts.

Data analysis
The files obtained from GeneCounts were imported to R (version 3.5.3), taking into account the strandness inherent to the sequencing protocol. Downstream analysis was performed using DESeq2 (version 1.22.2) (Love et al., 2014). Data from raw counts were normalized through a Regularized Log Transformation (rlog) to create the Principal Component Analysis plot and Heatmaps (Love et al., 2014). The log2FC provided by the standard DESeq2 model was shrunk using the ‘ashr’ algorithm (Stephens, 2017). Gene Information was obtained using the package org.Mm.eg.db. For the purposes of this study, genes were considered differentially expressed when the p value, adjusted using false discovery rate (FDR), was below 0.05. Functional clustering was performed using the DAVID Gene Functional Classification tool (https://david.ncifcrf.gov).

Liver metabolomics
Sample preparation
Liver samples (30–80 mg) were weighed and homogenized in 500 μL ice-cold methanol:acetoniitrile:Н2О (2:2:1, v/v) using a TissueLyser II. Homogenates were incubated at –80°C for 4 h and centrifuged at 20000 xg for 10 min at 4°C. The supernatant containing soluble fractions was stored at –80°C. The pellet was resuspended in 400 μL ice-cold 80% (v/v) methanol by vortexing for 1 min at 4°C. Samples were then incubated for 30 min at –80°C and centrifuged at 20000 xg for 10 min at 4°C. Supernatant was collected and combined with the previously obtained supernatant containing soluble fractions. Samples were centrifuged again and the supernatant stored at –80°C until analysis.

Untargeted metabolomics
Extracted samples were thawed on ice, centrifuged for 2 min at 15,000 xg, and diluted according to the different sample weight with 0.1% formic acid (RP, reversed phase) or 50% acetonitrile (ACN) (HILIC, hydrophilic interaction chromatography). 2.5 μL of each diluted sample were pooled and used as a quality control (QC) sample. Samples were randomly assigned into the autosampler and metabolites were separated on a SeQuant ZIC-pHILIC HPLC column (Merck, 100 × 2.1 mm; 5 μm) or a RP-column (Waters, ACQUITY UPLC HSS T3 150 × 2.1; 1.8 μm) with a flow rate of 100 μL/min delivered through an Ultimate 3000 HPLC system (Thermo Fisher Scientific). The gradient ramp up time takes 25 min from 10% to 80% B in HILIC (A: ACN; B: 25 mM ammonium bicarbonate (ABC) in water) and from 1% to 90% B in RP (A: 0.1% FA in water; B: 0.1% FA in ACN). Metabolites were ionized via electrospray ionization in polarity switching mode after HILIC separation and in positive polarity mode after RP separation. Sample spectra were acquired by data-dependent high-resolution tandem mass spectrometry on a Q-Exactive Focus (Thermo Fisher Scientific). Ioni-
50 mM KPi pH 7.5, 60 μM ubiquinone, 3 mg/mL BSA, 300 μM potassium cyanide (KCN), and 20 μg total protein. Complex II (succinate dehydrogenase) activity was determined by measuring the reduction of 80 μM dichloroindophenol sodium salt hydrate (DCPIP) at 600 nm in a reaction mixture containing 25 mM KPi pH 7.5, 20 mM succinate, 50 μM decylubiquinone, 1 mg/mL BSA, 300 μM KCN, and 10 μg total protein. Complex III (ubiquinol:cytochrome c oxidoreductase) activity was determined by measuring reduction of 75 μM cytochrome C at 550 nm in the presence of 25 mM KPi pH 7.5, 100 μM decylubiquinol (obtained by reduction of decylubquinone with potassium borohydride), 0.025% (v/v) tween-20, 100 mM EDTA, 500 μM KCN, and 1.5 μg total protein. Complex IV (cytochrome c oxidase) activity was determined by measuring oxidation of 60 μM cytochrome C (previously reduced with sodium dithionite) at 550 nm in the presence of 50 mM KPi pH 7.0 and 1.0 μg total protein. Citrate synthase (CS) activity was determined by following reduction of 100 μM 5,5¢-Dithiobis(2-nitrobenzoic acid) (DTNB) at 412 nm in a reaction mixture containing 100 mM Tris-HCl pH 8.0, 300 μM acetyl CoA, 0.1% (v/v) Triton X-100, 300 μM oxaloacetic acid, and 3 μg total protein. All reactions were performed at 37°C in 96-well plates (maximum of 12 simultaneous reactions) and absorbance was recorded using an Infinite M200 plate reader (Tecan). Unspecific activity of each complex was determined by performing a reaction in the presence of an appropriate inhibitor (rotenone for complex I, malonate for complex II, antimycin for complex III, and KCN for complex IV), which was then subtracted from the total activity of each sample. Enzymatic activity was calculated in nmol.min⁻¹.mg protein⁻¹, normalized for CS activity and expressed as percentage of the control.

Flow cytometry
HepG2 cells were incubated with doxycycline for 24 h, trypsinized and centrifuged at 200 x g for 5 min at room temperature. Cells were then stained with 50 nM Mitotracker Green FM (for total mitochondrial content) for 30 min at 37°C, or with 5 μM MitoSOX Red (for mitochondrial ROS) for 10 min at 37°C in PBS supplemented with 2% FBS and 10 mM EDTA. Cells stained with MitoSOX were centrifuged, washed and resuspended in buffer, while cells stained with Mitotracker Green were centrifuged and resuspended without washing. Flow cytometry data were acquired on FACSCalibur (Becton Dickinson) and analyzed using the FlowJo software package (version 887).

QUANTIFICATION AND STATISTICAL ANALYSIS
Mantel-Cox test was used for survival curve analysis. For infections with E. coli, mice with no changes in body temperature and weight within the first 24 h (temperature > 35°C and body weight > 95%) were excluded from the analysis. Mann-Whitney test was used for pairwise comparisons and two-way ANOVA with Tukey test was used for multiple comparisons. Statistical analysis was performed with Graphpad Prism 6.0 (GraphPad Software). The number of subjects used in each experiment is defined in figure legends. The following symbols were used in figures to indicate statistical significance: p < 0.05 (*); p < 0.01 (**); p < 0.001 (**); p < 0.0001 (**).
Supplemental Information

Tetracycline Antibiotics Induce Host-Dependent Disease Tolerance to Infection

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Figure S1, related to Figure 1. Characterization of doxycycline and chloramphenicol effects in vivo and in vitro.

Survival (A), rectal temperature (B) and % initial body weight (C) after infection with TetR CamR E. coli and treatment with chloramphenicol at the time of infection. (D) Growth curve of the TetR CamR E. coli strain in the presence or absence of 10 μg/mL doxycycline. (E) TNFα and Interleukin (IL)-6 concentrations in supernatant of bone marrow-derived macrophages incubated with doxycycline for 1h followed by stimulation with PFA-fixed E. coli (MOI=20) for 4h. (F) Percentage of initial weight in mice infected with a sublethal (100 pfu/mouse) dose of Influenza A PR/8 and treated with doxycycline at days 4, 5, and 6 post-infection. (G) Survival of mice infected with 5x10^6 CFU/mouse Candida albicans and treated with doxycycline at 0, 24, and 48h. Survival (H) and parasitemia (I) (% infected red blood cells, iRBC) in mice infected with 1x10^5/mouse Plasmodium berghei Anka and treated with doxycycline daily from days 0 to 8 post-infection.

(A), (B), and (C) represent pooled data from three independent experiments. (D) and (E) represent a single experiment with five technical replicates. (F) is representative from three independent experiments. (G), (H), (I) represent a single experiment. Mean±SD are shown in (B), (C), (D), (E), (F) and (I).
Figure S2, related to Figure 2. Lung RNA-Seq analysis.

(A) Top GO_BP annotation of genes up-regulated during infection (PBS-treated mice), log2 fold change>5; p<0.05. (B) Volcano plots of i.p. doxycycline versus PBS-treated mice in the absence or (C) presence of infection. Numbers indicate genes with log2 fold change <-5 or >5 and p<0.05. (D) Top GO_BP annotation of genes down-regulated with i.p. doxycycline treatment (non-infected mice), log2 fold change<-5; p<0.05. (E) Survival of Krt6a-depleted mice upon infection with E. coli and i.p. treatment with doxycycline. Krt6a-DTR mice were treated with 12 ng/g diphteria toxin by intra-tracheal administration 4 days before infection. Data represents a single experiment.
Figure S3

A

- inflammatory response
- immune response
- chemokine-mediated signaling pathway
- chemotaxis
- cellular response to interleukin-1
- cellular response to interferon-gamma
- neutrophil chemotaxis
- cellular response to lipopolysaccharide
- positive regulation of inflammatory response
- cellular response to interferon-beta
- defense response to virus
- response to lipopolysaccharide
- positive regulation of ERK1 and ERK2 cascade
- immune system process
- positive regulation of angiogenesis
- negative regulation of cell proliferation
- cellular response to tumor necrosis factor
- negative regulation of peptidase activity
- innate immune response
- positive regulation of cell proliferation

-B

Enrichment Score: 2.89

| Gene Name                               | log2 fold change | PAdj       |
|-----------------------------------------|-----------------|------------|
| collagen, type VI, alpha 3 (Col6a3)     | -1.44           | 8.1E-05    |
| collagen, type VI, alpha 2 (Col6a2)     | -1.11           | 1.2E-02    |
| collagen, type VI, alpha 1 (Col6a1)     | -1.26           | 2.7E-03    |
| collagen, type V, alpha 2 (Col5a2)      | -1.25           | 2.4E-03    |

C

D

E

Survival (%) vs Time (Days)
Figure S3, related to Figure 3. RNA-Seq analysis in liver.

(A) Top GO_BP annotation of genes up-regulated during infection (PBS-treated mice), log2 fold change>5; p<0.05. (B) Gene functional clustering of down-regulated genes upon doxycycline treatment (infected groups), log2 fold change <-1, p<0.05. (C) Survival of mice after infection with *E. coli* and treatment with perhexilene. (D) Survival of C57BL/6J mice after infection with *E. coli*. Mice were injected with AAV8 expressing Cpt2 shRNA (or scramble shRNA as a control) 7 days before infection. (E) Cpt2 mRNA expression in mouse liver 7 days after injection of AAV8 Cpt2 shRNA (or scramble shRNA as a control). (C) and (D) represent data from a single experiment. (E) represents 4 mice per group assayed in triplicate, bars indicate the mean.
Figure S4, related to Figure 4. Fatty acid oxidation and glucocorticoid signaling gain-of-function experiments.

(A, B, C) Survival of C57BL/6J mice after infection with E. coli. Mice were injected with AAV8-TBG expressing CPT1a (A) SLC25A20 (B), or a combination of CPT1a+CPT2+SLC25A20 (1:1:1) (C) one week before infection. Right-side panels represent mRNA expression of the target genes. (D) Survival of C57BL/6J mice after infection with E. coli and treatment with octanoic acid by oral gavage at 2, 8, 24, and 48h after infection. (E, F) Survival of C57BL/6J mice after infection with E. coli and treatment with CP868388 (PPARα agonist) (E) or dexamethasone (GR agonist) (F) by IP injection at the time of infection. (A) and (D) represent pooled data from three independent experiments. (B), (E) and (F) represent pooled data from two independent experiments. (C) represents data from a single experiment.
**Figure S5**

**A**
- Graph showing temperature plotted against time (Days)
- PBS (n=12) vs. Doxycycline (n=13)
- * denotes statistical significance

**B**
- Graph showing % weight plotted against time (Days)
- PBS (n=12) vs. Doxycycline (n=13)
- Statistical significance indicated

**C**
- Graph showing survival percentage plotted against time (Days)
- Ad.GFP (n=5) vs. Ad.Tufm (n=6)
- Statistical significance indicated

**D**
- Graph showing mRNA expression (A.U.)
- Ad.GFP vs. Ad.Tufm

**E**
- **MitoSOX red**
- Control vs. 1.25 µg/mL Doxy vs. 5 µg/mL Doxy

**F**
- **Mitotracker green**
- Control vs. 1.25 µg/mL Doxy vs. 5 µg/mL Doxy

**G**
- Graph showing survival percentage plotted against time (Days)
- Atm<sup>+/−</sup> Ad.Cre + PBS (n=5) vs. Atm<sup>−/−</sup> Ad.Cre + Doxycycline (n=6)
- Statistical significance indicated
Figure S5, related to Figure 5. Possible mechanisms of doxycycline-induced disease tolerance.

Rectal temperature (A) and % initial body weight (B) after infection of germ-free mice with TetR CamR E. coli and treatment with doxycycline at 0, 24 and 48h. (C) Survival of E. coli-infected mice and (D) Tufm mRNA expression in mouse liver. Mice were injected with AAV8-TBG expressing TUFM one week before infection/liver collection. (E) Mitochondrial peroxide (MitoSOX red) and total mitochondrial content (Mitotracker green, F) in HepG2 cells after incubation for 24h with the indicated concentrations of doxycycline. (G) Survival of mice with liver-specific ATM depletion upon infection with E. coli and treatment with doxycycline. Atmlox/lox mice were treated with AAV8-Cre one week before infection. (A) and (B) represent mean±SD pooled from four independent experiments. (C) and (G) represent data from a single experiment. (E) and (F) indicate the mean of three replicates representative of two independent experiments.
Figure S6, related to Figure 6. Protective effects of phenformin treatment and partial CRIF1 deletion.

Rectal temperature (A) and % initial body weight (B) after infection of $Crif1^{lox/lox}$ or $Crif1^{lox/-}$ mice with TetR CamR $E. coli$. Mice were previously injected with AAV8-TBG expressing Cre recombinase (or GFP as a control). Rectal temperature (C) and % initial body weight (D) after infection of C57BL/6J mice with $E. coli$ and treatment with a single administration of phenformin at the time of infection. (E) Survival after infection of C57BL/6J mice with $E. coli$ and treatment with phenformin at 0 and 24h after infection. (F) Enzymatic activity of the ETC complexes II, III, IV and CS in mouse liver collected 12h after PBS or phenformin treatment. ETC activity is expressed as % of PBS-treated control, normalized for CS activity. (G) TNFα, IL-6, IL-10, and IL12/IL23(p40) concentrations in mouse serum at the indicated time-points after infection. (H) Organ damage score and (I) representative images of Hematoxylin-Eosin stained slides from liver, lung, and kidney 24h after infection. Score 0 = no lesions; 1 = very mild; 2 = mild lesions. (A) and (B) represent pooled data from three independent experiments. (C) and (D) represent pooled data from four independent experiments. (E) represents data from a single experiment. (F) represents mean±SD of 5 mice/group from a single experiment. (G) represents data from a single experiment; squares represent individual mice and bars indicate the mean.
| Gene   | Fw                     | Rv                      |
|--------|------------------------|-------------------------|
| Gapdh  | AACTTTGGCATTGTGGAAGG   | CACACATTGGGGGTAGGAACA   |
| Cpt1a  | CTCCGCCTGAGCCATGAAG    | CACCAGTAGATGATGCCATTCT  |
| Cpt2   | CAAAAGACTCATCCGCTTTGTTC | CATCAGCAGACTGGGTTGGTA   |
| Slc25a20 | GCGCCCATCATGGGAGTCA | CACACCAGATAAACATCCAGC |
| Crif1  | TGCTCGCTTCCAGAAGACTATT | CATAGCAGCAATTCGTCCT |
| Actb   | GGCTGTATTCCCTCCATCG    | CCAATGGTAAACAATGGCATGT |
| Tufm   | GCAGCCACTCTATGCGAG     | CCCACCTGGAGGAGGCCATCC  |
| Adrb1  | CATCATGGGTGTGTTCACG    | GAAGACGAAGAGGCGATCC    |
| Adrb2  | GGTTATCGTCCTGGCCATCGTGTGTTTG | TGGTTGCTGAAAGATCAGCAAGATCT |
| Adra1a | TGCGAGGACTGAAGTCGCT    | CAGGGACGACTGGGGAATGG   |
| Adra2b | GAAGGAGTCTCGGAGCTAA    | GCCCTGACAGGTAAGATA     |
| Adrb3  | GGGGCTCTCTAGTCCAGAG   | TAGCCATCAAACCTGGTGGAGC |
| Adra1b | CGGTCATCTGGTGATGTACT   | TACAATGCGCCAGGGTTGGC   |