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The role of mitochondrial function and metabolism

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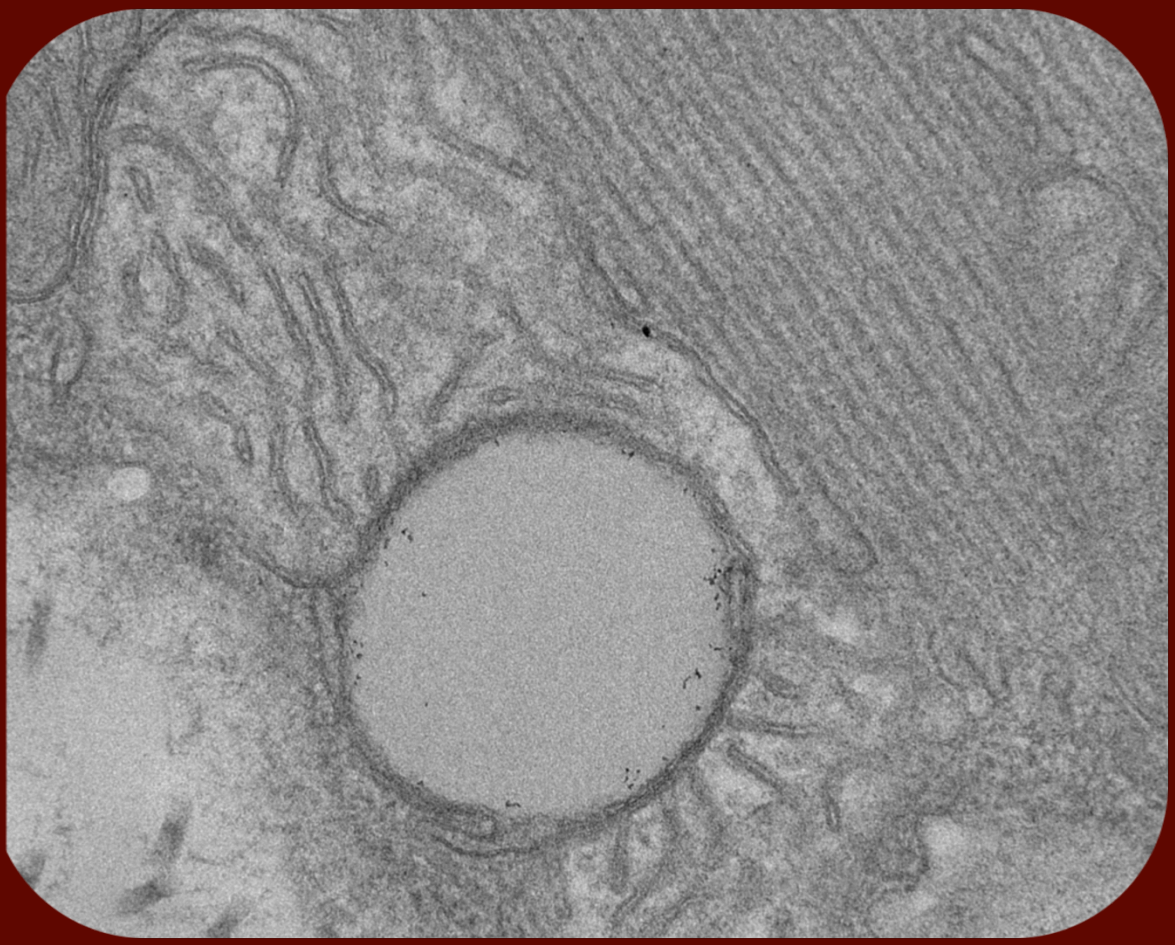

Dissertation presented to obtain the Ph.D degree in Integrative Biology and Biomedicine

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
January, 2020

UNIVERSIDADE NOVA DE LISBOA

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Research work coordinated by:

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Cover: transmission electron microscopy image of mouse skeletal muscle, showing one mitochondrion and a lipid droplet. Photo credits: Henrique Colaço and Sara Bonucci (Instituto Gulbenkian de Ciência).

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To my nephew Pedro,

a tiny scientist.

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Acknowledgments

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Summary

Optimal organismal function and survival in adverse conditions require robust homeostatic responses to challenging environmental conditions. Surviving a severe infection requires the synergy between resistance and disease tolerance, two evolutionarily conserved defense strategies that limit disease severity. Sepsis – a dysregulated host response to infection that leads to high risk of death – is a prime example of extreme homeostasis disruption and therefore constitutes an excellent model to study homeostasis and inter-organ communication principles.

An increasing number of studies have described cellular surveillance mechanisms that detect and correct deviations in homeostasis. How these protective programs can be harnessed to improve organismal fitness in extreme disruptions of homeostasis, such as sepsis, is still unknown. This thesis presents several lines of evidence for the protective effect of mild perturbations of core cellular functions in the context of infection. We began by gathering evidence from the literature that pathogen-induced homeostasis perturbations can be used by the host as an alarm sign for infection, thus triggering the appropriate defense responses.

We then hypothesized that pharmacologically targeting core functions of the cell might unveil protective effects that can be used as new therapeutic options for sepsis and other inflammatory conditions. Using a mouse model of bacterial sepsis, we tested the protective effect of clinically approved drugs known to perturb cellular functions. We found that tetracycline antibiotics – in particular doxycycline – robustly increase survival to sepsis by inducing disease tolerance, independently from their direct antibiotic properties. By analyzing the effects of doxycycline in the main targets of organ dysfunction during sepsis, we found improved fatty acid oxidation and glucocorticoid signaling in the liver, together with increased damage repair in the lung.

Mechanistically, we found that doxycycline – by binding to the mitochondrial ribosome and blocking the translation of mitochondrial-encoded transcripts –

decreases mitochondrial respiration *in vivo*, without compromising mitochondrial viability. Using a partial and acute deletion in the liver of CRIF1, a critical mitoribosomal component for protein synthesis, we found that mice are protected against bacterial sepsis. This observation is phenocopied by the transient inhibition of respiratory chain complex I activity by phenformin.

Together, we demonstrate that mitoribosome-targeting antibiotics are beneficial beyond their antibacterial activity. Furthermore, we prove that mitochondrial protein synthesis inhibition leading to respiratory chain perturbation is a novel mechanism for the induction of disease tolerance.

Resumo

A manutenção de funções corporais e a sobrevivência em condições adversas requerem respostas homeostáticas a condições ambientais adversas. A sobrevivência a uma infeção grave envolve a sinergia entre resistência e tolerância, dois mecanismos de defesa conservados ao longo da evolução que limitam a gravidade da infeção. A sépsis – uma resposta desregulada do hospedeiro a uma infeção, que conduz a um elevado risco de mortalidade – é um caso exemplar de desvio extremo da homeostasia e, como tal, constitui um modelo excelente para estudar os princípios fundamentais de homeostasia e comunicação entre órgãos.

Um número crescente de estudos tem descrito mecanismos celulares de vigilância que detectam e corrigem desvios nas condições de homeostasia. No entanto, a forma como estes programas celulares podem ser usados para melhorar a resposta do organismo a desvios extremos da homeostasia – como é o caso da sépsis – permanece desconhecida. Esta tese apresenta várias linhas que suportam a ideia de que perturbações ligeiras em funções celulares básicas têm um efeito protetor no contexto da infeção. Começamos por apresentar evidências publicadas na literatura de que perturbações da homeostasia provocadas por microrganismos patogénicos podem ser usadas pelo hospedeiro como um sinal de alarme que indicia uma infeção e ativa os mecanismos de defesa necessários.

Levantámos então a hipótese de que fármacos que perturbam as funções básicas das células podem ter efeitos benéficos que podem ser usados para novas opções terapêuticas na sépsis e em outras condições inflamatórias. Usando um modelo de sépsis bacteriana em ratinhos, testámos o efeito protetor de fármacos aprovados para uso clínico, conhecidos por perturbar determinadas funções celulares. Descobrimos que o grupo de antibióticos tetraciclinas – em particular, a doxiciclina – aumentam a sobrevivência à sépsis por indução de mecanismos de tolerância, que são independentes do efeito antibiótico destes fármacos. Ao analisar os efeitos da doxiciclina nos principais

órgão afectados pela sépsis, mostrámos que este fármaco melhora a oxidação de ácidos gordos e a resposta aos glucocorticóides no fígado, aumentado também a reparação das lesões pulmonares.

Em termos mecanísticos, reportámos que a doxiciclina – ao ligar-se ao ribossoma mitocondrial e ao bloquear a tradução de transcritos mitocondriais – diminui a atividade respiratória da mitocôndria *in vivo*, sem afectar a viabilidade mitocondrial. Usando uma deleção parcial e aguda da proteína CRIF1 no fígado, que tem um papel crítico na síntese proteica mitocondrial, descobrimos que os ratinhos ficam protegidos contra a sépsis bacteriana. Este resultado é replicado pelo o tratamento com fenformina, outra intervenção farmacológica que provoca uma inibição transitória na atividade do complexo I da cadeia respiratória.

Em conjunto, estes resultados demonstram que antibióticos que se ligam ao mitoribossoma têm um efeito benéfico que vai além da sua atividade antimicrobiana. Adicionalmente, provámos que a inibição da síntese proteica mitocondrial, que conduz a uma perturbação na atividade da cadeia respiratória, constitui um novo mecanismo de indução de tolerância à infeção.

List of abbreviations

2-DG	2-deoxyglucose
AAV	Adeno-associated virus
ADP	Adenosine diphosphate
ALT	Alanine transaminase
AMPK	5' AMP-activated protein kinase
ARSD	Acute respiratory distress syndrome
AST	Aspartate transaminase
AT	Ataxia telangiectasia
ATF	Activating Transcription Factor
ATFS	Activating Transcription Factor associated with Stress
ATGs	Autophagy related proteins
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BMDMs	Bone marrow-derived macrophages
C/EBPβ	CCAAT-enhancer-binding proteins
CACT	Carnitine-acylcarnitine translocase
cAMP	Cyclic adenosine monophosphate
CamR	Resistant to chloramphenicol
CFU	Colony-forming unit
CGN2	General control nonderepressible 2 kinase
CHOP	C/EBP homologous protein
CK	Creatine kinase
CLP	Cecal ligation and puncture
CLR	C-type lectin receptors
CoA	Coenzyme A
CPT	Carnitine palmitoyltransferase
CS	Citrate synthase
cSADDs	Cellular surveillance-activated detoxification and defenses
CSFs	Colony-stimulating factors
CXCL	Chemokine (C-X-C motif) ligand
DAMPs	Damage-associated molecular patterns
dsRNA	Double-stranded RNA
ECM	Extracellular matrix
eIF2α	Eukaryotic Initiation Factor 2
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ETC	Electron transport chain
ETI	Effector-triggered immunity
FA	Fatty acid
FAD	Flavin adenine dinucleotide
FAO	Fatty acid oxidation
FBS	Fetal bovine serum

FFA	Free fatty acid
FGF	Fibroblast growth factor
GDF	Growth Differentiation Factor
GF	Germ-free
GFP	Green fluorescent protein
GR	Glucocorticoid receptor
GTP	Guanosine-5'-triphosphate
HIF	Hypoxia-inducible factor
HMGB1	High mobility group box 1
HO-1	Heme oxigenase 1
HRP	Horseradish peroxidase
HSF	Heat shock factor
ICU	Intensive care unit
IFN	Interferon
IL	Interleukin
IMM	Inner mitochondrial membrane
ISR	Integrated stress response
JNK	c-Jun N-terminal kinase
KRT	Keratin
LCE	Late cornified envelope
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MAMPs	Microorganism-associated molecular patterns
MAVS	Mitochondrial antiviral-signaling protein
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MPC	Mitochondrial pyruvate carrier
mTORC	Mechanistic target of rapamycin complex
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor-κB
NLR	NOD-like receptors
NR	Nicotinamide adenine dinucleotide
NRF	Nuclear factor erythroid
OMM	Outer mitochondrial membrane
PAMPs	Pattern-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PDC	Pyruvate dehydrogenase complex
PEP	Phosphoenolpyruvate
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PPAR	Peroxisome proliferator–activated receptor
PRRs	Pattern recognition receptors
qPCR	Quantitative polymerase chain reaction

RLR	RIG-I-like receptors
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SCGB	Secretoglobin protein family
sh-RNA	Short hairpin RNA
SIRS	Systemic inflammatory response syndrome
SLC	Solute carrier family
SOFA	Sequential organ failure assessment
SPF	Specific pathogen-free
TBG	Thyroid hormone-binding globulin
TCA	Tricarboxylic acid
TCR	T-cell receptor
TetR	Resistant to tetracyclines
T_H	T-helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
UPR	Unfolded protein response
UPR^{mt}	Mitochondrial unfolded protein response
XBP-1	X-box binding protein 1
Wnt	Wingless-type MMTV Integration site family

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Chapter 1

Introduction

1.1 Introduction to homeostasis and stress responses

The notion that organisms regulate their body functions in order to maintain a stable environment that is resilient to changes from the outside dates back to ancient Greece. At the time, different 'humors' were thought to regulate organ function, such that any disease could be explained by an imbalance of these essential fluids, either caused by endogenous factors or environmental stimuli. In the 19th century, Claude Bernard founded the basis of modern physiology by proposing that the 'milieu intérieur' – the fluids that comprise the inner part of an organism, which regulate its balance and protect it from external stress[1] – are the most basic condition to support independent forms of life. This concept would be later explored by Walter B. Cannon, who coined the term homeostasis and proposed the existence of active mechanisms that work cooperatively to detect and correct even mild deviations from the setpoint[2].

In the subsequent decades, our understanding on how complex multicellular organisms regulate and maintain homeostasis has increased dramatically. We now know that mammals, for example, have evolved ways of maintaining temperature, pH, levels of glucose and ions, cell number, and countless other parameters, within very narrow ranges that result in optimal function of biochemical processes. Regulation of homeostasis encompasses two major steps: surveillance mechanisms that recognize deviations from setpoints, and compensatory responses – globally known as stress responses – that promote the adequate changes[3].

Surveillance mechanisms are represented at an organismal level (e.g. sensory neurons that detect changes in temperature and pressure, or pancreatic cells that sense blood glucose levels), but also at a cellular level (e.g. sensing of intracellular ATP levels by AMPK)[4]. Likewise, stress responses span from cell-autonomous to systemic reactions (Figure 1-1). While the former usually involve cellular signal transduction pathways, ultimately leading to changes in gene expression or protein function that correct the necessary deviations (e.g. heat stress leads to an increase in the expression of heat shock proteins that prevent

protein misfolding)[3]; the latter are typically mediated by the central nervous system, resulting in increased circulating levels of catecholamines, glucocorticoids, and other 'stress hormones' that mediate a complex set of biochemical and behavioral changes[5]. The integration between cellular and organismal stress responses through the release of 'danger signals' remains poorly understood[6,7], although a few elegant studies using the nematode *Caenorhabditis elegans* have provided valuable insights[8,9].

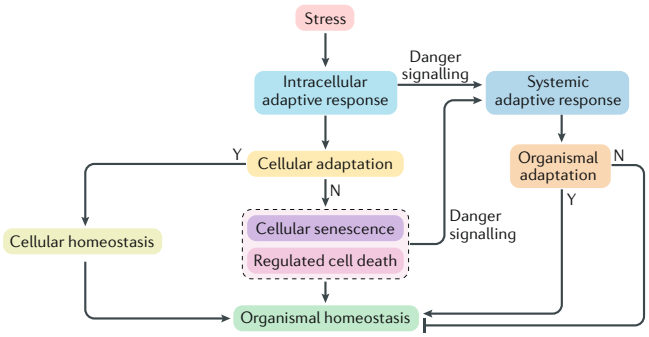

Figure 1-1 – Cellular and organismal stress responses that restore homeostasis (taken from[6]).
Local stress induces cellular stress responses aimed at restoring homeostasis. Affected cells that are unable to correct the defects are removed by senescence or cell death programs, thus sparing neighboring tissues from further damage. In some cases, local stress responses are communicated distally and trigger systemic changes.

Despite the advances in understanding basic mechanisms of physiology, the remarkable complexity of organisms – in which different organs need to be simultaneously independent and cooperative – and the myriad of cell autonomous and non-autonomous stress responses does not cease to motivate intense research efforts[10]. The next sections will provide a more detailed description of how animals respond to a major threat to homeostasis: invasion by pathogens. Later, we will come back to the concept of stress responses, and explore the central role of mitochondria in perceiving deviations in the environment and triggering compensatory changes in cellular function.

1.2 Host-pathogen interactions: an evolutionary arms race

Protection against invading pathogens is an essential component of any program of homeostasis maintenance. As metazoans strive to keep their interior sterile, a complex network of sensors and effectors is in place to: 1) detect the presence of microorganisms; 2) discriminate between commensal and pathogenic microbes; 3) assess the severity of the threat; and 4) start the adequate compensatory responses.

1.2.1 Pathogen sensing

1.2.1.1 Microorganism-associated molecular patterns (MAMPs)

The idea that specific molecules expressed by microbes (MAMPs) are recognized by specialized sensors of the immune system, named pattern-recognition receptors (PRR), was originally proposed by Janeway in 1989[11] and remains to this day as the most widely accepted mechanism for pathogen detection. Experimental proof to support this theory arouse from the discovery of the Toll protein in *Drosophila melanogaster*[12], soon followed by its mammalian homologue Toll-like receptor (TLR) 4[13]. PRRs recognize molecular signatures associated with basic biological functions of particular classes of microorganisms, such as components of the cell wall or nucleic acids. For example, binding of lipopolysaccharide (LPS) from gram-negative bacteria to TLR4 expressed at the surface of patrolling immune cells, such as macrophages, engages a signaling pathway that culminates with activation of innate immune responses. To this day, twelve TLR have been identified in mice, all localized at the cell surface or at the endosomal membrane and with a wide range of ligands that include proteins, lipoproteins, dsRNA and DNA from viruses and bacteria[14].

Besides TLR, a number of other PRR have been identified. NOD-like receptors (NLR) are cytosolic sensors that bind to a variety of ligands, most notably

bacterial peptidoglycan, and engage innate immunity pathways, such as the inflammasome[15]. RIG-I-like receptors (RLR) are cytosolic proteins specialized in the recognition of viral RNA, leading to the activation of antiviral programs such as the interferon (IFN) response[16]. Finally, C-type lectin receptors (CLR) are a large family of sensors that bind carbohydrates – the best characterized example being binding of β-glucan by dectin 1 – with an important role in antifungal responses[17].

While PRRs represent an effective tool to detect microbial components – as highlighted by the fact that they are conserved across most metazoans – the MAMP theory fails to grasp the complexity of pathogen sensing. In fact, MAMPs are expressed by commensals and pathogens alike, forcing hosts to employ a series of checkpoints and strategies to distinguish between harmful and innocuous threats[18]. Of particular interest is the strategic location of PRR in compartments where microbes are normally absent, such the basal surface of an epithelium rather than the apical, microbe-rich lumen, as proposed by the theory of patterns of pathogenesis[19].

1.2.1.2 Damage-associated molecular patterns (DAMPs)

The 'danger theory', first proposed by Matzinger to explain immune tolerance, postulated that activation of immune responses relied on the detection of danger signals rather than specific non-self molecules[20]. It became apparent in recent years that signals released by necrotic or seriously injured cells are perceived as danger signals (or DAMPs) by the immune system. These include molecules normally present inside functioning cells that become immunogenic once exposed (such as DNA, ATP or HMGB1), and also inflammatory molecules (also known as alarmins, e.g. IL-33) that are released upon cell lysis[21]. Interestingly, DAMPs and MAMPs target similar PRRs, indicating that both types of signals converge in the activation of downstream immune responses[22]. The selective activation of patrolling cells by either or both signals

might help to set the threshold for a full-blown response – for instance, detection of commensal bacteria might be tolerated as long as it causes no harm to the surrounding cells. Conversely, the absence of a microbial pattern in the presence of damage signals resulting from physical damage might help limiting an otherwise excessive immune response to a sterile stimulus.

1.2.1.3 Effector-triggered immunity (ETI)

First described in plants, the ETI is associated with detection of specific effectors expressed by pathogens, such as toxins and virulence factors. Of note, these inducers are not directly detected, but rather their adverse effects on host tissues. Such mechanisms not only allow for a more robust distinction between commensal and pathogenic microorganisms but also evolved as an additional defense against pathogens that escape from the traditional MAMP-PRR signaling[23]. Although there is currently limited evidence for ETI in animals – mostly confined to specific toxins produced by intracellular bacteria[24,25] – it is tempting to speculate that detection of pathogen effectors might play a role in detecting a large array of potential pathogens with a limited set of surveillance mechanisms[26].

1.2.2 Mechanisms of host defense

Host defense encompasses three categories of evolutionarily conserved responses: avoidance, resistance, and disease tolerance.

1.2.2.1 Avoidance

Avoidance consists of a set of behaviors that constitute the first line of defense against infection, as it allows animals to skip contact with noxious environments even before the interaction with pathogens occurs. Despite its importance for

survival, avoidance is the least studied of the mechanisms of host defense, in part due to the complex interaction of traits and trade-offs experienced by an animal in the wild, which are difficult to reproduce in the laboratory[27].

Avoidance results mainly from the integration of visual, olfactory, and gustatory cues that prevent animals from interacting with pathogens, either by avoiding contaminated food[28], environment[29] or conspecifics[30,31]. In *C. elegans*, although several Toll homologues have been identified, none of them was associated with inducible defense systems[32]. Instead, the *tol-1* gene was found to be involved in the discrimination of pathogenic bacteria and induction of avoidance behavior[32]. Following this observation, much of the work on avoidance mechanisms has been carried out in this bacteria-feeding nematode, which, despite its rudimentary nervous system, has evolved aversive behavior towards pathogenic bacteria[33] as well as defense and detoxification mechanisms[8]. Some of the sensory networks have been studied in other organisms, including mammals – examples include how the smell of rotting food impacts behavior[30] – and helped to shed light on the adaptive role of repulsion and disgust[34].

More broadly, this set of behaviors is not limited to healthy animals when they avoid contact with pathogens. In gregarious species, infected individuals present with a set of behavioral changes known as sickness behavior, characterized by social isolation, anorexia, loss of libido, lethargy, and changes in physical appearance, such as curling[35,36]. Anorexia is the most studied of these behaviors, with reports pointing to its adaptive role in the activation of immune responses[37] and energy saving programs[38]. For the large majority, however, the reasons for selecting such a complex set of behavioral traits in infected animals remain largely unknown; therefore, a putative role as an altruistic behavior to avoid contamination of healthy conspecifics cannot be ruled out[39].

1.2.2.2 Resistance

Resistance mechanisms include all responses aimed at protecting the host by reducing pathogen load. These properties of immune systems – from the simple antimicrobial peptide responses in *Drosophila* to an intricate crosstalk of innate and adaptive immunity in mammals – have dominated research in host-pathogen interactions over the past few decades[22,40].

Overview of innate and adaptive immunity

In vertebrates, specialized cells of the innate immune system (neutrophils, eosinophils, monocytes, macrophages, mast cells, natural killer cells, innate lymphoid cells, and dendritic cells) patrol blood and tissues, detect invading microbes, and start resistance programs. As previously discussed, activation of PRR typically engages the activity of transcription factors[41] – the most widely studied of which is nuclear factor-κB (NF-κB) – which in turn activate a specialized transcriptional program[42]. In the case of bacterial infections, within minutes from LPS detection by TLR4 at the surface of tissue-resident macrophages, NF-κB drives transcription of tumor necrosis factor α (TNFα), interleukin (IL)-1, IL-6 and other pro-inflammatory cytokines whose main function is to promote local changes in tissue function that facilitate killing of the invading pathogens[43]. These include vasodilation and increased expression of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), in vascular endothelial cells, which facilitate monocyte and neutrophil recruitment from blood vessels into the injury site. In turn, neutrophils respond to these cues by producing chemokines (namely CXCL1 and CXCL5) that enter circulation and participate in the recruitment of more neutrophils[44], thus propagating the inflammatory response.

In addition to cytokine and chemokine secretion, this acute phase response involves a variety of other mediators, such as vasoactive amines (histamine and serotonin, released by mast cells and platelets), vasoactive peptides (e.g. bradykinin and substance P), complement components, lipid mediators

(prostaglandins and thromboxanes), and proteolytic enzymes (e.g. elastin and matrix metalloproteinases)[45] (Figure 1-2).

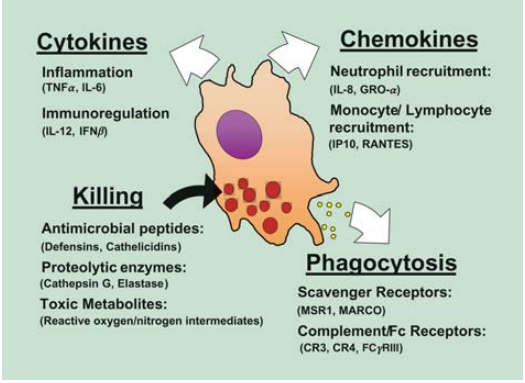

Figure 1-2 – Overview of innate immune responses triggered upon infection (taken from[46]).

The combined effect of these mediators causes dramatic changes in the vasculature, extracellular matrix, and surrounding sensory nerves, producing the classical signs of inflammation, such as swelling, pain, changes in temperature and behavioral alterations[46].

At the site of infection, resident and recruited leukocytes are now able to perform their specialized killing functions: macrophages and neutrophils take up bacteria by phagocytosis and digest them in vesicles through the action of reactive oxygen species (ROS) and reactive nitrogen species (RNS), as well as enzymes (such as neutrophil elastase) and antimicrobial peptides (such as defensins)[46]. Dendritic cells also perform phagocytic functions, but, unlike macrophages, preserve the structure of microbial antigens and subsequently express them at the cell surface by major histocompatibility complex (MHC) molecules, which are then recognized by $CD4^+$ T-helper (T_H) cells via T-cell receptors (TCR)[47]. This provides a link between the innate and adaptive immune systems, and starts a slower, antigen-specific response involving clonal selection and expansion of different subsets of T and B cells, and production of antibodies[48,49].

The innate immune response allows for a very fast (minutes to hours) control of infection even in first-time encounters with a pathogen, by starting resistance programs that target broad classes of microorganisms. In spite of its relatively unspecific function, the selective activation of PPRs, followed by secretion of specific cytokines and chemokines is of paramount importance to instruct the appropriate adaptive immune response – for example, secretion of IL-6 from macrophages upon bacterial infection engages a so-called type 1 immune response from T_H cells, whereas production of IL-13 upon helminthic infection leads to a type 2 response[48]. This depends on a complex communication within innate immune cell populations and between the innate and adaptive branches of immunity, which tailors a directed response from very early stages of infection[22,50].

Regulation of the immune response

As any stress response, activation of immunity aims to restore homeostasis – in this case, by clearing the underlying infection. The magnitude of the inflammatory response is dictated by the initial stimulus (i.e. the number of activated PRR, which is proportional to the pathogen load), but also by a number of regulatory mechanisms that avoid excessive reactions. Small, local infections are frequently ablated by tissue-resident populations alone, whereas slightly larger affected areas might result in paracrine cytokine signaling, which recruits leukocytes from the bloodstream and neighboring tissues[45]. In more severe cases, activated neutrophils, monocytes, and T_H cells can travel to lymph nodes and propagate inflammatory signals to their resident cells[51], thus starting a cascade of distal communication that constitutes the first step of a systemic inflammatory response.

Control of the magnitude of inflammation is exerted at the cellular level via production of anti-inflammatory molecules, the most important of which is IL-10[52]. This anti-inflammatory cytokine is produced by virtually any cell of the innate and immune system in response to PRR activation. Numerous pathways converge in IL-10 transcription (including NF-κB) making its production the

product of the integration of several cues. Importantly, inflammation-induced IL-10 secretion is an attempt to self-limit the inflammatory response by repressing antigen presentation, pro-inflammatory cytokine transcription, and type 1 T_H responses[52]. Other anti-inflammatory signaling molecules include transforming growth factor-β (TGF-β), cAMP, and nuclear hormone receptors, all of which act as brakes for the immune response in a tissue-specific manner[41].

At the organismal level, another level of regulation of inflammation is set centrally by the neuroendocrine system. Pro-inflammatory cytokines such as TNFα, IL-1β, and IL-6 stimulate the hypothalamic-pituitary-adrenal (HPA) axis, accounting for the central effects of inflammation (such as changes in body temperature and sickness behaviors, as previously described) and also stimulating the release of glucocorticoids from the adrenal cortex[53]. Increased circulating levels of glucocorticoids lead to inhibition of NF-κB signaling and constitute an essential negative feedback loop to systemically control inflammation[54], as highlighted by the fact that mice with impaired glucocorticoid signaling are unable to resolve inflammatory processes[55].

Consequences of inflammation

As discussed above, the initial steps of immune response provoke dramatic changes in tissue architecture that help fighting infection. In most cases, tissue remodeling is self-contained and is followed by repair mechanisms orchestrated by cytokines, chemokines, proteolytic enzymes, and growth factors, which ultimately restore normal tissue function[56].

Cell fate determination also plays a key role in the outcomes of inflammation. Cells infected with viruses or intracellular microorganisms, as well as old leukocytes, preferentially undergo programmed cell death mechanisms – most notably apoptosis, but also pyroptosis and necroptosis[57] – and are then removed by phagocytosis. In contrast, severely damaged cells may undergo necrosis and release their cellular contents into the extracellular matrix (ECM), thus providing DAMPs that contribute to aggravate, rather than resolve, the inflammatory response[58]. Failure to clear the infection and resolve inflammation

may have serious consequences for tissue function – examples include the formation of granulomas in cases where macrophages cannot effectively eliminate the pathogen; deposition of collagen in the ECM, leading to fibrosis and loss of tissue function; and persistence of large neutrophil infiltrates[59]. Despite all the checks and brakes, inflammation always comes at a cost for the host. Not only is it energetically expensive, but it also involves the use of highly toxic and reactive species (namely ROS and RNS) to kill pathogens, which cause some degree of tissue damage – these collateral effects are collectively known as immunopathology[60]. As a consequence, regulation of the immune response entails a trade-off between the necessary effector mechanisms (to clear the infection) and damage control tools. The level of immunopathology correlates with the magnitude and duration of inflammation, and can pose a dramatic threat to host physiology and survival when not properly controlled, as we will discuss later (section 1.4.2).

1.2.2.3 Disease tolerance

The concept of tolerance – a mechanism of host protection that does not exert a direct negative impact on pathogen load – was first introduced in the field of plant-pathogen interactions to explain how survival of crops to fungal pests could be uncoupled from resistance mechanisms[61]. Over the past century, theoretical models and experimental evidence have supported the importance of these mechanisms in plant ecology and crop productivity[62] as well as its impact on pathogen evolution[63].

In animals, research on host-pathogen interactions has been largely dominated by studies on the function of the immune system and resistance mechanisms, which led to remarkable success in fighting infectious diseases, for example using vaccines and antibiotics. It was not until the beginning of the 21st century that a study identified genetic variants that confer protection to malaria in mice independently of the pathogen load[64]. By analyzing a collection of inbred mouse

strains with considerable genetic variability and plotting disease severity (anemia and weight loss, in this case) against pathogen load, the authors successfully identified genotypes that correlate with better infection outcomes despite high parasitemia, therefore applying the theoretical principles of plant disease tolerance to animal models[64]. The first mechanistic insights followed soon with the identification of heme oxigenase 1 (HO-1) as an essential component for survival to severe malaria, by minimizing the oxidative damage resulting from hemoglobin release upon infection[65,66]. In *D. melanogaster*, a genetic screen identified several mutants conferring protection to *Listeria monocytogenes* infection independently of immune functions[67], while in *C. elegans* the proteostasis regulator XBP-1 was deemed essential to maintain fitness upon activation of immunity[68]. These initial observations helped to strengthen the notion that, regardless of the studied infection model and host, resistance mechanisms are not sufficient to explain infection outcomes. Most likely, disease tolerance mechanisms co-evolved with immune systems to limit infection-related damage. From that point on, more research groups became committed to the intense effort of uncovering the underlying mechanisms of tissue protection – a complex network that we are only beginning to understand[69–71].

At this point, it is important to distinguish between the similar, and somewhat confusing, terminologies of immunological tolerance and disease tolerance. Immunological tolerance is an old concept to explain the lack of reactivity of T and B cells towards self antigens[72]. While there are some common mechanisms between immunological tolerance and disease tolerance, these are two distinct concepts, with the latter referring exclusively to the context of infectious stimuli.

Disease tolerance and stress responses

Globally, disease tolerance mechanisms encompass any host response that 1) reduces damage caused by an infectious agent; 2) limits an excessive immune response; and/or 3) reduces immunopathology[69]. As seen by the examples above, tolerance mechanisms are tightly connected to stress responses,

meaning that any compensatory response that restores host homeostasis, for example, by controlling tissue damage[73], accelerating tissue repair[74], or reprogramming metabolism[75] could potentially be part of a tolerance program. At the cellular level, several stress responses have been associated with activation of disease tolerance programs[71,73].

Protein misfolding is a common feature of infection-induced tissue damage. As a consequence, activation of proteotoxic stress responses from the endoplasmic reticulum (unfolded protein response, UPR)[68] or cytosol (regulated by heat shock factor 1, HSF-1)[76] have been proven essential to tolerate bacterial infections. Other cellular stress responses required for tissue homeostasis during infection include the antioxidant response (coordinated by the transcription NRF2)[77], and the hypoxia response (regulated by the HIF family of transcription factors)[78].

Pharmacological activation of DNA damage responses by anthracyclines, a class of anticancer drugs that cause double-stranded DNA breaks, constitutes a promising strategy to induce disease tolerance against polymicrobial sepsis in mice[79]. The proposed mechanisms for this protective effect include the activation of the ATM kinase and transcriptional repression of inflammatory genes[79,80].

Another intriguing aspect of stress-induced disease tolerance relates to metabolic sensing and reprogramming in parenchymal cells. In a mouse model of sepsis, pharmacological activation of the cellular energy sensor AMPK results in reduced inflammation and tissue damage, although a mechanistic connection with the metabolic functions of AMPK is still missing[81]. Likewise, reprogramming of glucose metabolism has been shown to promote disease tolerance to sepsis[38,82] and malaria[83].

At an organismal level, centrally regulated stress responses seem to provide another level of activation of less understood tolerance programs. Changes in body temperature are a hallmark of systemic inflammation – which can be reflected in either fever or hypothermia, depending on the host and the causal agent – and have been proposed to play an adaptive role in tissue

homeostasis[84]. Only very recently, a study by Chawla and co-workers started to bring some mechanistic insight into this idea. By exposing mice to different temperatures and performing transcriptional and metabolic tissue profiling upon LPS injection, the authors concluded that inflammation-induced metabolic changes lead to energetic trade-offs that result in hypothermia[85]. Remarkably, this hypometabolic, hypothermal state confers disease tolerance during bacterial infections, as shown by the fact that mice housed at thermoneutral (30ºC) conditions show worse disease outcomes for similar pathogen loads when compared to conventional housing (22ºC)[85].

Consequences of disease tolerance mechanisms

The general principles of disease tolerance described above entail two important implications:

1) The activation of the appropriate stress responses is context-dependent, as different pathogens as well as different affected tissues may have very diverse patterns of tissue damage, immunopathology, and metabolic needs. As a consequence, tolerance mechanisms need to be as diverse as the underlying pathologies – for example, an effective anti-oxidant defense may be essential to tolerate infections that course with hemolysis (such as malaria)[65], whereas mechanisms of programmed cell death may be more important to contain tissue damage associated with viral infections[86]. Not surprisingly, a beneficial stress response on a given infection may have a neutral, or even detrimental, effect in a different context – as exemplified by the impact of nutrition and metabolism during bacterial and viral infections[38].

2) Tolerance programs that act on immune cells may have a negative impact on resistance mechanisms[67]. Examples include the metabolic reprogramming of macrophages[87] and T_H cells[88] into anti-inflammatory phenotypes that dampen an otherwise excessive inflammatory response. This highlights the need for a fine balance between conflicting tolerance and resistance programs, as excessive immunosuppression may delay pathogen clearance and predispose to

secondary infections[69]. It is worth noting, however, that tolerance and resistance are not necessarily mutually exclusive and can be used together to improve host fitness. Such is the case of ibuprofen treatment in a mouse model of tuberculosis, which simultaneously leads to anti-inflammatory effects (via inhibition of leukotriene synthesis) and a decrease in bacterial loads in the lung[89].

Perspectives in disease tolerance research

As made clear from some of the examples above, pharmacological activation of disease tolerance pathways presents a novel, promising way to promote host fitness during infection. In many cases, this is achieved by drug-induced perturbations of homeostasis, which activate adaptive stress responses that help limiting tissue damage during infection – a phenomenon known as hormesis or preconditioning[90]. While a much deeper understanding of the molecular mechanisms that explain these drug-induced protective stress responses is required before considering them for clinical application, it is undeniable that research on new therapies for infection can no longer rely on resistance mechanisms alone. Combining resistance and tolerance approaches to treat infectious diseases would tremendously expand the available therapeutic options, especially in face of the increasing problems with antibiotic resistance, as well as in cases where antimicrobials are not enough to guarantee recovery from infection.

This approach presents a few challenges for future studies. First, it involves a paradigm shift from an immune cell-centered mindset to a holistic perspective that combines tissue-specific pathology, inter-organ communication, metabolism, and immunity[69]. Another challenge regarding experimental design and interpretation is the intricate connection between resistance and tolerance – the two can have additive or antagonistic effects on pathogen load, as discussed – making it difficult to disentangle the contribution of each to host fitness. Strategies to circumvent this problem include the control of pathogen

loads with antimicrobial treatments, or the careful quantification of pathogens along with indicators of host fitness throughout the course of infection[71]. These monitored parameters can be used to plot 'health curves' that can help to predict disease susceptibility and the relative contributions of resistance and disease tolerance for survival and recovery[91].

1.3 Mitochondria as signaling and metabolic hubs

1.3.1 History, structure, and function of mitochondria

Mitochondria are intriguing organelles. The first observation of mitochondria on the microscope dates from the 1840s and they were first associated with energy production by Kingsbury in 1912[92]. After that, mitochondria proceeded to dominate the eukaryote bioenergetics field for most of the 20th century. Early studies with electron microscopy revealed the famous rod-shaped structure bound by an outer mitochondrial membrane (OMM), which regulates ion and metabolite transport; and a large, convoluted inner mitochondrial membrane (IMM), which regulates energy production and encloses the mitochondrial matrix, where most biochemical reactions occur[92]. Yet, the most intriguing aspect of mitochondria to this day is probably how their bacterial origin shaped their current function.

It is now clear from mitochondrial DNA (mtDNA) analysis that mitochondria originated from α-proteobacteria, a single phylum of ancestral bacteria[93]. The original mtDNA suffered massive reduction throughout evolution, with most of its functions being taken over by the nucleus[94]; yet the exact processes that dictated the emergence of this endosymbiont interaction remain elusive[93]. The immediate implication of this interaction is that it requires an intricate communication between the nucleus and mitochondria to ensure maintenance of homeostasis.

The human mitochondrial genome encodes for 2 mitochondrial rRNAs, 22 tRNAs and 13 proteins[95]. Mitochondrial-encoded rRNAs are assembled together with nuclear-encoded rRNAs and proteins to form the mitochondrial ribosome (or mitoribosome) consisting of a large (39S) and a small (28S) subunit[96]. Transcripts encoded by the mitochondrial DNA are translated by the mitoribosome and subsequently integrated with nuclear-encoded proteins to form functional complexes of the electron transport chain (ETC)[97].
The mammalian mitochondrial proteome comprises over 1000 proteins[98,99], only 1% of which are mitochondrial encoded. The remaining 99% are synthesized in the cytosolic ribosome and need to be processed and imported into the mitochondria in a highly regulated process[100]. Not surprisingly, a large portion of mitochondrial proteins still have unknown function, and the study of such complex interactions using high-end technologies has just begun[101].

Decades of research following Kingsbury's description of the role of mitochondria in "reducing substances concerned in cellular respiration"[92] allowed for a very complete picture of mitochondrial energy generation, which was awarded the Nobel prize on three different occasions[102]. By the early 1990's, most of the mitochondrial biology seemed to have been uncovered, yet this idea would be challenged by the observation that release of cytochrome C from mitochondria induces apoptosis[103]. This finding triggered the 'comeback' of mitochondria in research[102,104] and eventually led to the realization that mitochondrial functions go way beyond ATP production.
Intermediate metabolites of mitochondrial function provide the building blocks for most cellular biosynthetic pathways, such as nucleotides, amino acids, cholesterol, or heme[105]. Many of these metabolites, together with calcium, ROS and other molecules originated at the mitochondrial matrix provide vital signals to regulate cellular function[106]. Mitochondrial function orchestrates the decision between catabolic (energy saving) and anabolic (energy consuming) programs, which in turn impacts cell fate, proliferation, and differentiation[107] with immense

implications for organismal fitness[108]. This is highlighted by the role of mitochondria in the regulation of immunity[109], aging[110], or cancer progression[111], just to name a few examples. Indeed, mitochondria remain as intriguing as they were a century ago, and will likely occupy a central role in our future understanding of homeostasis regulation.

1.3.2 Overview of mitochondrial bioenergetics

Popularly known as the 'powerhouses of the cell', mitochondria are the single most important component of eukaryotic cells regarding energy generation. This section briefly reviews how the major carbon sources of the cell are oxidized in aerobic conditions in the mitochondrial matrix to produce ATP (Figure 1-3)

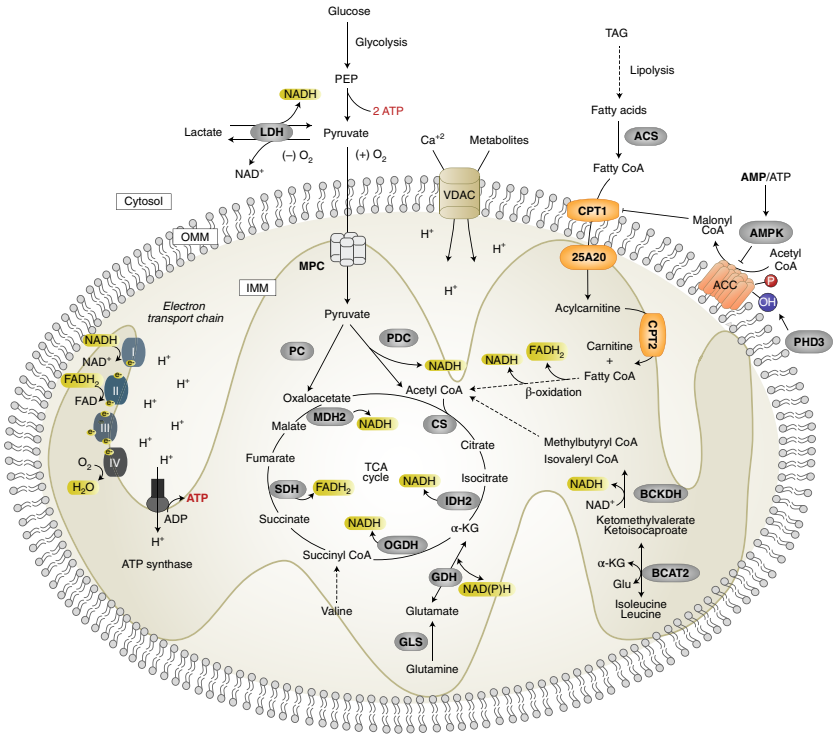

Figure 1-3 – Overview of mitochondrial bioenergetics (taken from[105]).

The major carbon sources pyruvate (originated from glucose) and fatty acyl-CoA (from lipids) enter the mitochondria through specialized transport systems. This is followed by a series of oxidation steps, which originate the electron-rich molecules NADH and $FADH_2$. Electrons are then transferred along the respiratory chain complexes and protons are pumped to the intermembrane space. This proton gradient is ultimately used to generate ATP.

1.3.2.1 TCA cycle and oxidative phosphorylation

Under normal oxygen and nutrient availability, oxidation of glucose to carbon dioxide (CO_2) is the major driver for ATP production. Each 6-carbon glucose molecule is initially oxidized in the cytosol to two pyruvate molecules (3 carbons each) with the production of 2 ATP molecules, in a process called glycolysis[112]. Pyruvate is then shuttled into the mitochondria by the mitochondria pyruvate carrier (MPC). Once in the mitochondrial matrix, pyruvate can either be carboxylated to yield oxaloacetate by pyruvate carboxylase (PC), or decarboxylated and conjugated with coenzyme A (CoA) to yield acetyl CoA via the pyruvate dehydrogenase complex (PDC)[113]. Both oxaloacetate and acetyl CoA enter the tricarboxylic acid (TCA) cycle (or Krebs cycle)[114] for further oxidation steps.

From a strictly bioenergetic point of view, the purpose of the TCA cycle is to generate GTP (or ATP), NADH, and $FADH_2$, which will feed the ETC for ATP production[112]. However, most of the TCA intermediates have other roles in biosynthetic pathways. Succinyl-CoA, for example, can be used for porphyrin synthesis, which will then be used to produce heme, while α-ketoglutarate can be used for glutamate production, and oxaloacetate for aspartate production[105]. Citrate has an important role in anabolic reactions, such as fatty acid, cholesterol and ketone bodies synthesis. This happens in the cytosol, upon export from the mitochondria via the malate-citrate antiporter[105].

While most of the TCA cycle reactions are bi-directional, substrate availability drives the equilibrium towards citrate consumption and oxaloacetate production in the majority of cases. Overall, one 6-carbon citrate molecule suffers two sequential decarboxylation steps resulting in the production of two molecules of

CO_2. At the end of the cycle, the 4-carbon oxaloacetate molecule is regenerated and restarts the cycle (Figure 1-3). The net effect of the oxidation of one citrate molecule is the production of one GTP (or ATP), one $FADH_2$, and three NADH molecules[112].

The NADH and $FADH_2$ molecules generated by the TCA cycle are then used as electron donors for the redox reactions of the ETC, ultimately driving ATP synthesis. The ETC consists of four multiprotein complexes (CI, CII, CIII, CIV) inserted in the IMM, which use redox cofactors and electron transfer reactions to drive proton translocation to the intermembrane space (Figure 1-4). This proton gradient is then dissipated via the F_1F_O-ATP synthase (or complex V) and the associated electrochemical energy used to synthesize ATP from ADP and inorganic phosphate (P_i). Structure and organization of the respiratory chain complexes follow a strict balance of nuclear and mitochondrial encoded proteins and also include higher-order structures combining multiple complexes, named supercomplexes or respirasome[115,116].

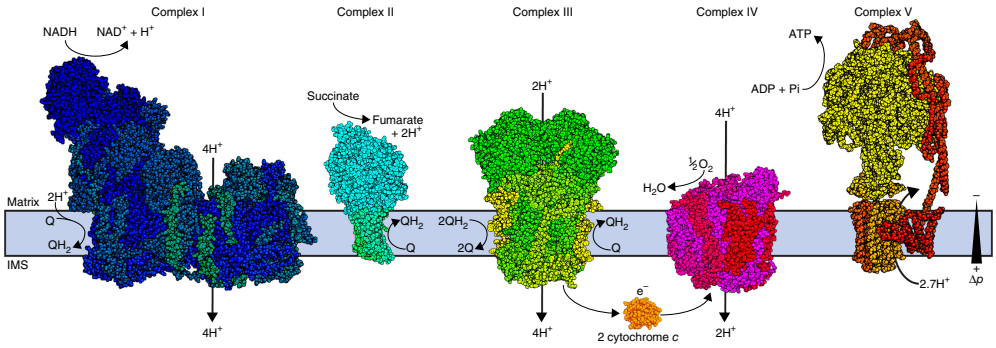

Figure 1-4 – The mammalian mitochondrial ETC (taken from[117]).

In the first step of the respiratory chain, electrons are transferred from NADH to ubiquinone in CI (NADH-ubiquinone oxidoreductase)[118] and protons are translocated to the intermembrane space. CI is the largest and more elaborate

of the ETC complexes, in which electron transfer and proton translocation are physically separated and mediated by a series of conformational changes[118].

CII (succinate–quinone oxidoreductase)[119] is the smallest complex and the only one comprised only of nuclear encoded proteins. It provides a unique link between the ETC and the TCA cycle. Oxidation of succinate to fumarate in the mitochondrial matrix (TCA cycle) generates $FADH_2$, which is then used to reduce ubiquinone to ubiquinol. Although this reaction does not result in proton translocation across the membrane, complex II contributes to the ETC gradient by providing an additional entry point of electrons and increasing the ubiquinol pool, which is essential for CIII activity[119].

Ubiquinol resulting from reduction of ubiquinone in CI and CII is then used in CIII (cytochrome bc_1)[120] to reduce cytochrome C with direct translocation of protons to the intermembrane space, through heme and iron-sulfur clusters[120,121].

In CIV (cytochrome C oxidase)[122], reduced cytochrome C originated in CIII is finally oxidized by transferring electrons to O_2 with production of H_2O. Similar to CIII, proton translocation in CIV is coupled to electron transfer, in this case mediated by heme-copper centers[121,122].

The protons accumulated in the intermembrane space are finally transported to the matrix through the F_O unit of the ATP synthase[123]. This flux drives rotation of one of the F_O subunits and the generated energy is used by the F_1 unit to synthesize ATP at an estimated rate of 1 ATP molecule per 2.7 translocated protons[123,124].

As made clear above, the pools of ubiquinone/ubiquinol and reduced/oxidized cytochrome C are regulated within the ETC and depend mostly on the crosstalk between different complexes. Therefore, the main exogenous regulators of the ETC activity are NADH and O_2. In addition, the respiratory capacity of the cell can be adjusted and optimized by the dynamic formation of supercomplexes, in a process still far from understood[117].

1.3.2.2 Fatty acid oxidation

Most of the fat reserves in the body are stored in adipocytes in the form of triglycerides, consisting of three fatty acid moieties linked to a glycerol molecule by ester bonds. Under stress or starving conditions, epinephrine, glucagon, and glucocorticoids activate lipases, which sequentially cleave the triglyceride ester bonds to release free fatty acids (FFAs) and glycerol[125]. FFAs are taken up by cells through specialized transporters and conjugated with CoA to form fatty acyl CoAs, which can then be used for membrane synthesis and a variety of signaling functions[126]. Alternatively, the high energy levels contained in FFAs can be made available to the cell through β-oxidation in the mitochondria[127].

Small and medium chain fatty acids are thought to diffuse freely across the double membrane of mitochondria, while long chain fatty acids (>10C) – which constitute the majority of lipids derived from diet – require a dedicated transport system, known as the acylcarnitine shuttle[128,129] (Figure 1-3). To this end, fatty-acyl CoAs are conjugated with the amino acid carnitine at the cytosolic face of the OMM by palmitoylcarnitine transferase 1 (CPT1), originating acylcarnitines. These are then transported across the OMM by the carnitine-acylcarnitine translocase (CACT, coded by the gene *Slc25a20*) in exchange for a carnitine molecule. At the IMM, CPT2 catalyzes the reverse reaction of CPT1, releasing acyl CoAs into the matrix[130]. Conjugation with carnitine by CPT1 is considered to be the rate-limiting step in β-oxidation as it commits fatty acids to oxidation. As a result, perturbations in downstream steps of β-oxidation, which result in slower rates of oxidation, are often reflected in the accumulation of acylcarnitines in the cytosol[131].

Once in the mitochondrial matrix, acyl CoAs undergo a series of oxidation steps, each consisting of the cleavage of the β carbon (i.e., the carbon adjacent to the carboxyl group) with release of an acetyl CoA molecule. This is done by a series of dehydrogenases that show preference for carbon chains of a specific size

(short, medium, long and very long-chain acyl CoA dehydrogenases)[127]. The β-oxidation process is repeated until only two or three carbons are left, for even and odd chain fatty acids, respectively. The resulting acetyl CoA can be further oxidized in the mitochondria through the TCA cycle or converted to ketone bodies in the mitochondria or cholesterol in the cytosol. Propionyl CoA generated from the oxidation of odd chain FA can be converted in succinyl CoA that enters the TCA cycle[132]. Each round of β-oxidation also generates NADH and $FADH_2$ that will work as electron donors for the ETC, thus highlighting the extremely high energetic potential of lipids.

Regulation of lipid metabolism involves an intricate crosstalk between glucose and fatty acid catabolism to guarantee maintenance of energetic supplies in a tissue- and context-specific manner[133]. Transcriptional control of β-oxidation is driven by the peroxisome proliferator–activated receptor (PPAR) family of transcription factors, which translocate to the nucleus upon binding to FFAs, and regulate the expression of genes involved in all aspects of lipid transport, uptake, storage, and oxidation[134].

PPARα is the most widely studied member of this family and plays an essential role in regulating liver β-oxidation upon fasting[135]. Besides regulating lipid and glucose metabolism[136], PPARα has wider implications in organismal function, as highlighted by its negative role in inflammation[137,138]. PPARβ/δ has similar roles to PPARα and an important function in β-oxidation in skeletal muscle and heart[139], while PPARγ regulates adipocyte differentiation and fatty acid uptake in peripheral tissues[140].

At the post-transcriptional level, β-oxidation is mainly regulated at the level of CPT1 activity, consistent with its rate-limiting effect in FAO[141]. Malonyl CoA, the first metabolite in the fatty acid synthesis pathway, binds CPT1 and inhibits its activity, thus providing a negative feedback loop that links lipid catabolism and anabolism. The *de novo* synthesis of fatty acids is in turn regulated by the cellular energy sensor AMPK. When cellular ATP levels drop, AMPK detects increased levels of AMP and inhibits the first step of fatty acid synthesis, thus

lowering malonyl CoA levels and increasing CPT1 activity (Figure 1-3). Simultaneously, AMPK activation promotes glycolysis in a concerted attempt to restore ATP levels during nutrient deprivation[142].

1.3.2.3 Alternative sources for ATP production

Under hypoxia conditions, reduced ETC activity leads to accumulation of NADH, which inhibits NADH-producing enzymes of the TCA cycle. Simultaneously, the hypoxia sensor HIF1α transcriptionally up-regulates glycolytic enzymes while repressing the activity of PDC[143]. As a result, pyruvate is diverted from mitochondrial oxidative metabolism and reduced to lactate in the cytosol, by lactate dehydrogenase (LDH). The reductive activity of LDH replenishes NAD^+ that is essential to sustain ATP production in the upstream steps of glycolysis.

When the major carbon sources glucose and FFA are depleted, amino acid catabolism helps sustaining cellular energy demands. Glutamine, the most abundant amino acid in serum, can be converted to glutamate in the mitochondria, which is then converted to α-ketoglutarate to feed the TCA cycle[144]. Likewise, the branched chain amino acids leucine, isoleucine, and valine can be converted into succinyl CoA or acetyl CoA in starving conditions[145]. Alanine can be used to produce pyruvate by alanine transaminase (ALT), while aspartate is used to generate oxaloacetate by aspartate transaminase (AST)[146] (Figure 1-5).

In starving conditions, acetyl CoA (produced either by FAO or amino acid catabolism) is used to produce ketone bodies in the mitochondria of hepatocytes[147]. The ketone bodies acetoacetate, β-hydroxybutyrate, and acetone freely diffuse from the liver to the blood and travel to target tissues (most notably the brain), where they can be oxidized back to acetyl CoA, thus providing a key source of energy when glucose is not readily available[148].

Since low glucose levels in the blood are a major threat for survival, energy-generating pathways need to be balanced with *de novo* glucose production in starved cells. The major catabolic pathway for glucose production is called gluconeogenesis and takes place in the liver, kidney, and muscle.

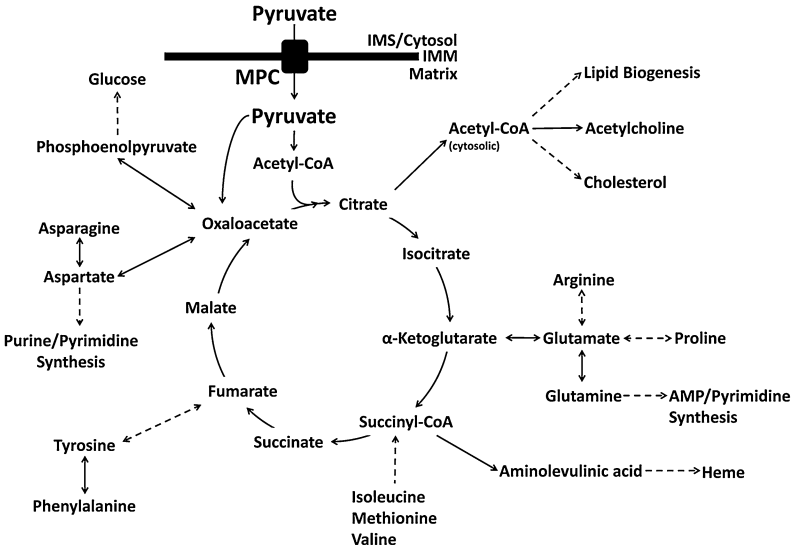

Figure 1-5 – Integration of the major cellular carbon sources (taken from[113]).

Initiation of gluconeogenesis includes the conversion of lactate, alanine, or glycerol to pyruvate, which is then imported to the mitochondria. Pyruvate is converted to oxaloacetate by PC and reduced to malate, which is exported back to the cytosol where it can be converted in phosphoenolpyruvate (PEP) by the enzyme PEPCK. PEP is then metabolized to glucose in a series of ATP-consuming steps that revert the glycolysis reactions[149].

1.3.3 Mitochondria as signaling organelles

1.3.3.1 Signaling molecules

Reactive oxygen species

ROS are an interesting example of the emerging concept of signaling functions associated with mitochondrial-derived molecules. For a long time, these were considered toxic species involved in aging and numerous diseases, which led to the rise in antioxidant therapies and supplements. As these approaches started to fail[150], new lines of research began to identify adaptive signaling roles associated with controlled levels of ROS[151].

Mitochondrial ROS are a natural consequence of the electron trafficking in the ETC and are produced when single electrons are transferred to O_2 resulting in the formation of the highly reactive species superoxide, hydrogen peroxide, and hydroxyl radical. Production of mitochondrial ROS occurs at the CI[152], CII[153] and CIII[154] of the ETC and depends on oxygen availability, the redox state of the ETC complexes, membrane potential, and the availability of ETC substrates[106,153].

Mitochondrial ROS were first associated with signaling functions in the context of hypoxia. ROS generated in CIII during oxygen deprivation result in stabilization of the hypoxia factor HIF1α and consequent up-regulation of genes involved in adaptation to hypoxia[155,156]. Other signaling roles of ROS include the oxidation of cysteine residues that participate in transduction pathways, such as the activation of the stress-responsive kinase JNK[157] or the autophagy regulator ATG4[158]. ROS have also been found to provide important signals for cell differentiation[159,160], migration and survival[161]. Very recently, a study showed that a transient increase in ROS levels during *C. elegans* development modulates epigenetic signaling, leading to increased stress resistance and prolonged life span[162].

Calcium

Mitochondria are the gatekeepers of intracellular calcium pools and a tight control of calcium trafficking into the mitochondria is essential for numerous cellular functions[163]. Similar to ROS, perturbations in mitochondrial function lead to calcium-mediated adaptive stress responses[164] and have an impact in cell fate[165,166].

Metabolites

Due to their overwhelming importance in bioenergetic and biosynthetic processes, the signaling role of metabolites, in particular the TCA cycle intermediates, has only very recently become the focus of research groups. Acetyl CoA has a fundamental role in epigenetics by providing the substrate for histone acetylation, which regulates gene expression[167]. In fact, acetyl CoA levels have been associated with cell growth and proliferation signaling[168] as well as epigenetic control of the synthesis of metabolic enzymes[169]. Furthermore, many histone deacetylases are dependent on NAD, thus imposing another layer of gene expression regulation that depends on the metabolic and redox status of the cell (expressed in this case as the ratio $NADH/NAD^+$)[170]. Very recently, lactate production, otherwise considered a toxic waste product of glycolysis, has been shown to modify histones (in a process called histone lactylation) and induce the expression of genes involved in homeostatic processes such as wound healing[171].

Metabolic signaling seems to be particularly relevant when it comes to the activation of immune responses, as highlighted by studies linking lipid signaling and defense against pathogens in *C. elegans*[172,173]. In mammals, signaling through TCA intermediates has been recently brought to stage due to its role in inflammation[174]. Accumulation on succinate in macrophages leads to pro-inflammatory signaling mediated both by HIF1α stabilization[175] and generation of ROS[176]. An opposing role has been attributed to itaconate, a derivative of the TCA cycle metabolite aconitate[174]. Production of itaconate in macrophages has an anti-inflammatory effect, which has been mechanistically linked to decreased

succinate oxidation[177] and activation of the NRF1-induced antioxidant reponse[178].

The field of metabolic signaling in the context of immunity has exploded in the past few years and provided advances in our understanding of how immune cells shift fuel usage to regulate effector responses[109,179]. It should be noted, however, that many of these studies are conducted *in vitro*, very far from physiological conditions[180]. This has been recently illustrated by the dramatically different metabolic needs and effector responses of T cells isolated from mice when compared to cultured cells[181]. Our mechanistic insight on the intricate regulation of metabolism and immunity at the organismal level is still limited and will require intense research efforts that take into account complex interactions of organismal homeostasis.

1.3.3.2 Transcriptional control of mitochondrial stress responses

As mentioned before, the dual origin of the mitochondrial proteome requires a tightly controlled communication between the nucleus and the mitochondria to maintain homeostasis. This communication serves two main purposes: 1) to maintain the adequate balance between nuclear and mitochondrial encoded proteins; and 2) to ensure that mitochondria meet the required metabolic needs (e.g. substrate availability and protein function) before the cell commits to a new biological process. To this end, the nucleus instructs mitochondrial function (anterograde signaling) and the mitochondria respond by sending signals back to the nucleus, causing changes in nuclear gene expression that influence cellular function (retrograde signaling)[182]. The examples below illustrate how mitochondrial stress impacts on nuclear transcriptional programs.

Energetic stress

As previously discussed, AMPK is the major sensor of metabolic stress, as it detects ATP depletion and triggers adaptive changes to correct it. Activated

AMPK phosphorylates the master regulator of mitochondrial biogenesis PGC1α[183]. This results in the up-regulation of nuclear-encoded genes of the ETC, as well as genes involved in mitochondrial transcription and translation, in an attempt to increase respiratory capacity of the mitochondria[183,184]. Activated PGC1α also interacts with PPARα to promote transcription of FAO genes[185].
Depending on the severity of metabolic stress and on the integration of several signals, AMPK may promote autophagy to ensure recycling of cellular components into energy substrates[186,187]. Furthermore, AMPK negatively regulates mTORC resulting in decreased protein synthesis and cell proliferation[188] as well as increased ketogenesis[189].

Proteostasis stress

Proteotoxic stress in the mitochondria can be caused by accumulation of misfolded proteins (for example, due to heat stress), protein damaging by ROS, or an imbalance between nuclear and mitochondrial-encoded proteins of the respiratory chain. All of these promote retrograde signaling and activate a transcriptional program known as mitochondrial unfolded protein response (UPR^{mt}), which aims at increasing the expression of nuclear-encoded mitochondrial proteostasis genes[190,191].
The induction of UPR^{mt} was first observed in mammalian cells depleted of mtDNA. Upon treatment of hepatoma cells with ethidium bromide, the authors noticed an up-regulation of the mitochondrial chaperones HSP10 and HSP60, indicating a nuclear-orchestrated attempt to restore mitochondrial proteostasis[192]. In the following years, the molecular mechanisms of UPR were intensely studied in *C. elegans* by the use of genetic or pharmacological perturbations to the ETC function[9,193], the mitochondrial folding capacity[194,195], or by generation of mitochondrial ROS[196].
In nematodes, UPR^{mt} is regulated by ATFS, a protein that includes both mitochondrial and nuclear targeting sequences. In normal conditions, ATFS is directed to the mitochondria and constantly degraded by mitochondrial proteases. When proteostasis stress surpasses the capacity of mitochondrial

proteases, ATFS accumulates and gets shuttled to the nucleus, where it acts as a transcription factor to induce stress responsive genes (including chaperones, proteases, and antioxidants)[197].

In mammals, UPR^{mt} is mediated by the C/EBPβ and CHOP transcription factors, which have been also implicated in ER stress responses[198,199]. Mitochondrial protein misfolding in human cells has been shown to up-regulate nuclear-encoding mitochondrial chaperones and proteases, while slowing down mitochondrial transcription and translation, therefore restoring folding capacity specifically at the mitochondrial matrix[200].

However, the mechanisms that regulate mammalian UPR^{mt} are still poorly understood and how mitochondrial stress is communicated to the nucleus in mammals is still a matter of debate. A recent study proposed a central role for ATF5 in mediating mitonuclear communication (similar to ATFS in *C. elegans*)[201], while a large multi-omics screen identified ATF4 as the master regulator of UPR^{mt} [202].

The fact that most regulators of mammalian UPR^{mt} are also activated upon ER stress points to a network of cellular stress responses that link different organelles. In fact, UPR^{mt} is increasingly recognized as part of a broader integrated stress response (ISR)[203]. The ISR relies on sensors for diverse perturbations such as ER stress, amino acid deprivation, or heme deficiency, all of which regulate cytosolic translation through phosphorylation of eIF2α. Increased protein levels of ATF4 resulting from eIF2α activation lead to transcriptional up-regulation of stress response genes such as CHOP and ATF5[203] (Figure 1-6). The tight link between UPR^{mt} and ISR has become apparent as different studies found increased eIF2α phosphorylation upon genetic or drug-induced mitochondrial stress[204,205].

In recent years, more intricate forms of communication between mitochondria and cytosol following proteotoxic stress have been identified – these include the lipid-mediated mitochondrial-to-cytosolic stress response (MCSR) in *C. elegans*[206] and the UPR activated by mistargeting of proteins (UPR^{am}) in

yeast[207] – suggesting that we are still far from fully understanding the integration of different stress responses in mammals.

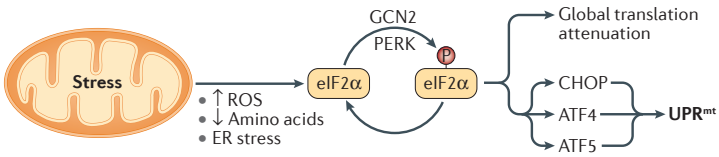

Figure 1-6 – Mitochondrial stress signaling: UPR^{mt} and the ISR (taken from[191]).

At the end of the spectrum of mitochondrial proteostasis stress, we find cases where stress responses are not sufficient to restore homeostasis. In this case, UPR^{mt} may activate mitophagy to remove severely damaged mitochondria, although the mechanisms of coordination between the two are still unclear[208].

Over the past twenty years, the idea that mild perturbations in mitochondrial function induce protective stress responses – including, but not limited to UPR^{mt} – which promote organismal fitness, has been gaining increasing popularity. The first observation resulted from a genetic screen in *C. elegans*, in which a mutation in the iron sulfur protein 1 (*isp-1*) subunit of complex III was associated with increased longevity[209]. At the time, this beneficial effect was attributed to decreased oxygen consumption and increased resistance to ROS[209]. In the following years, this concept would be further explored by the pioneer work of Dillin and co-workers, who eventually established a causal link between UPR^{mt} and prolonged lifespan[9,210,211]. In recent years, hormetic mitochondrial responses – also known as mitohormesis[212] – have been intensely studied in the context of longevity across all model organisms[110,191].

Our current understanding of how perturbations in mitochondrial function improve fitness is now expanding beyond the classical activation of UPR^{mt} markers. Future studies will need to integrate a complex network of signals that is highly context-dependent and includes not only mitokines, but also ROS[213] and calcium[214] signaling, together with metabolic reprogramming[215,216] and other

still unknown factors that promote organismal homeostasis upon mitochondrial dysfunction.

1.4 Sepsis: a challenge for healthcare and biology

1.4.1 Definition and clinical importance of sepsis

Sepsis is characterized by a heterogeneous set of signs and symptoms that has complicated its definition, diagnosis and clinical management. The first international consensus on sepsis dates from the early 1990's and defined sepsis as a systemic inflammatory response syndrome (SIRS) in the presence of an infection[217] (Table 1-1). Patients in which SIRS was accompanied by organ dysfunction were diagnosed with severe sepsis, whereas cases including persistent hypotension were qualified as septic shock[217].
Over the following decades, it became apparent that this definition does not fully grasp the complexity and heterogeneity sepsis. In 2016, the third international consensus (Sepsis 3) defined sepsis as a 'life-threatening organ dysfunction caused by a dysregulated host response to infection'[218]. Of note, the classical signs of inflammation (fever and leukocyte counts) are no longer required for diagnosis and organ dysfunction became the major defining feature of sepsis – in fact, tissue damage accounts for most of the mortality and long term morbidity of sepsis, even after infection has been cleared. According to the new definition, the terms SIRS and severe sepsis were replaced by the single term 'sepsis', while maintaining the term septic shock to define persistent hypotension that requires administration of vasopressors[218] (Table 1-1).

Sepsis constitutes a major healthcare problem and socio-economical burden worldwide. In the USA, incidence of sepsis is estimated to range between 894,013 and 3,110,630 cases per year (0.3-1% population)[219], although precise

numbers are difficult to obtain due to the variety of definitions and case registration methods. The worldwide incidence is even more difficult to calculate, due to the lack of data for low-income countries. Still, a recent estimate based on the extrapolation of data from high-income countries pointed to 19-34 million cases annually, with 5 million potential deaths[220].

Although most large-scale studies in developed countries indicate a decrease in mortality of sepsis patients in recent years[219,221], the total number of cases is instead increasing[222]. This can be attributed to increased awareness and more accurate diagnosis, but also to the aging of the population and consequent increase in co-morbidities[219,222].

1991 consensus conference[217]	
Diagnosis	**Signs and symptoms**
Systemic inflammatory response syndrome (SIRS)	Patients experiencing at least two of the following symptoms: • Body temperature >38ºC or <36ºC • Heart rate >90 beats per minute • Respiratory rate >20 breaths per minute or arterial CO_2 <32 mmHg • White blood cell count >12 × 10^9 L^{-1} or <4 × 10^9 L^{-1}, or >10% immature forms
Sepsis	Systemic inflammatory response syndrome and proven or suspected infection
Severe sepsis	Sepsis and acute organ dysfunction
Septic shock	Sepsis and persistent hypotension after fluid resuscitation
2016 Sepsis-3[218]	
Diagnosis	**Signs**
Sepsis	• Life-threatening organ dysfunction caused by a dysregulated host response to infection • Organ dysfunction can be identified as an acute change in total SOFA score of ≥2 points
Septic shock	• Sepsis in which the underlying circulatory and cellular and/or metabolic abnormalities are marked enough to substantially increase mortality • Clinically defined as sepsis with persisting hypotension that requires vasopressors to maintain the mean arterial pressure at ≥65 mmHg and with a serum lactate concentration >2 mmol.L^{-1}

Table 1-1 Evolution of the definitions of sepsis (adapted from[223]).
SOFA – sequential organ failure assessment score, based on six different scores for disease severity (respiratory, cardiovascular, hepatic, coagulation, renal and neurological systems).

In addition to its economic burden – it has been considered the most expensive condition for USA hospitals[224] – sepsis has a devastating social impact, due to

its high incidence in the pediatric population, the lengthy recovery and the long-term physical and cognitive impairment of survivors[225].

The need for initiatives to prevent and fight sepsis has been increasingly recognized worldwide. In 2017, the World Health Organization issued a resolution urging for policy makers, research funding agencies, healthcare professionals, and all involved parties to improve the diagnosis and management of sepsis[226]. In the meantime, international guidelines have been published providing recommendations for evidence-based clinical management of septic patients[227], while several public health initiatives strive to raise public awareness about sepsis and implement preventive measures[228].

1.4.2 Sepsis pathophysiology

1.4.2.1 Etiology

Sepsis is a syndrome, not a disease. This means that any microbial agent – bacterial, fungal, viral, or protozoan – can trigger a dysregulated response to infection that courses with systemic inflammation, metabolic dysfunction, and epithelial barrier failure, which are hallmarks of sepsis. Likewise, the initial focus of infection varies among patients, with respiratory infections being the most prevalent, followed by abdominal, bloodstream, and genitourinary[229].

The factors that determine the risk of developing sepsis from an infection are complex and include microbial pathogenesis, host genetic susceptibility to develop acute organ dysfunction, as well as environmental factors. It is well established that infants and the elderly are more likely to develop sepsis, as are immunosuppressed patients and people with chronic diseases such as cancer, chronic obstructive pulmonary disease, or cirrhosis[230,231].

1.4.2.2 Immunopathology

As discussed in section 1.2.2, pathogen invasion triggers an acute inflammatory response, which needs to be tightly balanced with repair and anti-inflammatory mechanisms to allow return to homeostasis. Sepsis is one of the most prominent cases of dysregulation of this balance, leading to severe immunopathology. Upon the discovery that TNF-blocking antibodies increased survival in a model of septic shock in baboons[232], the initial pro-inflammatory response became the cornerstone of sepsis research and a major promise for therapies. However, it is becoming clearer that organismal dysfunction is much more complex and involves dramatic reprogramming in cellular functions. This is reflected in simultaneous inflammation and immunosuppression, metabolic collapse, and failure to return to homeostasis[223].

Excessive inflammation

Initial stimulation of PRR in leukocytes (in particular macrophages and neutrophils) triggers a fast and abundant secretion of cytokines, including TNFα, IL-1β, IL-6, IL-12, IL-17, and IL-18 – a phenomenon known as 'cytokine storm'[233,234]. In humans, injection of endotoxin results in a peak of cytokine and chemokine secretion within 2 to 4 hours[235]. In addition, colony-stimulating factors (CSFs) play a role in the cytokine storm by promoting leucocyte differentiation and stimulating pro-inflammatory functions in mature immune cells[236]. Other less well-known players of immunopathology include the IL-3-producing innate response activator B cells. IL-3 works as a cytokine and a myeloid growth factor that mediates an aberrant inflammatory response and has been associated with worsened sepsis prognosis in humans[237].

The cytokine storm is a self-propagating state, in which more leukocyte recruitment will further aggravate the inflammatory response. Moreover, high cytokine levels cause damage to bystander cells, leading to the release of DAMPs, which will further activate PRRs and propagate this vicious cycle[233].

In addition to leukocyte-derived cytokines, chemokines and growth factors, other components contribute to the initial exacerbation of inflammation. Endothelial cells, which play a key role in activating and trafficking leukocytes during inflammation, also become highly dysfunctional in sepsis. High levels of cytokines, metalloproteinases, and DAMPS (especially HMGB1) contribute to disrupt the endothelial barrier, causing leakage of serum and proteins out of the vascular compartment. This leads to widespread edema and impaired perfusion, which are particularly serious when affecting the brain[238,239].

Vascular problems during sepsis are further aggravated by an excessive activation of the coagulation system. Platelet activation by inflammatory mediators, together with circulating neutrophil extracellular traps (NETs), contributes to disseminated intravascular coagulation, which can lead to vascular occlusion and ischemia. Paradoxically, excessive coagulation can also be the cause of hemorrhage, resulting from the exhaustion of platelets and coagulation factors[240,241].

Immunosuppression

The initial hyper-inflammatory phase of sepsis is followed by an immunosuppressive state that poses additional threats to the host. The immunosuppressive phase can be explained by the emergence of a compensatory anti-inflammatory program and an exhaustion of immune cell function. The former is now recognized as a highly dynamic crosstalk between pro- and anti-inflammatory programs, rather than a simple temporal activation of the two. In fact, anti-inflammatory genes (such as *Il10*) are transcribed within the first few hours of infection, in close proximity with pro-inflammatory ones[235,242]. The exhaustion and apoptosis of lymphocytes, in turn, occur at later stages of unresolved infections: massive apoptosis occurring in lymphoid tissues causes depletion of $CD4^+$, $CD8^+$ and B cells[243], while surviving leukocytes dramatically reduce production of cytokines[244]. This results in a prolonged immunosuppressive state that predisposes patients to secondary infections and is ultimately responsible for large rates of late mortality after sepsis[244].

1.4.2.3 Metabolic dysfunction

Catabolism and anabolism in sepsis

As previously discussed, metabolic regulation of immune cells shapes their effector functions, and often dictates the balance between pro- and anti-inflammatory phenotypes. The initial inflammatory response in sepsis is associated with a shift towards glycolysis in human peripheral blood mononuclear cells (PBMCs). This serves not only as a source of ATP but also of intermediate metabolites (such as NAPDH and glucose 6-phosphate), which are essential for anabolic pathways including lipid, protein, and nucleotide synthesis to support rapid proliferation of leukocytes[245]. In contrast, the late immunosuppressive stage of sepsis is characterized by defects in both glycolysis and oxidative phosphorylation, which help to explain paralysis of immune functions[246].

Outside of the hematopoietic compartment, sepsis causes dramatic changes in fuel utilization that result in a global decrease in ATP levels and a consequent up-regulation of catabolic pathways[247]. The energetic collapse associated with sepsis has been increasingly recognized as a driver of pathology and organ failure, as well as a promise for new therapies[248].

Since the initial observation, more than half a century ago, that sepsis causes morphological abnormalities in mitochondria[249], several studies have linked impaired mitochondrial function to sepsis pathophysiology[250]. Despite normal (or even increased) oxygen delivery to tissues, cells are unable to use oxidative metabolism to produce ATP – a phenomenon known as cytopathic hypoxia[251].

Increased levels of ROS and nitric oxide produced during the inflammatory response inhibit the activity of all ETC complexes[252,253], while PDC activity is repressed, resulting in decreased mitochondrial oxidation of pyruvate[254]. As a consequence, ATP production is sustained by glycolysis, a less efficient

process that leads to overall depletion of ATP in parenchymal tissues. A study in recently admitted intensive care unit (ICU) patients revealed a correlation between low ATP levels in skeletal muscle and mortality[253].

As a result of impaired oxidative phosphorylation, pyruvate produced in glycolysis is ultimately reduced to lactate in the cytosol. Accumulation of lactate is in turn deleterious and can lead to metabolic acidosis, arrhythmia, and coma – in fact, high levels of lactate in the blood are a well-known indicator of bad prognosis in patients[255].

In summary, maintenance of mitochondrial structure and function has been long recognized as an essential component of recovery from sepsis. For example, increased levels of mitochondrial biogenesis genes at early stages of infection have been associated with higher chances of survival[256]. These findings encourage the search for new therapeutic strategies that restore the lost balance in mitochondrial biology during infection, a challenging mission that remains unsolved to this day[257,258].

Glucose metabolism

Another well-established component of energetic failure in sepsis is the disturbed distribution and metabolism of the major energy-generating nutrients – glucose, fatty acids, and amino acids.

Hyperglycemia is commonly found in sepsis patients, presumably due to insulin resistance induced by catecholamines, cytokines, and glucocorticoids[259]. Whether this is an adaptive or maladaptive response is not clear – mild hyperglycemia may be advantageous to fuel neurons and leukocytes, while severe hyperglycemia is life threatening[260]. As a result, after years of debate on clinical management of glucose levels, the most recent guidelines advocate for a less strict control compared to the past[227,248].

In contrast to humans, sepsis models in laboratory mice are characterized by persistent hypoglycemia[38,82]. The differences in glucose utilization between mice are humans are poorly understood and may include thermoregulation programs[85], and infection-induced anorexia in mice[38] (which is counteracted in

humans by parenteral feeding in the ICU). As in humans, the benefits of controlling glucose levels in mice are unclear and seem to be highly context dependent. A recent study reported that glucose supplementation is beneficial in viral infections and detrimental in bacterial sepsis[38], while a different study showed a protective effect for glucose supplementation and induction of hepatic gluconeogenesis in avoiding lethal hypoglycemia during bacterial infection[82].

Lipid metabolism

Upon the action of stress hormones (catecholamines and glucocorticoids) during infection, triglycerides stored in adipocytes are mobilized and broken down, resulting in high levels of FFAs in circulation[261]. FFAs then serve as important sources of ATP production and also as intermediates for lipid inflammatory signals, such as arachidonic acid[260].

In sepsis patients, however, it has become apparent that oxidation of fatty acids is compromised, and may contribute to energetic failure. Decreased expression of *Ppara* (the gene coding for PPARα)[262] and accumulation of acylcarnitines[263] have been observed in patients and correlated with disease severity, thus highlighting the importance of β-oxidation in sepsis pathophysiology. The same principles seem to apply in mice, as seen in two recent studies exploring mechanisms of liver fatty acid metabolism during sepsis. Impaired liver β-oxidation was shown by the accumulation of acylcarnitines in mouse liver and blood upon LPS treatment[85], while hepatic PPARα was proven essential for survival to *E. coli*-induced sepsis[264]. Inhibition of β-oxidation during sepsis is problematic not only because it aggravates the deficit in ATP production, but also because it leads to accumulation of toxic lipid species, which cause mitochondrial dysfunction and tissue damage[248,265].

Amino acid metabolism

In line with changes in glucose and lipid metabolism, sepsis is characterized by increased protein catabolism. This is particularly evident in the skeletal muscle and explains muscle wasting observed in patients[266]. Amino acids resulting from

muscle protein catabolism can be shuttled to immune cells, to support proliferation and production of inflammatory mediators, or to the liver, where they can be used for energy production[259].

Several amino acids, most importantly glutamine, glutamate, and alanine, can enter the TCA cycle and be used for ATP production (unlikely, since mitochondrial function is impaired), or enter the gluconeogenesis pathway[248]. Alternatively, amino acids such as lysine or leucine can be used for the synthesis of ketone bodies[85]. Ketones are important sources of energy during starvation and have been deemed essential for survival to sepsis[38]. In face of defective glucose and fatty acid oxidation during sepsis, muscle-derived amino acids have been proposed to partially sustain energy production through ketogenesis[85].

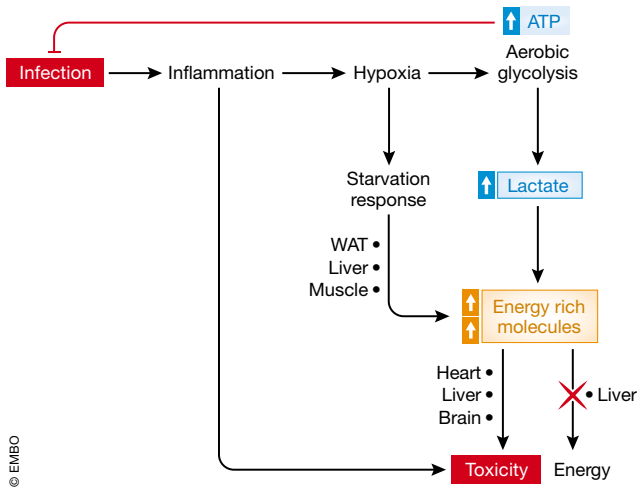

Figure 1-7 – Metabolic dysregulation in sepsis (taken from[248]).

In summary, catabolic programs are an adaptive response to stress conditions that support tissue function during starvation or hibernation, for example[75]. In sepsis, the correlation between these metabolic alterations and disease tolerance mechanisms that limit tissue damage has just begun to be investigated. In mouse models of bacterial sepsis, it is now clear that both

anorexia[38] and hypothermia[85] support beneficial metabolic changes that are reflected, for example, in increased ketogenesis.
Paradoxically, while catabolic pathways release high-energy molecules, cellular oxidative metabolism is impaired during sepsis. This leads not only to energetic failure but also to accumulation of toxic species such as lactate and FFAs, which further aggravate inflammation-induced tissue damage[248] (Figure 1-7).

1.4.2.4 Organ failure

A small fraction of sepsis patients develop a fulminant, TNF-driven septic shock characterized by hypotension, cardiac failure and ischemic necrosis. In most patients, however, sepsis develops as a more progressive and persistent organ failure condition[267] (Figure 1-8). Remarkably, organ failure is characterized by low levels of cell death and rather seems to be a consequence of cellular reprogramming that impairs communication and tissue function[239].

As previously discussed, inflammation causes an increase in endothelial permeability, resulting in widespread edema and loss of barrier functions in all tissues. In the lung, interstitial edema in the alveoli perturbs gaseous exchanges and leads to acute respiratory distress syndrome (ARDS), a common complication of sepsis[268].
Loss of intestinal barrier results in bacterial translocation that further aggravates the infection; while in the liver, altered transport of lipids and bilirubin leads to cholestasis[230]. In the kidney, a combination of vascular and energetic defects results in loss of ion gradients across tubules, leading to acute kidney injury[269].
The central nervous system is particularly sensitive to edema, ischemia, and hemorrhage caused by vascular defects, which very commonly results in encephalopathy that leads to long-term cognitive problems[230].

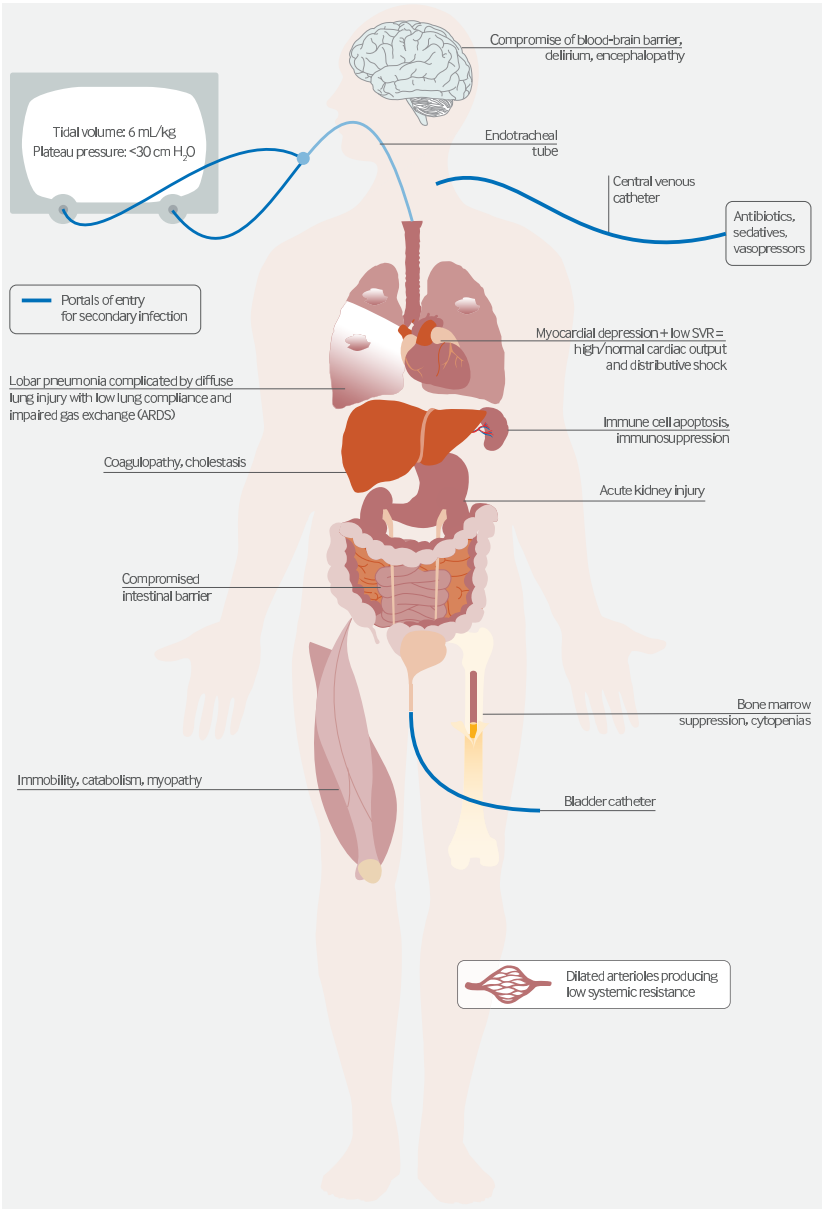

Figure 1-8 – Organ failure in sepsis patients (taken from[230]).
ARDS – acute respiratory distress syndrome.

More generally, multiple organ failure in sepsis has been described as a syndrome in which cellular processes become restricted to the minimum that supports survival. As low-priority functions like cell-to-cell and inter-organ

communication start to fail, tissue functions that require coordination are lost, and organismal homeostasis crumbles[239,267].

1.4.3 Therapeutic approaches and failures

1.4.3.1 Current support measures

There are currently no specific treatments for sepsis. Standard management of patients includes the administration of antimicrobials to treat the underlying infection, together with lung ventilation, intravenous fluids, and vasopressors in the case of septic shock[230,267]. While these organ support measures allowed for a significant reduction in mortality over the past decades, no therapy has successfully addressed all of the dysfunctional features of sepsis, despite intense research efforts and decades of clinical trials[270]. While disappointing, this is a hardly surprising result, considering the heterogeneity of the population and the complex interplay of immunity, metabolism, and organ communication.

1.4.3.2 Targeting inflammation and immunosuppression

The long-standing view of sepsis as a primarily hyper-inflammatory condition led to numerous attempts to block the initial cytokine storm. Several clinical trials using blocking antibodies for TNFα showed no convincing improvement in survival[230]. Other blocking antibodies are still being investigated and may be useful in dampening immune activation in particular cases – these include anti-IL1β receptor[271] and anti-CD28 (which blocks T-cell activation)[272].

Glucocorticoids are another well-studied tool, due to their potent anti-inflammatory effect and their role in metabolic adaptation (namely by increasing gluconeogenesis and β-oxidation). However, a recent, large-scale study in septic shock patients failed to show improvement in any of the analyzed parameters after treatment with hydrocortisone[273]. This can be partly explained

by glucocorticoid resistance – high levels of endogenous corticoids produced during sepsis eventually cause patients to stop responding to glucocorticoid administration[274].

A number of therapeutic approaches to improve endothelial function and inhibit disseminated intravascular coagulation have also shown limited protective effect, although new clinical trials are ongoing[223,267]. More recent strategies that are starting to be evaluated in the clinics include the technologies to filter bacterial toxins from the blood[275]; and the injection of mesenchymal stromal cells, which have immunomodulatory, antimicrobial, and barrier-preserving effects[276].

The failure of anti-inflammatory therapies led to a more recent focus on approaches that correct the immunosuppressive phase of sepsis, with the aim to reduce the incidence of secondary infections. Treatment with the colony-stimulating factors G-CSF and GM-CSF, which increase the production and maturation of neutrophils and macrophages, yielded some initial promising results but ultimately failed to demonstrate reproducible clinical improvement of patients[277].

Other targets under investigation include the use of immune-stimulating cytokines, such as IL-7, IL-15, and IFNγ[223]. A recent clinical trial using recombinant IL-7 showed beneficial effects in reversing sepsis-induced loss of T cells, although the long-term effect in survival and incidence of infections still needs investigation[278].

Immune checkpoint blockers, namely PD1 and PDL1 blocking antibodies, which have been successfully used to boost T cell function against cancers, have shown promising results in pre-clinical studies and are currently being tested in clinical trials[223,244].

In summary, modulating the immune response during sepsis presents a challenging balance between the control of acute inflammatory response and the risk of immunosuppression – a conflict that decades of research failed to

resolve[279]. While there is still hope for new adjuvant therapies in this area, future clinical trials need to include careful stratification of patients and the use of treatments that are tailored to individual needs[223].

1.4.3.3 Targeting metabolism

Metabolism in sepsis has been increasingly acknowledged as a driver of pathology and a regulator of the immune response. While this has resulted in a profusion of recent studies using animal models of sepsis, the clinical translation of metabolic therapies is still lagging far behind their immune-centered counterparts[248].

Sepsis-induced mitochondrial dysfunction is characterized by increased production of ROS, which turned them into attractive therapeutic targets in the past. However, clinical trials using supplementation of antioxidants such as vitamins C and E, or the ROS scavenger N-acetylcysteine failed completely[280]. This is not surprising in light of the current knowledge on the critical role of ROS as signaling molecules, as previously discussed. In line with this idea, NRF2, a transcription factor activated in conditions of oxidative stress, has been associated with beneficial effects in sepsis. In a group of pediatric septic shock patients, increased expression of NRF2-linked genes was associated with metabolic benefits and improved disease outcomes[281], while in a mouse model of pneumonia NRF2-induced mitochondrial biogenesis was associated with improved lung pathology[77].

Lactate accumulation as a result of oxidative metabolism impairment is one of the most prominent features of sepsis. In mice, targeted deletion of HIF-1α, one of the main transcriptional drivers of glycolysis, results in protection against endotoxin shock[282,283]. Likewise, administration of 2-deoxyglucose (2-DG), a glycolysis inhibitor, decreases lactate production and inflammation in mouse models of sepsis and septic shock[38,284].

Promoting β-oxidation, in particular through the master regulator PPARα, poses another promising strategy by counteracting energetic failure and lipotoxicity. Genetic up-regulation of PPARα expression improved metabolic profile and cardiac function upon LPS injection in mice[285]; whereas the PPARα agonist fenofibrate increased survival in bacterial sepsis, although the proposed mechanism was related to reduced inflammation rather than improved metabolism[286]. Supplementation with ketone bodies, which represent readily available alternative sources of ATP, may also have a beneficial effect. A recent study reported improved muscle function and regeneration in a mouse model of polymicrobial sepsis upon treatment with the ketone body β-hydroxybutyrate, although no differences were found in survival[287].

In spite of the encouraging results with reprogramming of metabolic dysfunction in sepsis, these are still the early days of this field and more detailed mechanistic knowledge is required before any therapies are translated into clinical practice. Of note, drugs that target metabolic function need to overcome the dynamic, and sometimes opposing, tissue-specificity of metabolism. For example, metabolic changes that increase the fitness of parenchymal tissues may reflect negatively in immune cell function and aggravate infection outcomes – such is the case of metformin treatment in a model of *Candida albicans* infection, which increases pathogen load and mortality[246].

1.4.4 Sepsis and fundamental research

1.4.4.1 Animal models of sepsis

The extensive use of animal models of sepsis has allowed for a deep understanding of host responses to severe, systemic infection, as well as providing valuable therapeutic targets to be translated to clinical practice.

The vast majority of sepsis models are performed in laboratory mice and rats, due to their small size and easy, inexpensive maintenance. In addition, the use of inbred strains and the possibility to generate knockout and transgenic strains has been of paramount importance to uncover genetic determinants of disease outcomes[288]. Larger mammals, such as sheep and pigs, are preferred for more advanced pre-clinical studies, as they more closely replicate the clinical features of multi-organ failure occurring in human sepsis. The larger size of these animals facilitates monitoring of clinical parameters – such as cardiac output and biochemical parameters obtained from repeated blood sampling. Moreover, their genetic heterogeneity more closely represents the diversity of human patients[289].

Endotoxemia models of sterile sepsis are easy to perform and widely used to study the initial inflammatory response. A single intraperitoneal or intravenous injection of LPS (the most common), CpG DNA, or zymosan causes an acute and amplified cytokine response, accompanied by hypotension, decreased cardiac output, and hypothermia[288]. These somewhat resemble the features of fulminant septic shock in humans, although mice show higher cytokine levels and a faster progression of the disease compared to humans, with high mortality levels within 24h[290].

For a more accurate reproduction of human sepsis, live bacterial models are preferred. Cecal ligation and puncture (CLP), a surgical model of polymicrobial peritonitis, is considered the 'gold standard' of sepsis research. In this model, the cecum is ligated, perforated with a needle, and placed back in the abdomen, causing peritonitis that gradually progresses to systemic organ dyfunction[291]. Limitations of this model include technical variations that can influence reproducibility, and animal welfare issues, due to its highly invasive nature[289]. Moreover, differences in intestinal microbiota between mice can have a dramatic effect in disease outcome. A viable alternative to circumvent this issue is the use of cecal slurry models, in which the cecal contents of donor mice are

intraperitoneally injected in recipient mice, thus providing a more standardized source of infection[292].

Other models of infection with live microorganisms involve the administration of pure cultures of a single species of bacteria or fungi. This allows for a more controlled and flexible experimental design, as different strains with varying degrees of pathogenicity can be tested. Furthermore, a variety of infection routes can be used – the most common being the lung, peritoneal cavity, and blood – all with distinct features of disease progression and outcome[288,289]. The major criticism towards these models is the fact that a rapid inoculation with a single pathogen does not reflect the slow development of human sepsis. In addition, injected bacteria may be rapidly lysed by complement, which can result in an endotoxemia model rather than a live infection model[289].

1.4.4.2 Pre-clinical research in sepsis: lessons and perspectives

There has been an intense debate on the validity and usefulness of animal models of sepsis. Not only are they a major cause of concern for animal welfare, but also the applicability of basic research findings to the clinical practice has been rather limited. Countless therapies failed to show benefits in clinical trials despite robust validation in pre-clinical animal models, supporting the idea that even the best controlled experimental settings fail to reproduce the complexity of human disease[288]. Reasons for this include the fact that sepsis in animal models tends to progress very rapidly, relying mostly on the hyper-inflammatory phase and ignoring late-stage immunosuppression, incidence of secondary infections, and long-term organ failure. Furthermore, experiments are typically performed in inbred, young, and healthy animals that are very far from patients with co-morbidities and extensive genetic variability. Finally, laboratory animals have intrinsic differences in physiology compared to humans (e.g. mice tolerate LPS doses more than 1000-fold higher than humans), which are aggravated by the lack of supportive interventions in animal models – patients

receive pharmacological, nutritional, and other forms of support at the ICU[230,290,293].

All these differences make it very hard to extrapolate results obtained in pre-clinical models directly to humans. While this should not compromise the existence of pre-clinical studies, there is room for improvement in the future[294]. Promising new therapies may be tested in outbred strains, using multiple models of infection, and sequentially validated in large mammals using experimental settings that resemble the ICU[293,294].

Looking further than clinical translation, basic research in sepsis has brought outstanding contributions to biology. Being at the far end of the spectrum of homeostasis failure, sepsis provides a window to better understand complex whole-body interactions. This is illustrated by the recent discovery of disease tolerance programs, which, through a myriad of still poorly understood mechanisms, help restoring the lost balance of biological functions[295]. Recent studies have helped to understand how cellular stress responses[79], metabolic programs[82], or neuro-metabolic interactions[38,85] help maintaining fitness in face of the extreme challenge of sepsis.

Earlier in this chapter, we discussed how cellular surveillance programs communicate and resolve homeostasis perturbations. Initially uncovered in *C. elegans*[8], these mechanisms are gradually being acknowledged in mammals, especially in the context of severe inflammation[296]. A deeper understanding of such mechanisms will likely have an impact not only on the field of sepsis, but also in aging, metabolic disorders, and virtually any disruption of homeostasis.

1.5 Thesis aims and outline

An increasing number of studies have described how cellular surveillance mechanisms correct deviations in homeostasis. How these protective programs

can be harnessed to improve organismal fitness in extreme disruptions of homeostasis, such as sepsis, is still unknown.

Following the observation that low doses of anthracyclines trigger a DNA damage response that confers disease tolerance in a mouse model of sepsis[79], we hypothesized that drugs that perturb core functions of specific organelles will activate a stress response that is protective against infection. In particular, we focused on perturbations of mitochondrial function, due to the pivotal role of these organelles in signaling, metabolism, and cell fate determination. The aims of this study were:

- To identify drugs that improve sepsis outcomes in mice through organelle-specific perturbations.
- To explore the multifaceted mechanisms of protection induced by these drugs, including tissue-specific and organismal effects.
- To establish a link between mitochondrial function, metabolism and disease tolerance mechanisms in mouse models of sepsis.

Several aspects of the protective role of homeostasis perturbations are addressed throughout this thesis:

Chapter 2 presents a model of innate immune activation driven by perturbations of homeostasis. Here, we gather recent evidence from the literature linking stress responses and the activation of immune functions. We argue that cellular surveillance systems perceive pathogen invasion not only by classical PRR activation but also by detecting pathogen-induced changes in cellular functions.

In **Chapter 3** we tested the hypothesis that drug-induced perturbations in organelle homeostasis can trigger compensatory responses that induce disease tolerance in sepsis. In particular, we show how the mitoribosome-targeting drugs doxycycline and chloramphenicol increase survival and induce tissue protection in infected mice independently of their antibiotic effect. The protective effect of doxycycline is associated with transient perturbations in mitochondrial

ETC activity, therefore supporting the hypothesis of hormetic organelle perturbations.

Chapter 4 focuses on the role of mitochondrial function and metabolism in sepsis. We show that infected mice present with severe metabolic dysfunction in the liver during sepsis, namely impaired fatty acid transport and oxidation in the mitochondria and defective glucocorticoid signaling. Both of these defects are improved by doxycycline treatment, thus providing mechanistic insight into the protective effect of mild perturbations in mitochondrial function. Notably, this effect can be replicated by phenformin, a non-antibiotic drug that inhibits ETC complex I activity, and by mild genetic perturbations in ETC function.

Chapter 5 presents a different aspect of doxycycline-induced tissue protection, which relates to lung physiology. We report a surprising role of doxycycline in promoting lung repair, which may explain faster recovery from infection.

A specific discussion of the findings is included throughout the thesis along with the data of each chapter. **Chapter 6** presents a more general discussion and unified framework of this thesis, including perspectives and suggestions for future studies in the topic.

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Chapter 2

Initiation of innate immune responses by surveillance of homeostasis perturbations

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Author contributions

LFM conceived the hypothesis. HGC and LFM contributed ideas, researched the literature, and wrote the manuscript.

2.1 Abstract

Pathogen recognition, signaling transduction pathways, and effector mechanisms are necessary steps of innate immune responses that play key roles in the early phase of defense and in the stimulation of the later specific response of adaptive immunity. Here, we argue that in addition to the direct recognition of conserved common structural and functional molecular signatures of microorganisms using pattern recognition receptors, hosts can mount an immune response following the sensing of disruption in homeostasis as proximal reporters for infections. Surveillance of disruption of core cellular activities leading to defense responses is a flexible strategy that requires few additional components and that can effectively detect relevant threats. It is likely to be evolutionarily very conserved and ancient because it is operational in organisms that lack pattern recognition triggered immunity. A homeostasis disruption model of immune response initiation and modulation has broad implications for pathophysiology and treatment of disease and might constitute an often overlooked but central component of a comprehensive conceptual framework for innate immunity.

2.2 Introduction

Innate immunity refers to first-line host defense mechanisms that limit damage in the early stages of homeostatic disruption, more often caused by exposure to microorganisms. In addition to its early role in containment of disease, innate immunity is central for the initiation and orchestration of the later but specific, diverse, memory enabled, and nonself-reactive adaptive immunity of vertebrates[1].

Innate immunity relies on physical and chemical barriers, cellular effectors (e.g., neutrophils, macrophages, dendritic, and natural killer cells), protein effectors

(including complement), and regulators of cellular activity like cytokines and chemokines. Innate immunity has evolved to recognize signature patterns of targets rather than specific components that distinguish related molecules or structures.

Innate immune responses can be shaped by anatomic and compartment information. The anatomic location informs on the presence and level of threat posed by pathogens to the host. For example, microorganisms on the gut lumen are less likely to be pathogenic and therefore do not usually trigger inflammation, contrary to those that have crossed the epithelial layer and characteristically induce an inflammatory response. Accordingly, innate sensors are strategically positioned in the baso-lateral but not on apical cell surface of gut epithelial cells. Additional information can be generated by the presence of tissue-specific cell and molecular sensors either in the cell surface or subcellular compartments. Microorganisms that reach the bloodstream are identified by multiple molecular and cellular sensors and signal a higher level of threat that triggers a more vigorous response that often leads to an over-response like in the case of sepsis. At a subcellular level, a strong immune response can be initiated against agents that invade the cytosol, where the host cell expects no microbial products. This has come to be known as the patterns-of-pathogenesis hypothesis[2] and is exemplified by the strongest immune responses against cyto-invasive pathogens like *Listeria* spp[3].

The pioneering and insightful work of Metchnikoff[4] proposed that one of the primary functions of the immune system was to preserve homeostasis in addition to protect from infection. After him, and for many decades, the study of immune responses was largely focused on the adaptive arm of the immune system and innate responses were thought to consist mostly of physical barriers, cellular phagocytic effectors, and soluble factors with a dominating role for complement. It was only in the mid-1990s that the field of innate immunity took center stage, after the paradigm shift proposed by Janeway[5]. This novel conceptual framework was inspired by the need to address the then limitations of clonal selection theory and formalized the already generally perceived

requirement for additional signals for lymphocyte activation as earlier suggested by the work of Coutinho and others[6,7]. The now standard model of how the innate immune system detects microbial infections to immediately initiate a defense and later generate long-lasting adaptive immunity predicted that microorganism-associated molecular patterns (MAMPs) representative of different groups of pathogens are recognized by pattern recognition receptors (PRRs). Their engagement leads to the activation of immune signaling pathways, immediate effector mechanisms tailored to each pathogen group, and to the generation of long-lasting adaptive immunity[5]. This model is now overwhelmingly supported by data collected by a growing number of research laboratories in the last two decades but fails to explain how the host can respond to pathogens with which it has no evolutionary history, as the repertoire of PRRs is limited in chemical specificity, even for those with chemical promiscuity[8]. Critically, it is insufficient to describe the interaction with commensal organisms that populate epithelial barrier surfaces (e.g., lung, skin, and gut) and how vertebrate hosts discriminate between avirulent and virulent microorganisms that display overlapping MAMPs[8].

A complementary hypothesis, proposed and popularized by Matzinger, widely known as the danger model, originally predicted that contextual cues to the innate immune response were provided by molecular components that resulted from pathogen-induced cell lysis leading to damage-associated molecular patterns (DAMPs) that could activate cellular receptors and their downstream signaling pathways[9]. The underlying message was that the immune system recognizes the damage caused by pathogens, not the pathogens that cause it[9]. While this model is clearly useful in the context of sterile inflammation, doubts remain as to the relevance of immune activation by DAMPs in a context of an infection, especially for its initiation step.

A related hypothesis, limited to the case of pathogenic microorganisms, has been referred to as effector- triggered immunity (ETI), and originally defined in plants as a protective immune response induced by the detection of microbial effectors[10,11]. In vertebrates, this form of activation of innate immunity covers the

ability of the immune system to recognize pathogens not through their structural features using PRRs to detect their group-specific ligands, but through the sensing of their virulence factors. This is best exemplified by the activation of the inflammasome by pore- forming exotoxins. This is an additional attractive concept to explain innate immune sensing as it can inform the host on the presence of a threat because PPRs that detect typical signatures of groups of microorganisms are insufficient to distinguish between commensals and pathogens. However, not all substantial cellular physiological perturbations caused by pathogens are due to the direct or indirect effects of their virulence factors or effectors that have intracellular targets, a central assumption of this hypothesis[12]. Instead, the deviations can also be the result of a foreign microorganism that stresses the host by taking advantage of the host physiological processes and resources to complete the different steps of its life cycle. In addition, contrary to the case of plants, there is no evidence for the direct sensing of virulence factors in vertebrates[12]; an impossibility for most given their number and lack of common signature molecular motifs, which would require a very large number of additional molecules, incompatible with a germline-encoded repertoire. By contrast, many microorganism effectors have been shown to have immune-inhibitory activity[13], rather than to boost an immune response. In fact, pathogens that lack one or several virulence factors are more likely to be effectively eliminated by the host. While ETI is certainly operational and is an important form of immune detection in vertebrates, it might be a special case of detection of pathogens based on the direct sensing of disruptions of homeostasis induced by virulence factors.

2.3 Homeostasis perturbation-induced immune response

In this assay, we focus on an emerging conceptual framework pointing to a critical role for substantial deviations in homeostasis in the initiation and

direction of innate immune responses. Disruption of homeostasis is for many groups of pathogens, contrary to commensals, a necessary consequence of the pathogen's invasion and it might be (a) necessary for the completion of one of the steps of the pathogen's life cycle, (b) due to collateral changes to cellular physiology caused by the abnormal presence of a pathogen and competition for the limited resources of the host, or (c) the result of the direct and purposeful targeting of core cellular functions to inhibit a host effective immune response (ETI). *Caenorhabditis elegans,* which lacks *bona fide* pattern recognition receptors, has evolved to detect the presence of pathogenic bacteria by sensing changes in core cellular functions triggered by their presence[8], which might point to the possibility that this sensing component of innate immunity is evolutionarily older than PRR-triggered immunity. It has been shown in *C. elegans* that disruption of core cellular activities by toxins and virulence factors might enable organisms to detect invading pathogens and to trigger avoidance behaviors, detoxification pathways and innate immune responses of different categories[8,14,15]. Avoidance is one of the three key defense strategies, collectively known as ART[16], in addition to the classic and more thoroughly studied resistance mechanisms and the emerging tolerance mechanisms that limit the negative impact of infection on the host without affecting the pathogen load[17].

Interestingly and significantly, substantial and continued deviations to homeostasis have been proposed to be a root cause of chronic debilitating conditions that invariably are accompanied by inflammation, including obesity, type 2 diabetes, and atherosclerosis. This tight connection is underscored by the long implication of macrophages as sensors of homeostasis deviations[18,19]. This theme has been elegantly conceptualized and described by Ruslan Medzhitov and will not be extensively discussed here[20,21]. Below we describe and explore the main processes and mechanisms for which there are currently data for a role of homeostasis disruption leading to the initiation of an immune response.

2.4 Translation inhibition

Viruses need to use the host translation machinery to complete their life cycle. Host translation inhibition is a critical component of antiviral responses. It is therefore not surprising that sensing of host translation inhibition can signal for the initiation of an innate immune response. While this would be expected in the case of viruses, it is perhaps less intuitive for other groups of pathogens with their own translation machineries but data are now accumulating, especially in the case of bacteria[22]. At least in specific cases, like *C. elegans* infection by *S. aureus*, translation inhibition is sufficient to trigger the expression of immune effectors, 80% of which can be transcribed by a single transcription factor (HLH-30)[8,23]. This expression signature is conserved in vertebrate macrophages, where there is considerable functional synergy with PRR activation[23].

Inhibition of host translation and elongation has been proposed to be a virulence mechanism used by pathogens to prevent the expression of antimicrobial peptides. The evolutionary arms race between host and microbe dictated that translation inhibition would be a signal of pathogen invasion. In *C. elegans,* infection with *P. aeruginosa* results in translation inhibition by the bacterial exotoxin A. Paradoxically, translation of the transcription factor ZIP-2 is enhanced, leading to the activation of downstream genes that are crucial for the animal's immune response[24]. Notably, such defense mechanism proved to be a response to translation inhibition rather than the toxin itself[25], suggesting a role as a surveillance system for a wide range of toxins and microbes. In fact, a recent study by Ruvkun and coworkers[26] corroborated this hypothesis by showing that hygromycin and G418, two bacterial toxins with translation inhibitory activity, activate genes involved in immune and detoxification responses that are also activated in translation-defective mutant worms. In this study, the systemic response to local damage was found to rely on lipid signaling pathways, namely bile acid synthesis[26]. Therefore, it is likely that bile acids function as messengers that mediate immune response as well as the metabolic adaptation required to cope with insult caused by infection. In

mammals, a similar line of evidence has arisen from studies in mice macrophages infected with *Legionella pneumophila.* A subset of toxins that inhibit host translation was found to be essential for the activation of the MAP kinase signaling pathways that mediate innate immunity[27]. Moreover, these effectors mediate NF-κB signaling, as translation inhibition prevents synthesis of IκB, a short-lived NF-κB inhibitor, whereas the long-lived NF-κB remains active[28].

2.5 DNA stress and damage

DNA is the key molecule to store genetic information, therefore its accurate replication and repair is critical for the survival of the organism. Many factors can contribute to its change or damage, including errors during replication and direct damage through chemical or physical factors. Additional sources of DNA damage to consider are the lesions caused to DNA either resulting from collateral effects of cellular defense mechanisms against intracellular pathogens, like the generation of ROS to kill phagocytized bacteria, or the lesions induced by bacteria[29,30] and viruses[31], that in some cases are required for their life cycle[32,33].

DNA damage responses are critical for the preservation and accuracy of the structure and information of DNA, a fact underscored by a large, complex, and accurate machinery of components and signaling pathways present in all eukaryotic life forms[34]. Ataxia telangiectasia mutated (ATM) kinase is a central component of the DNA damage response machinery required for the repair of double-strand breaks[34]. Loss-of-function mutations in ATM are the causal mechanism of the ataxia telangiectasia (AT) syndrome characterized by neuro-degeneration and substantial increased risk of (mostly hematologic) cancer[35]. Interestingly, while AT patients are known to be more susceptible to some respiratory bacterial infections[36–38] and chronic herpes virus infections[39,40], which

presumably result from defects in the adaptive arm of the immune response pointing to a role of ATM in the biology of B cells and antibody generation, these patients are also known to be remarkably resistant to several and severe systemic viral infections[36–38]. It has also been appreciated that type I interferons (IFNs) required for viral control can be produced constitutively in wild-type mice, in the absence of an ongoing infection[41]. Through the combined analysis of AT patients and ATM-deficient mice, Gekara et al.[42] have recently shown that in both cases, there was an accumulation of unrepaired DNA lesions that triggered the induction of type I IFNs leading to increased antiviral and antibacterial responses[42]. In addition to ATM, other DNA damage responsive factors such as p53 have been shown to regulate inflammatory responses[43,44] and another DNA damage sensor, MRE11, can recognize cytosolic double-strand DNA to induce type I interferon by the regulation of STING trafficking. The spontaneous production of type I IFNs by ATM-deficient cells was due to the release of altered DNA species to the cytoplasm, where they were sensed by the STING-mediated pathway[42]. The same researchers also found that ATM defects prime cells to mount a stronger response to other PRR engagement, including TLR-induced type I IFN induction[42]. This work shows that disruptions in homeostasis leading to DNA lesions caused by sterile factors, including chemical, physical, and metabolic stressors, or defects in the DNA damage response machinery can lead to spontaneous IFN responses. Because at least bacteria and virus are known to cause DNA damage, not necessarily through the use of virulence factors, this work also supports the hypothesis that a response to infection can be initiated by sensing DNA damage, which might provide information on the subcellular localization and type of pathogen in addition to the detection of the presence of a pathogen. This work is also an indication that pathogen detection via PRRs likely synergizes with homeostasis disruption sensing to optimize an innate immune response based on the perceived level of threat. DDR-initiated immune responses can potentially be explored therapeutically, including for the induction of tolerance to tissue damage like that caused by severe infections, such as sepsis[45].

In addition to the ability of nuclear DNA damage to initiate an immune response, mitochondrial DNA (mtDNA) has also been shown to constitute a cell- intrinsic trigger of antiviral signaling[46]. For example, herpesvirus can cause mtDNA stress and lesion leading to ISG expression and antiviral priming[46]. The monitoring of mtDNA homeostasis is likely to constitute an important surveillance mechanism capable of signaling the presence of a viral infection and to trigger an antiviral response required to cooperate with other classical sensing mechanisms for a full-blown antiviral response.

2.6 Unfolded protein response

2.6.1 Mitochondria

As autonomous organelles with a transcriptional program, mitochondria must tightly regulate their protein homeostasis. In particular, the balance of mitochondrial- and nuclear-encoded proteins that form the electron transport chain complexes needs to be maintained. Therefore, perturbations in protein folding, import, or function trigger a transcriptional adaptation, which was named mitochondrial unfolded protein response (UPR^{mt}). In *C. elegans*, the model organism used for most studies, a major mechanism of UPR^{mt} activation has been proposed, which involves a sensor of cytosol-to-mitochondria traffic efficiency. The transcription factor ATFS, which is normally imported into the mitochondria and degraded, accumulates in the cytosol in case of mitochondrial dysfunction that alters the import efficiency. In such cases, ATFS is transported to the nucleus, where it binds to the promoters of several genes involved in mitochondrial chaperone production and antioxidant defense, which constitute the UPR^{mt} machinery[47]. In mammals, however, no 'mitochondrial stress sensor' has been identified, and very little is known about how UPR^{mt} is induced and regulated. Perturbations of mitochondrial function, such as accumulation of

ROS or respiratory chain inhibition, cause mitochondrial signaling to the nucleus through a process not completely understood. This process culminates in the activation of the transcription factor CHOP, which regulates expression of genes with protective function[48].

Activation of the UPR^{mt} has been proposed to have a beneficial role in aging[49], xenobiotic detoxification, and stress resistance in general[50]. Both the endoplasmic reticulum (ER) and UPR^{mt} are surveillance systems that contribute to restore homeostasis in stress conditions[51]. Therefore, it is tempting to speculate that mitochondrial stress could have a role in triggering an immune response. Infection is a well- known source of mitochondrial stress, mostly due to accumulation of ROS. Accordingly, a recent study showed that *C. elegans* activates the UPR^{mt} when exposed to H_2O_2-treated *E. coli*, revealing a new role for mitochondria in sensing oxidative stress in the environment in anticipation of changes in cellular homeostasis[52]. The first report on the activation of immune response involving mitochondrial stress, by Pellegrino et al.[53], showed that pathogen exposure in *C. elegans* results in ATFS1-mediated UPR^{mt} leading to the activation of not only mitochondrial protective genes but also of antimicrobial peptides and lysozyme, which culminates in improved resistance to *P. aeruginosa* infection. Very recently, two other studies extended this role to mammalian innate immunity. Mitochondrial DNA damage was found to result in mtDNA escape to the cytosol, where it activates the STING-IRF3 signaling that results in type I IFN production and increased resistance to viruses[46]. Bronner et al.[54] reported that infection-induced ER stress involves crosstalk with the mitochondria to allow inflammasome activation. Interestingly, integrators of cytosolic antiviral signaling, such as MAVS, are known to co-localize with the mitochondrial membrane and are functional links between the mitochondria and the mitochondria-associated endoplasmic reticulum membrane[55]. Although the signaling pathways that connect ER and mitochondria were not yet identified, this unprecedented observation highlights the importance of these two organelles in an integrated model of response to infection based on surveillance of cellular homeostasis.

2.6.2 Endoplasmic reticulum

Protein homeostasis is tightly regulated in the ER. Deviations from steady-state protein synthesis and folding levels trigger signaling cascades that mediate an unfolded protein response (UPR). In basal conditions, the three ER lumen sensors IRE-1, PERK, and ATF6 are bound in an inactive state to the master regulator of UPR BiP (GRP78). When unfolded proteins accumulate in the ER lumen, the binding equilibrium causes IRE-1, PERK, and ATF6 to be released and activated. IRE-1 phosphorylation activates its endonuclease domain, which cleaves the XBP-1 mRNA to produce its active form, sXPB-1. The active sXBP-1 protein is a transcription factor that activates a number of cytoprotective genes, such as chaperones. Activation of the kinase PERK causes the cytoplasmic eukaryotic initiation factor (eIF2a) to be phosphorylated, leading to translation inhibition and hence reduction in the unfolded protein overload in the cell. Phosphorylated eIF2a also regulates the transcription factor ATF4, involved in oxidative stress resistance and apoptosis. Finally, upon dissociation from BiP, ATF6 is transported to the Golgi, where it is cleaved and processed to its transcription factor form. Mature ATF6 then moves to the nucleus and regulates lipid synthesis and the expression of chaperones.

The interaction between pathogens and the ER has been extensively studied, especially in the context of viral infections. By hijacking the protein synthesis and folding machineries of the host cell, viruses perturb the folding capacity of the ER, leading to UPR activation[56,57]. Such host protective mechanisms may in turn be exploited by the pathogens to their own advantage: the activation of UPR not only increases the production of chaperones, which facilitate folding of viral proteins but it also leads to overall host cell survival, thus supporting pathogen subsistence. However, a new hypothesis is emerging in the field suggesting that virus-induced perturbations in the ER function as an alarm signal. This places the ER at the core of a complex surveillance system that detects invasion by pathogens and activates the appropriate immune responses[56,58]. Evidence to support this view is growing and expanding to other

intracellular pathogens and, more interestingly, to toxins secreted by extracellular bacteria. *Listeria monocytogenes*, a facultative intracellular pathogen, has been shown to trigger ER stress prior to invading the host cell by means of the toxin listeriolysin, which activates the three branches of UPR[59]. Although a direct link between UPR and innate immunity is missing in this study, the authors showed that ER stress induced by thapsigargin or tunicamycin decreases the intracellular pathogen load. Cho et al.[60] provided more compelling evidence of ER-mediated innate immunity by showing that a portion of the cholera toxin is able to bind IRE1a and induce endogenous mRNA degradation (RIDD). The resulting RNA fragments subsequently activate the virus detection system RIG-I, leading to the production of interferon and NF-κB.
Other independent studies suggested several links between one or more arms of the UPR and inflammation. For instance, upon activation of the IRE-1a branch, spliced XBP-1 binds to the IL-6 and TNFα promoters, and is essential for sustained cytokine production[61]. Interestingly, IRE-1a phosphorylation is enhanced upon TLR activation, showing interplay between PRR- and UPR-dependent immunity.
All these data support a model of integrated surveillance in which both PRR and UPR systems cooperate to achieve a fast and effective immune response, as well as to activate cytoprotective mechanisms (such as translation inhibition, chaperone production, and antioxidant defense) that improve infection outcome.

2.6.3 Cell non-autonomous activation of the UPR

While most studies discussed so far focus on the cell autonomous activation of the UPR, the idea of an immune activation mediated by stress responses across distant tissues is particularly appealing. There is now evidence that mitochondrial and ER stress can autonomously be perceived and activated by cells located far away from the original stress focus. Upon the finding that mitochondrial stress in *C. elegans* neurons can be perceived by intestinal cells,

the term ‘mitokine’ was proposed to describe a putative molecule responsible for this cell non-autonomous signal[62]. The identity of such molecules and their role in the systemic regulation of protein homeostasis and innate immunity is still a matter for debate and intense research efforts[63].

2.7 Additional homeostasis perturbations leading to initiation of immune responses

2.7.1 Barrier disruption

The lung, skin, and intestine constitute key and extensive surfaces that are not only in constant contact with a wide array of commensal microorganisms but are also the first barrier faced by pathogens. They are first-responders to pathogenic invasion, which requires constant sensing and decision on what is a commensal and what is a pathogen. Standard immune recognition using PRRs is insufficient to provide this decision as these sensors will identify broad classes of microorganisms but not if they are beneficial, neutral, or likely to cause disease. Disruption of epithelial cell core physiology pathways and functions is therefore likely to play a central role informing on this decision. Evidence for this principle is exemplified in *C. elegans*, where pathogen-caused structural damage to epithelial cells can be sensed through hemidesmosomes that regulate AMP transcription through association with STAT proteins[64]. Interestingly, hemidesmosome disruption in HEKa cells induces β-defensin antimicrobial peptides transcription[64].

2.7.2 Metabolic and signaling pathways

Immune cells reprogram their metabolism to activate their responses to fight pathogens[65,66]. Lipid metabolism seems to have a particular important role[67].

Bensinger and coworkers[68] have identified a metabolic-inflammatory circuit linking perturbations of cholesterol biosynthesis with initiation of antiviral immunity in macrophages. This coordination allows for the adjustment of metabolic requirements to immune activation. Interestingly, perturbing cholesterol synthesis initiates type I IFN signaling through a STING/TBK1 pathway making mice more resistant to a viral challenge when the circuit is reprogrammed in macrophages in vivo[68]. More generally, we can expect the composition of the plasma membrane to be changed when the viral production is very high, not only in terms of cholesterol content but also due to modifications in the concentration and aggregation capacity of signaling complexes. This will predictably have massive implications for cell physiology. Perhaps, it will produce plasma membrane patterns that can be sensed and trigger a cell autonomous response or be sensed by innate immune patrolling cells.

In addition to cholesterol biosynthesis, it is conceivable that substantial deviations in other controlled metabolic flows can be sensed and serve as indicators of a particular group of pathogens as they are known to have particular requirements that vary according to their groups and type of life cycle. A pathogen-induced shift toward glycolysis[69–72], causing substantial changes in the profile of metabolic intermediates, has been reported and is sure to have a profound impact on cellular physiology. In addition, both bacteria and viruses have been shown to cause amino acid depletion that can be sensed and interpreted by the host as the presence of a pathogen [73,74]. The case where dendritic cells can be reprogrammed by the viral-dependent activation of the general control nonderepressible 2 kinase (GCN2), a sensor of amino acid starvation in mammals, to initiate autophagy and enhance antigen presentation to CD4+ and CD8+ T cells is particularly striking[74].

2.7.3 Rho-GTPases

Rho-GTPases constitute frequent targets for pathogens. Recent elegant examples include the modulation of mitochondrial dynamics by *Vibrio cholerae* T3SS effector VopE through Miro GTPases[75] and triggering protective immunity via activation of Rac2 and IMD or Rip kinase signaling pathway *of D. melanogaster*, by the cytotoxic necrotizing factor 1 of *E. coli*[76].

2.8 Conclusions and perspectives

The sensing of pathogen-induced disruption of homeostasis is likely to be a key component for detecting the presence of a disease-causing microorganism (Figure 2-1).

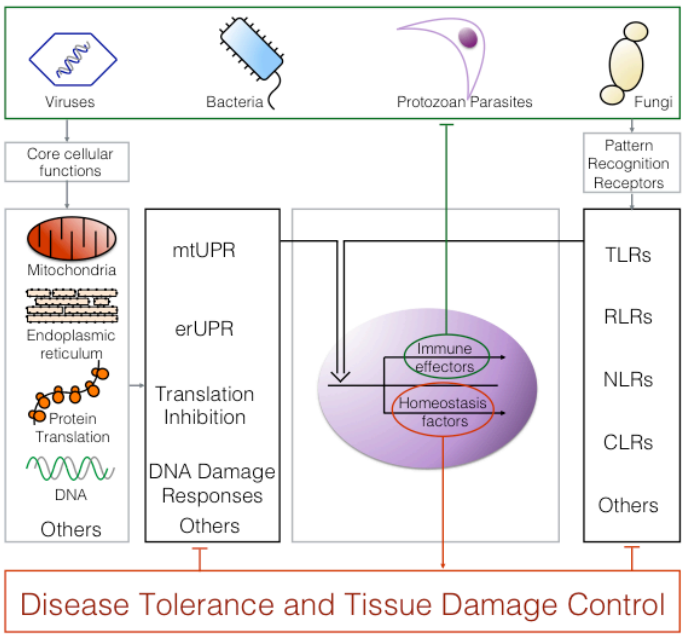

Figure 2-1 - Major groups of pathogens, including viruses, bacteria, protozoan parasites, and fungi can induce homeostasis disruption of mitochondria, endoplasmic reticulum, protein translation, DNA, or other core cellular functions in addition to the activation of pattern recognition receptors such as Toll-like receptors, RIG-I-like receptors, NLR-like receptors, and C-type lectin-like receptors. Signaling pathways triggered by both events synergize in the production of immune effectors leading to resistance mechanisms tailored to specific classes of

pathogens and of homeostasis promoting factors that not only restore homeostasis but also limit tissue damage caused by infection and initiate tissue repair.

These pathways should synergize with the sensing capability of PRRs not only to potentiate the resulting feed-forward mechanisms that contribute to the initiation of innate immunity and inflammation but also to inform the host on the level of threat posed by specific challenges. The early events triggered by disruption of homeostasis might also have a fundamental role in the counter-regulatory mechanisms aimed at the later negative-feedback pathways to effectively terminate the inflammatory response and, critically, to activate tissue damage repair, without which tissues cannot return to steady state. Disease tolerance, the defense strategy that limits the negative impact of infection on the host without affecting the pathogen load[17], is likely to be closely dependent on the mechanisms induced by homeostasis disruption[45,77]. While in this assay we have focused predominantly on the consequences of disruption of homeostasis by pathogens, this conceptual framework might also be relevant in the context of innate immune responses to tumors.

The mechanisms of homeostasis perturbation-induced immune responses are still considerably unexplored but their characterization is likely to open a complete new field of opportunity to molecularly understand core surveillance mechanisms of basic cellular processes with a critical role in the regulation of organ function and explain how organisms deal with stress, age, and set limits to their lifespan. Their activation can ultimately promote health and expand longevity. The exploration of this theme also raises the possibility of pharmacologically targeting the pathways involved, which predictably might be useful to more effectively fight infections not only by increasing resistance to pathogens but also by increasing disease tolerance and tissue damage control[16]. This last effect is likely to be particularly important in the specific case of sepsis[78], but more generally in dealing with multiple causes leading to

multiple organ failure, which carries a very high mortality rate and for which novel and effective strategies are urgently needed.

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Chapter 3

Host-dependent induction of disease tolerance to infection by tetracycline antibiotics

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Author contributions

I was responsible for planning, executing and analyzing data from all experiments in this chapter. Ana Neves-Costa and Dora Pedroso performed the isolation and differentiation of bone marrow-derived macrophages. Ana Neves-Costa provided assistance in the *in vitro* studies. André Barros, Elsa Seixas, Tiago Velho and Dora Pedroso provided assistance in the *in vivo* work. Elsa Seixas also provided assistance in flow cytometry analysis of HepG2 cells. Luís Ferreira Moita supervised the project and reviewed this chapter.

3.1 Abstract

Sepsis is a life-threatening organ dysfunction condition caused by a dysregulated host response to infection, which still lacks specific therapeutic interventions. It has been proposed that in addition to the current standard therapies, strategies that induce disease tolerance might constitute the necessary missing treatment complement.

Following the observation that drug-induced DNA damage elicits a protective response that confers tolerance to sepsis, we hypothesized that pharmacologically targeting core functions of the cell might unveil new therapeutic options for sepsis and other inflammatory conditions.

Here, we used a mouse model of bacterial sepsis to test the protective effect of clinically approved drugs known to perturb cellular functions. We found that doxycycline and chloramphenicol, two drugs that inhibit mitochondrial protein synthesis, increase survival independently of their antibiotic effect.

Doxycycline treatment improves tissue pathology during sepsis without an impact on pathogen load. This is associated with changes in mitochondrial function, namely decreased mitochondrial respiration *in vivo*, without compromising mitochondrial viability. Our findings provide new insights into disease tolerance mechanisms induced by homeostasis perturbations.

3.2 Introduction

Infection presents an immense challenge to host physiology that demands a concerted response to limit disease severity. Dysregulation of host homeostasis is particularly evident in the case of sepsis, a major healthcare problem defined as a life-threatening organ dysfunction caused by the host response to infection[1]. Hallmarks of sepsis include an acute burst in pro-inflammatory cytokine production[2] and metabolic failure[3], both leading to severe tissue

damage and high mortality rates. Current management of critically-ill patients is limited to control of infection with antibiotics and organ support measures, with most attempts to modulate immune response resulting in failure[4]. This is in line with the idea that host resistance mechanisms – which rely on the immune response to clear pathogens – are not enough to guarantee recovery from infection. Indeed, a number of strategies have been recently proposed to promote disease tolerance – a host defense strategy that limits the negative impact of an infection without affecting pathogen load[5] – thus opening perspectives for new therapies based on tissue damage control during sepsis[6–8].

All eukaryotic organisms are equipped with surveillance mechanisms to detect and correct perturbations in homeostasis. Organelle dysfunction caused by pathogens, toxins, drugs, physical insults or nutritional changes can be rapidly communicated to the nucleus, where a compensatory transcriptional response will be generated[9]. Activation of such stress responses is associated with numerous beneficial effects, such as the initiation of an effective immune response[10] and lifespan extension[11]. Remarkably, locally induced cytoprotective stress responses can be communicated to distant organs, therefore generating whole-body beneficial effects[12,13].

Mitochondria, having a pivotal role in bioenergetics, metabolism, and cell signaling are strictly surveyed organelles notably associated with stress-induced cytoprotection[14]. Pioneer work in *C. elegans* revealed that mild perturbations in mitochondrial function induced both by genetic defects in the ETC[15] or by inhibition of mitochondrial translation[16] resulted in extended lifespan. In mice, several studies have pointed to metabolic benefits arising from inhibition of ETC activity in the context of obesity and insulin resistance[17–19] with no significant effects in longevity[20]. While the molecular mechanisms of mitochondrial stress responses remain poorly understood in mammals, it is generally accepted that perturbations in mitochondrial function involve: 1) retrograde signaling to the nucleus, which activates a transcriptional program known as the mitochondrial

unfolded protein response (UPR^{mt})[16,21], and 2) metabolic adaptation, which may increase fitness in adverse conditions[18].

Besides mitochondria, perturbation in other core cellular functions such as insulin/insulin growth-factor signaling[22], mRNA translation[23], and ER protein homeostasis[24] have shown beneficial effects in numerous experimental models. In the context of sepsis, we have previously reported that the activation of DNA damage responses by the DNA damaging drugs anthracyclines promotes disease tolerance[6].

In this study, we set out to identify novel drug-induced stress responses that confer protection in mouse models of sepsis. Our data indicate that doxycycline and chloramphenicol, two antibiotics previously reported to affect mitochondrial function, increase survival in a model of bacterial sepsis. Doxycycline-treated mice present reduced tissue damage, in spite of similar bacterial loads in blood and organs, suggesting a link between mitochondrial stress responses and disease tolerance.

3.3 Methods

3.3.1 Experimental Models

3.3.1.1 Mice

All animal studies were performed in accordance with Portuguese regulations and approved by the Instituto Gulbenkian de Ciência ethics committee (reference A002.2015) 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, except if otherwise stated. Mice were maintained under specific pathogen-free (SPF) or germ-free (GF) conditions with 12h light/12h 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.

3.3.1.2 Primary cell cultures

Bone marrow-derived macrophages (BMDMs) were differentiated from adult (typically 8 week-old) C57BL/6J male mice. After euthanasia by CO_2 inhalation, the mouse skin was sterilized with 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 x*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 x 10^6 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% (v/v) 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. 24h-48h afterwards, 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 x*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) Pyruvate, 1% (v/v) L-Glutamine, 1% (v/v) Non-essential amino acids, 1% (v/v) Hepes buffer, 0.05 M of 2-Mercaptoethanol.

3.3.1.3 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 37ºC with 5% CO_2. Three to five days before experiments, medium was changed to DMEM with 10% FBS with no addition of antibiotics.

3.3.1.4 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 Balbontín 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.

3.3.1.5 Fungal cultures

Candida albicans (Robin) Berkhout[25] were cultured in yeast culture medium (YPD) for 16-20h at 30°C, 180 rpm.

3.3.2 Method details

3.3.2.1 *E. coli*-induced sepsis model and drug treatments

A starter culture from a single *E. coli* colony was incubated overnight (12-16h) at 37°C, 200 rpm. The next morning, the culture was diluted 1:50 in LB+doxy and incubated for 2.5h until late exponential phase was reached (OD_{600nm} = 0.8-1.0).

The culture was then centrifuged at 4400 x*g* for 5 min at room temperature, washed with PBS and resuspended in PBS to obtain an OD_{600nm} = 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 48h after infection. The following drugs were dissolved in PBS, except if otherwise stated, and injected intraperitoneally (200 µL/mouse) at the time of infection and at the indicated concentrations: chloramphenicol (vehicle: 5% cyclodextrin, dose 50 µg/g); metformin hydrochloride (100 µg/g body weight), menadione sodium bisulfite (20 µg/g), trifluoperazine dihydrochloride (5 µg/g), bortezomib (10 ng/g), nicotinamide adenine dinucleotide (NR) (16 µg/g and 80 µg/g).

Body weight and rectal temperature were determined 0, 24 and 48h 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 CO_2 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 x*g* for 5 min and serum collected and stored at -80°C.

3.3.2.2 Other infection models

Infection with GFP-transgenic *Plasmodium berghei* ANKA was performed as described[26]. Briefly, female mice 8-12 weeks old 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).

C. albicans cultures were grown to exponential phase, washed and resuspended in PBS to obtain an OD_{600nm} = 0.5, corresponding to $5x10^6$ CFU/mL. Female mice 8-12 weeks old were infected by an intravenous injection of 100 µL in the tail vein. Mice were treated with 1.75 µg/g body weight doxycycline at 0, 24 and 48h after infection.

3.3.2.3 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 16h.

3.3.2.4 Biochemical assays in mouse serum and supernatant from BMDMs

Cytokine levels were determined using the following ELISA kits, according to the manufacturer's instructions: mouse TNF-α (#430902, Biolegend), mouse IL-6 (#431302, Biolegend). Serological makers of organ damage were determined using the following colorimetric assays, according to the manufacturer's instructions: QuantiChrom Creatinine (#DICT, Bioassay Systems), QuantiChrom Lactate Dehydrogenase (#D2DH, Bioassay Systems), EnzyChrom Creatine Kinase (#ECPK, Bioassay Systems), EnzyChrom Alanine Transaminase (#EALT, Bioassay Systems), EnzyChrom Aspartate Transaminase (#EASTR, Bioassay Systems). All absorbance readings were performed in 96-well plates using an Infinite M200 plate reader (Tecan). Glucose and lactate levels were

measured in freshly collected heparinized whole blood using the GEM Premier 3000 system (Instrumentation Laboratory).

3.3.2.5 Histopathology

Mouse liver, lung, and kidney were collected 30h after infection and immediately fixed in 10% (v/v) buffered formalin. Samples were then embedded in paraffin, sectioned (3 μm) and stained for hematoxylin and eosin according to standard procedures. 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.

3.3.2.6 Transmission Electron microscopy

Mice were euthanized 24h 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 fixated for 1h 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 1h. Samples were then dehydrated in a graded series of ethanol dilutions 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.

3.3.2.7 Immunoblotting

Cultured cells were rinsed with PBS and lysed with RIPA buffer containing protease and phosphatase inhibitor cocktails. Homogenates were centrifuged at 20000 x*g* for 10 min at 4°C, the supernatant was collected and proteins quantified by the Bradford method. SDS-PAGE was performed by loading 20 µg total protein onto 12% polyacrylamide gels. Proteins were then transferred onto nitrocellulose membranes, blocked with 5% low-fat milk and incubated with primary antibodies for 16h at 4°C. HRP-conjugated secondary antibodies were incubated for 1h at room temperature and developed with ECL Prime. Chemiluminescence was acquired with GE Amersham Imager 680. Band density was analyzed with Fiji version 1.52n.

3.3.2.8 Gene expression analyses

Liver samples (~50 mg) were homogenized in 500 µL Trizol using a TissueLyser II (Qiagen). Homogenates 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 an 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 $2^{-\Delta\Delta Ct}$ relative to a control gene (*Actb* or *Gapdh*).

3.3.2.9 Electron transport chain (ETC) complex activity

Enzymatic activity of ETC complexes in mouse liver was performed as previously described[27]. Briefly, frozen liver samples (50-100 mg) were homogenized in 1 mL homogenization buffer containing 8 mM Tris, 16 mM KCl,

0.8 mM EGTA and 250 mM sucrose using TissueLyser II. Lysates were centrifuged at 20000 x*g* for 10 min at 4°C, the supernatant collected and proteins quantified by the Bradford method. Samples were then diluted in homogenization buffer for a final concentration of 1 mg/mL. Complex I (NADH:ubiquinone oxidoreductase) activity was determined by following oxidation of 100 µM NADH at 340 nm in the presence of 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 decylubiquinone with potassium borohydride), 0.025% (v/v) tween-20, 100 µM 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) 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 37ºC 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.

3.3.2.10 Flow cytometry

HepG2 cells were incubated with doxycycline for 24h, tripsinyzed 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 37ºC, or with 5 µM MitoSOX Red (for mitochondrial ROS) for 10 min at 37ºC 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).

3.3.2.11 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 24h (temperature >35ºC and body weight >95%) were excluded from the analysis.
Mann-Whitney test was used for pairwise 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: $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***); $p < 0.0001$ (****).

3.4 Results

3.4.1 Mitochondrial ribosome-targeting drugs confer protection against bacterial sepsis

To explore the role of drug-induced organelle perturbations in sepsis, we began by selecting a panel of clinically approved drugs previously reported to impose stress to cellular processes. These drugs were intraperitoneally injected in male C57BL/6J mice at the time of infection with an *E. coli* strain carrying resistance to tetracyclines (TetR) and chloramphenicol (CamR). This model of bacterial sepsis was chosen to circumvent the direct antibiotic effect of some of the tested drugs on the infection agent, thus focusing on host-dependent effects. From the panel of tested drugs, treatment with low-dose doxycycline, a tetracycline antibiotic known to bind to the mitochondrial ribosome and block translation of mitochondrial-encoded mRNA[28], revealed a robust increase in survival (Figure 3-1A).

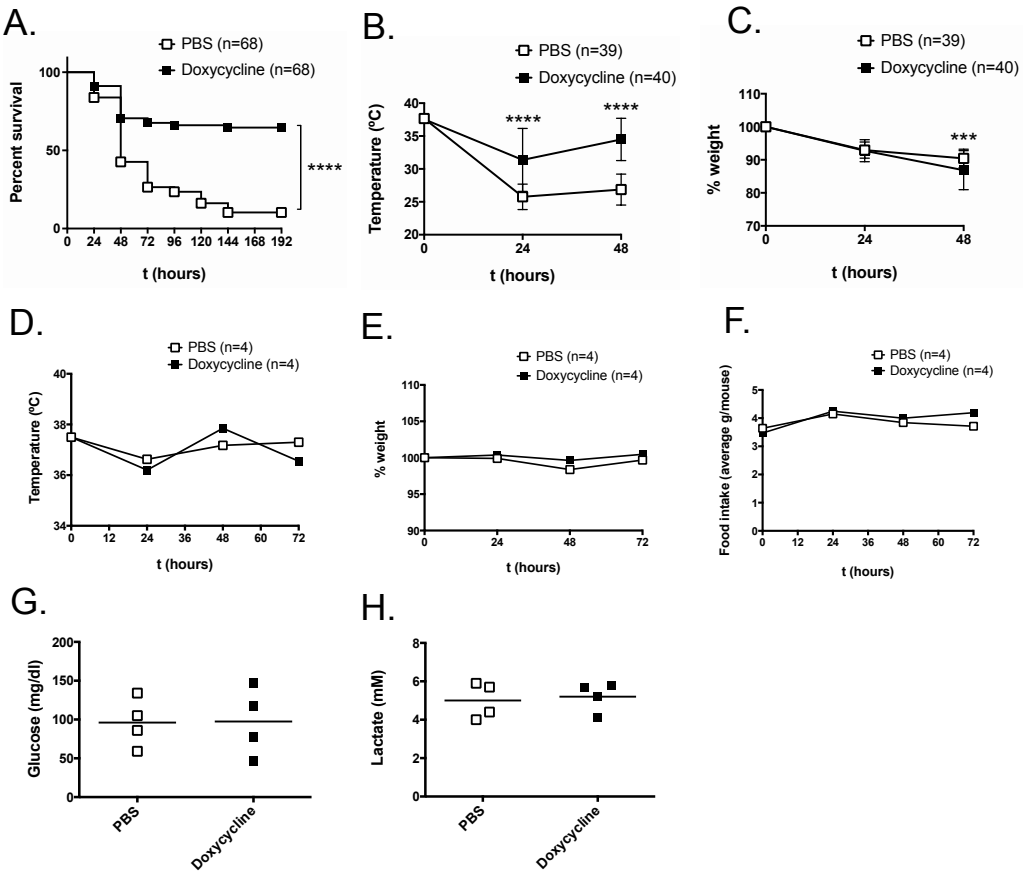

Figure 3-1 - Doxycycline confers protection in a mouse model of bacterial sepsis.

Survival (A), rectal temperature (B) and % initial body weight (C) after infection of male C57BL/6J mice with $3x10^8$ CFU/mouse TetR CamR *E. coli* and treatment with 1.75 µg/g body weight doxycycline (or PBS as a control) at 0, 24 and 48h. Rectal temperature (D), % body weight (E), and food intake (expressed as average food consumption in 24h) (F) recorded in non-infected, doxycycline-treated mice. Glucose (G) and lactate (H) levels determined 24h after infection in freshly collected blood. (A) represents pooled data from 12 independent experiments. (B) and (C) represent mean±SD pooled from six independent experiments. (D), (E), and (F) represent mean±SD from a single experiment. In (G) and (H), squares represent individual mice, bars represent the mean obtained from a single experiment.

We then used rectal temperature and body weight measurements to assess disease severity and found that doxycycline-treated mice present less severe hypothermia than PBS-treated controls and increase body temperature close to normal levels within 48h of infection (Figure 3-1B). Only marginal differences in body weight were found between groups, with doxycycline-treated mice presenting slightly lower body weight at 48h (Figure 3-1C).

While these results suggest a role for doxycycline in thermal regulation and nutritional and metabolic status, this seems to be a specific adaptive response to infection, as no changes were found in temperature, body weigh, and food consumption in non-infected, doxycycline-treated mice (Figure 3-1D, 3-1E, 3-1F). Additionally, no differences were found in glucose or lactate levels 24h after infection with *E. coli* (Figure 3-1G, 3-1H).

Notably, treatment with a single dose of chloramphenicol, an antibiotic structurally unrelated to doxycycline but with similar effect on mitochondrial mRNA translation inhibition, results in a similar effect in sepsis outcome (Figure 3-2).

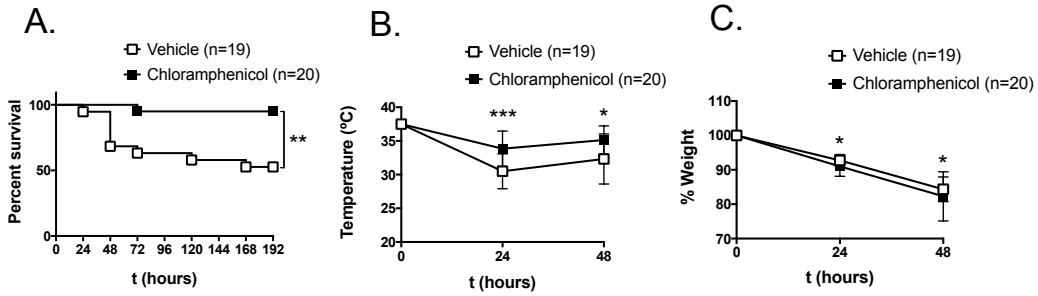

Figure 3-2 Chloramphenicol confers protection in a mouse model of bacterial sepsis.

Survival (A), rectal temperature (B) and % initial body weight (C) after infection of male C57BL/6J mice with $3x10^8$ CFU/mouse TetR CamR *E. coli* and treatment with 50 µg/g body weight chloramphenicol (or 5% cyclodextrin as a control) at the time of infection. (B) and (C) indicate mean±SD. Graphs represent pooled data from three independent experiments.

Other tested drugs included menadione, which generates ROS in the mitochondria (Figure 3-3A); metformin, which activates AMPK signaling (Figure 3-3B); bortezomib, a proteasome inhibitor that causes proteotoxic stress in the ER (Figure 3-3C); trifluoperazine, an antagonist of calcium-mediated intracellular trafficking (Figure 3-3D); and nicotinamide adenine dinucleotide (NR), which increases NAD^+ levels (Figure 3-3E), none of which led to significant improvement in survival at the tested doses.

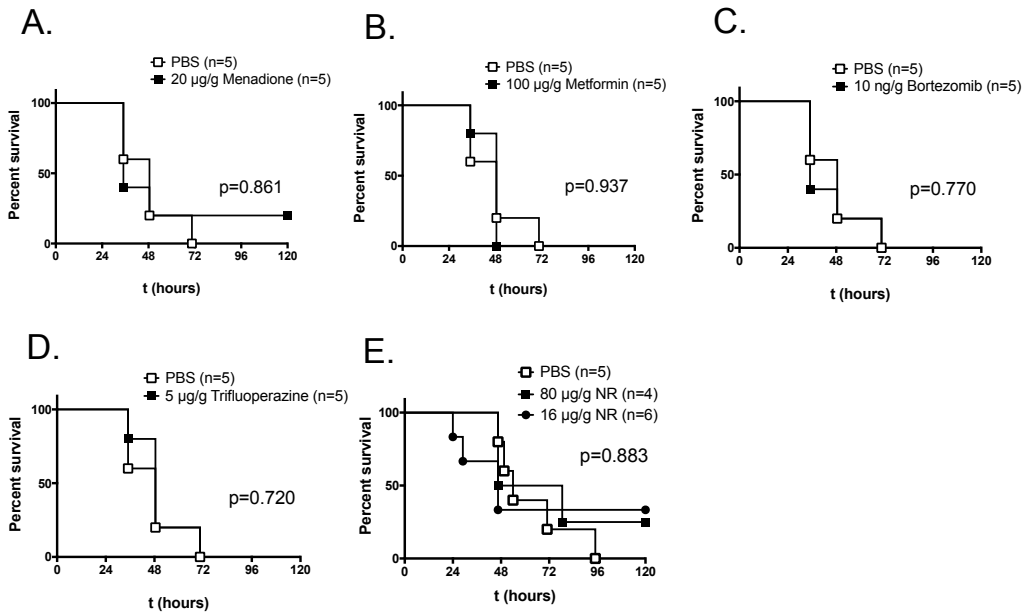

Figure 3-3 - Survival curves of male mice treated with a panel of cellular stress-inducing drugs.

Male mice were infected with $3x10^8$ CFU/mouse TetR CamR *E. coli* and treated with 20 µg/g body weight menadione (A), 100 µg/g body weight metformin (B), 10 ng/g body weight bortezomib (C), and 5 µg/g body weight trifluoperazine (D) at the time of infection, or 16 µg/g and 80 µg/g body weight nicotinamide adenine dinucleotide (NR) (E) at 0, 24 and 48h. Data were obtained from a single experiment.

3.4.2 Doxycycline treatment affords tissue protection in sepsis independently of pathogen load

Upon intraperitoneal injection of $3x10^8$ CFU *E. coli*/mouse, we were able to recover high levels of viable bacteria from blood, liver, lung, and kidney of infected mice for at least 30h (Figure 3-4A). By plating samples collected at 6, 12, and 30h after infection and determining the number of CFU, we found no differences in the bacterial loads of doxycycline-treated mice, except for a modest reduction in kidney CFU at 30h (Figure 3-4A). These results rule out a direct antibiotic effect of doxycycline on the TetR *E. coli* strain used for this study, also indicating no impact of doxycycline on host resistance mechanisms that act to clear the infection. Instead, doxycycline induces disease tolerance by limiting disease severity without affecting pathogen load[5].

Remarkably, despite similar bacterial burden between groups, doxycycline-treated mice show reduced levels of tissue damage in the major target organs of sepsis (Figure 3-4B, 3-4C, 3-4D).

Serum levels of the liver damage markers aspartate transaminase (AST) and alanine transaminase (ALT) determined at different time-points after infection revealed significantly reduced AST levels at 12h, with no significant differences in ALT levels (Figure 3-4B). The kidney damage marker creatinine was markedly reduced at 30h, whereas the muscle damage marker creatine kinase (CK) showed slight, non-statistically significant differences (Figure 3-4B). Lactate dehydrogenase, an unspecific damage marker, showed markedly reduced levels at 12h (Figure 3-4B). Reduced tissue damage was also observed 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 (Figure 3-4C, 3-4D). In all analyzed tissues, doxycycline-treated mice globally showed lower scores of damage and a higher number of animals with no visible damage (score 0) (Figure 3-4D). These changes were more pronounced in the liver, where necrotic areas of doxycycline-treated mice

were markedly reduced; and in the lung, in which we observed reduced neutrophil infiltration, hemorrhage, and thickening of the alveolar wall upon doxycycline treatment (Figure 3-4C).

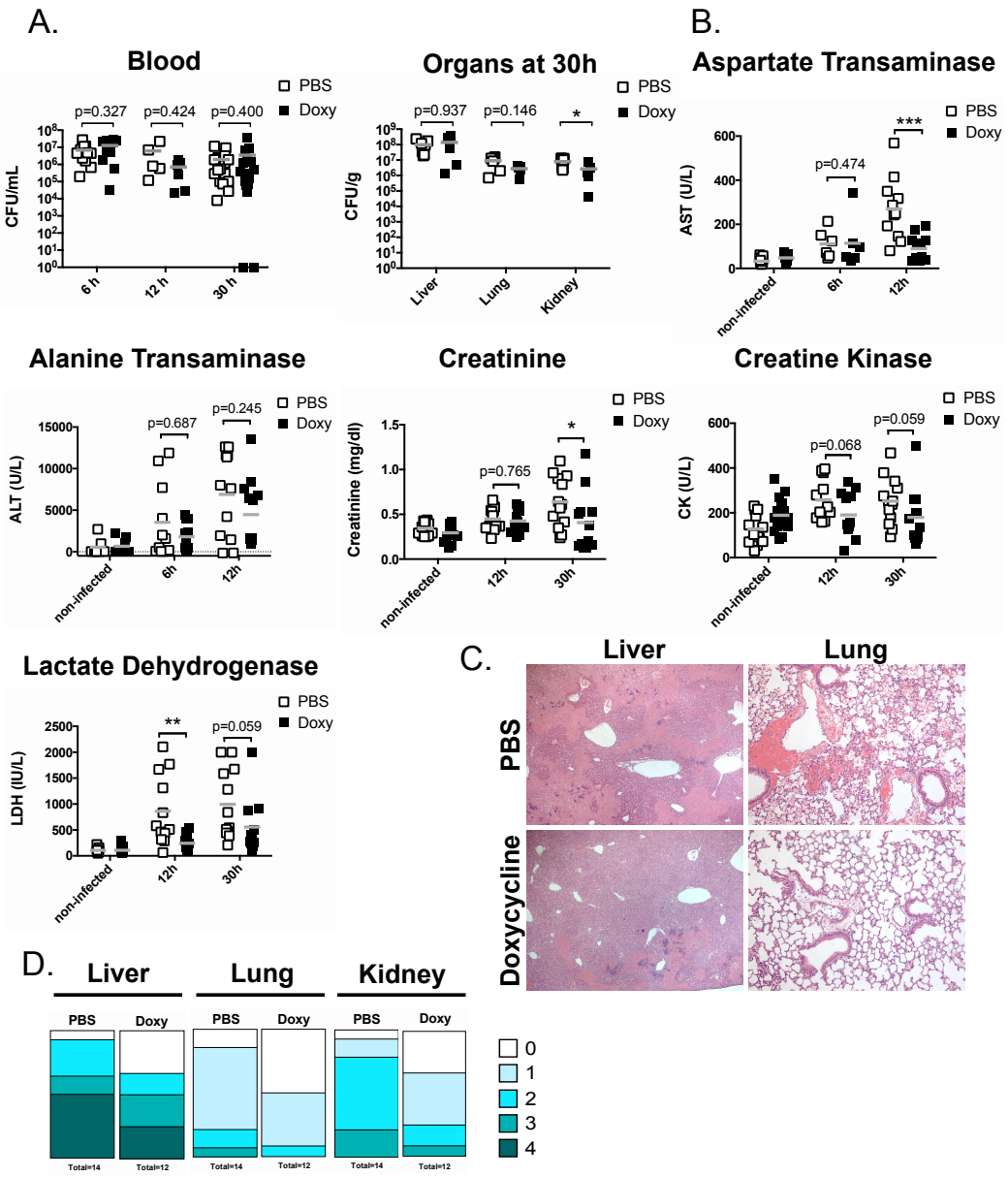

Figure 3-4 - Doxycycline treatment affords tissue protection in sepsis independently of pathogen load.

(A) Bacterial load in mouse blood, liver, lung, and kidney at the indicated time-points after infection. (B) Levels of the organ damage markers Aspartate Transaminase (AST), Alanine Transaminase (ALT), Creatinine, Creatine Kinase (CK), and Lactate Dehydrogenase (LDH) at the indicated time-points after infection. (C) Representative images (n=12-14 mice/group) of Hematoxylin-Eosin stained liver and lung 30h after infection. (D) Organ damage score in Hematoxylin-Eosin stained slides from liver, lung, and kidney 30h after infection (n=12-14 mice/group). Score 0 = no lesions; 1 = very mild; 2 = mild; 3 = moderate; 4 = severe lesions. (A), (B), and (D) represent pooled data from at least two independent experiments; squares represent individual mice and gray bars indicate the mean.

Finally, we analyzed levels of the pro-inflammatory cytokines tumor necrosis factor α (TNFα) and interleukin 6 (IL-6), which are quickly and strongly increased during sepsis. In serum of *E. coli*-infected mice, TNFα levels were found significantly reduced at 12h, while IL-6 levels showed slight, non-statistically significant reduction (Figure 3-5A). In mouse BMDMs treated with doxycycline and stimulated with *E. coli*, no differences were found in cytokine levels (Figure 3-5B), suggesting that doxycycline does not directly modulate the inflammatory response during sepsis, but point instead to the role of the drug in tissue protection mechanisms that induce disease tolerance.

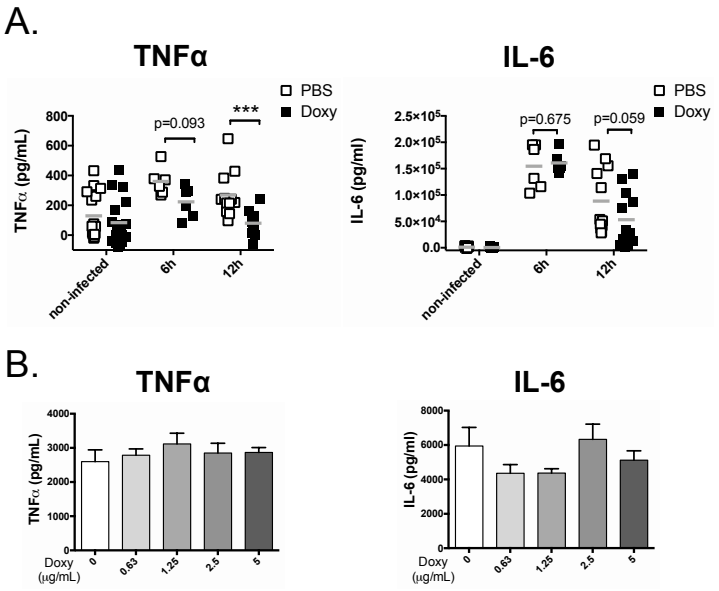

Figure 3-5 - Effect of doxycycline treatment on pro-inflammatory cytokine secretion.
(A) TNFα and IL-6 levels in mouse serum at the indicated time-points after infection. (B) TNFα and IL-6 levels in supernatant of bone marrow-derived macrophages incubated with doxycycline

for 1 h followed by stimulation with PFA-fixed *E. coli* (MOI=20) for 4 h. (A) represents pooled data from at least two independent experiments; squares represent individual mice and gray bars indicate the mean. (B) represents mean±SD from a single experiment assayed in triplicate.

3.4.3 Protective role of doxycycline is independent of its effect on host microbiota

The role of microbiota in host physiology has been increasingly acknowledged, with extensive research focusing on its therapeutic potential in sepsis[29] including its role in disease tolerance[30]. As an antibiotic, doxycycline likely induces changes in microbiome composition that may affect host fitness. To address the contribution of microbiota in doxycycline-induced protection against sepsis, we applied the TetR *E. coli* sepsis model and doxycycline treatment in C57BL/6J mice raised and maintained in germ-free conditions. Both survival (Figure 3-6A) and body temperature (Figure 3-6B) were significantly improved in doxycycline-treated mice whereas no differences were found in body weight (Figure 3-6C). These results largely phenocopy the protective effect obtained on conventionally raised, specific pathogen-free mice (Figure 3-1A, 3-1B, 3-1C), thus demonstrating a host-dependent disease tolerance mechanism.

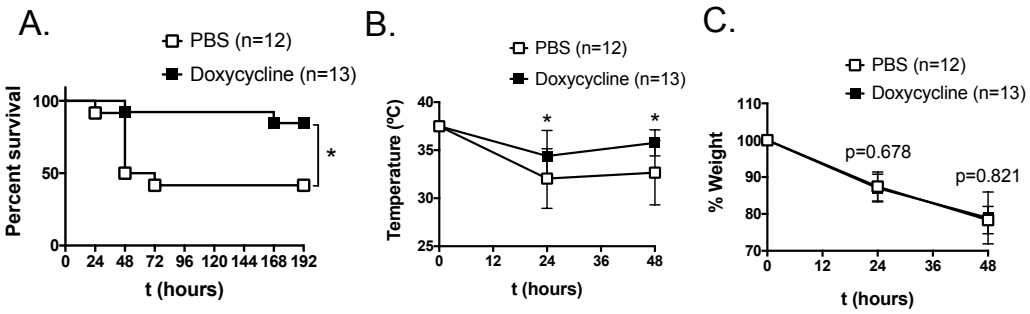

Figure 3-6 - Protective role of doxycycline is independent of its effect on host microbiota. Survival (A), temperature (B), and % body weight (C) after infection of germ-free mice with TetR CamR *E. coli* and treatment with 1.75 µg/g body weight doxycycline (or PBS as a control) at 0, 24 and 48h. Graphs represent pooled data from four independent experiments.

3.4.4 Effect of doxycycline treatment in non-bacterial infection models

Encouraged by the salutary effects of doxycycline in a model of bacterial sepsis, we next tested its effect in non-bacterial infection models. In a mouse model of cerebral malaria induced by *Plasmodium berghei* Anka, no differences were found in survival rates despite reduced percentage of infected red blood cells upon doxycycline treatment (Figure 3-7A), in line with previously reported anti-malarial effects of the drug[31].

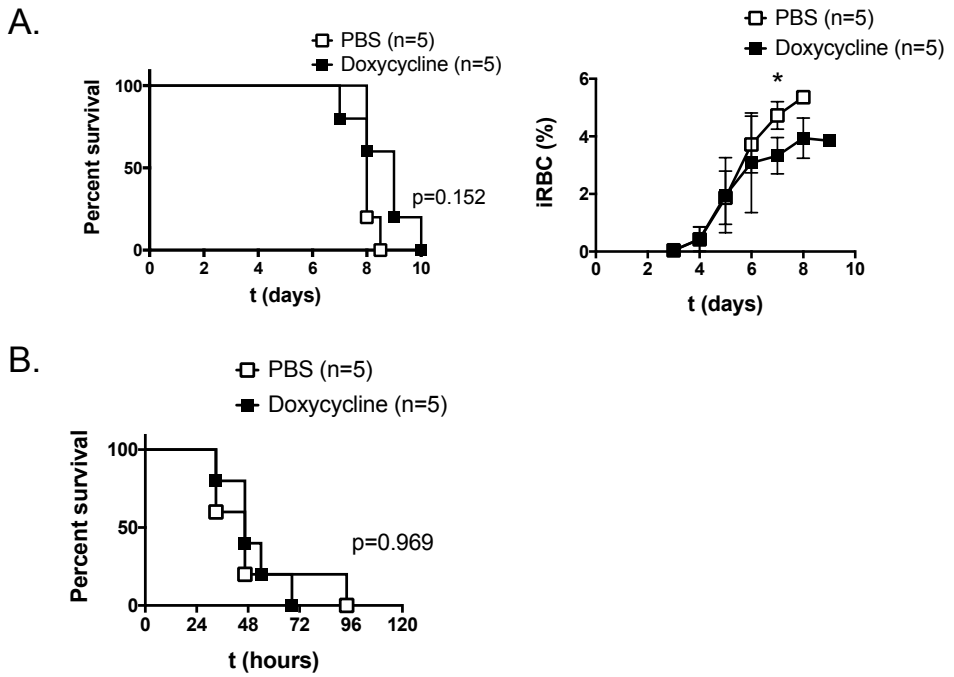

Figure 3-7 - Doxycycline treatment shows no effect in non-bacterial infection models. (A) Survival and parasitemia levels (% infected red blood cells, *iRBC*) in female mice infected with $1x10^5$/mouse *Plamodium berghei* Anka and treated with 1.75 µg/g body weight doxycycline (or PBS as a control) daily from days 0 to 8 post-infection. (B) Survival of female mice infected with $5x10^5$CFU/mouse *Candida albicans* and treated with 1.75 µg/g body weight doxycycline (or PBS as a control) at 0, 24, and 48h. Data were obtained from a single experiment.

In addition, no survival advantage was found in a model of systemic fungal infection induced by intravenous injection of *Candida albicans* (Figure 3-7B). These results suggest doxycycline-induced mechanisms of disease tolerance that are specific for bacterial infections.

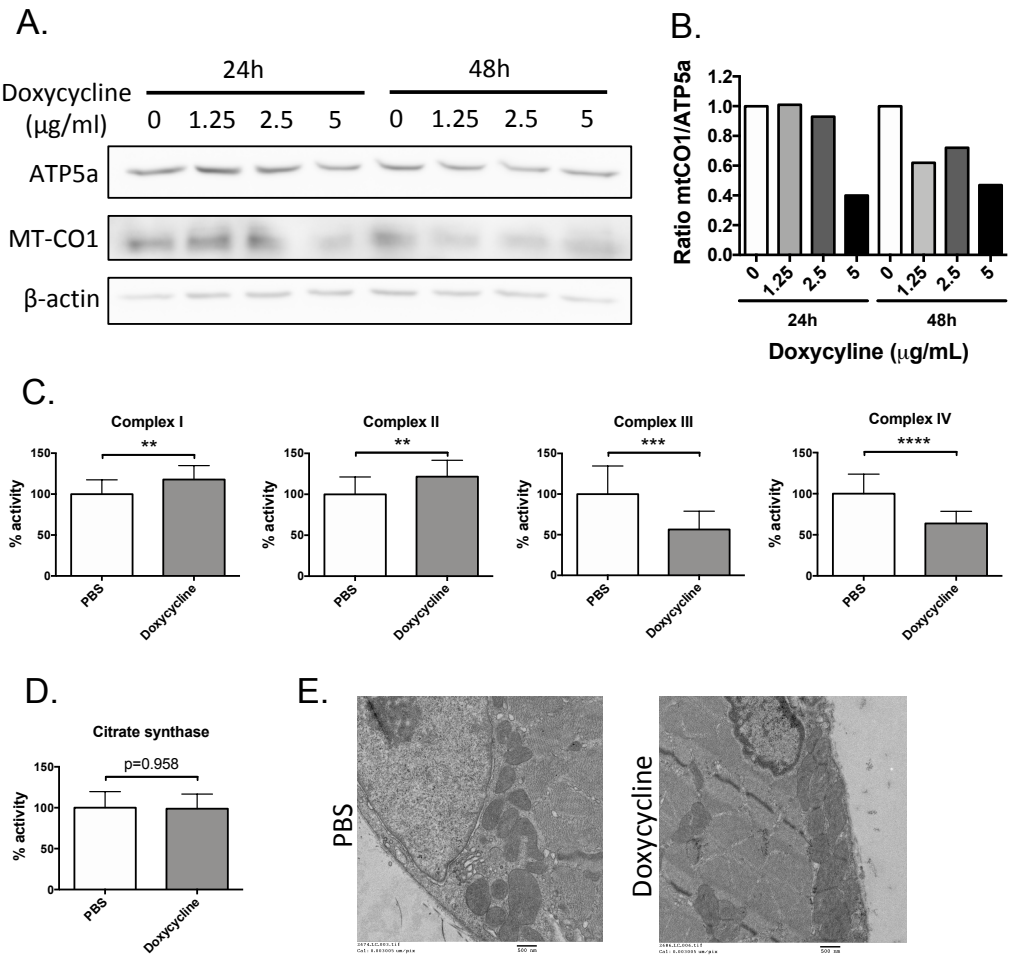

Figure 3-8 - Low-dose doxycycline affects mitochondrial function in vivo.
(A) Immunoblot of HepG2 cells incubated with varying concentrations of doxycycline for 24 or 48h and probed for nuclear (ATP5a) or mitochondrial (MTCO1)-encoded proteins of the ETC. (B) MT-CO1/ATP5a ratio determined by densitometry in (A). (C) Enzymatic activity of the ETC complexes I, II, III, and IV in mouse liver collected 12h after doxycycline treatment. Enzymatic activity is expressed as % of PBS-treated control, normalized for citrate synthase (CS) activity. (D) CS activity (expressed as % of control) in mouse liver collected 12h after PBS or doxycycline treatment. (E) Representative images (n=3-4 mice/group) of transmission electron microscopy in mouse skeletal muscle 24h after PBS or doxycycline treatment. Scale bar = 500

nm. Data in (C) and (D) represent mean±SD of 12 mice/group from two independent experiments.

3.4.5 Doxycycline decreases ETC activity

Doxycycline has been reported to block synthesis of mitochondrial-encoded proteins at relatively high doses across several model organisms, including mammalian cells[32,33]. We used the human hepatocellular carcinoma cell line HepG2 to examine the abundance of proteins of the ETC encoded by the nucleus (ATP5a) or by the mitochondrial DNA (MT-CO1) upon incubation with doxycycline. Even low concentrations of the drug (1.25 to 5 µg/mL) revealed decreased MT-CO1 levels, while ATP5a levels remain unchanged (Figure 3-8A, 3-8B), showing that physiologically relevant doses of doxycycline have an impact in mitochondria.

Remarkably, doxycycline treatment also results in changes in mitochondrial function *in vivo*. By measuring the enzymatic activity of the mitochondrial ETC complexes in mouse liver collected 12h after doxycycline treatment, we found strongly reduced activity of complexes III and IV (54% and 60% of the control, respectively) and a slight activation of complexes I and II (115% and 118% of the control, respectively) (Figure 3-8C). These changes in function were, however, not reflected in impaired mitochondrial integrity as judged by citrate synthase (CS) activity (Figure 3-8D), or in morphology, analyzed by transmission electron microscopy (Figure 3-8E).

We then measured the expression of the major UPR^{mt} markers *Hspd1*, *Atf5*, *Atf4*, and *Clpp* by qPCR in mouse liver 8h after doxycycline treatment (Figure 3-9A). We found no significant differences in mRNA levels of doxycycline-treated mice, except for an up-regulation (1.8x) of *Atf4* (Figure 3-9A). In HepG2 cells, a 24h-incubation with doxycycline followed by staining with mitochondrial specific

probes Mitotracker green and MitoSOX red revealed no differences in the number of mitochondria and a very mild increase in mitochondrial ROS levels, respectively (Figure 3-9B).

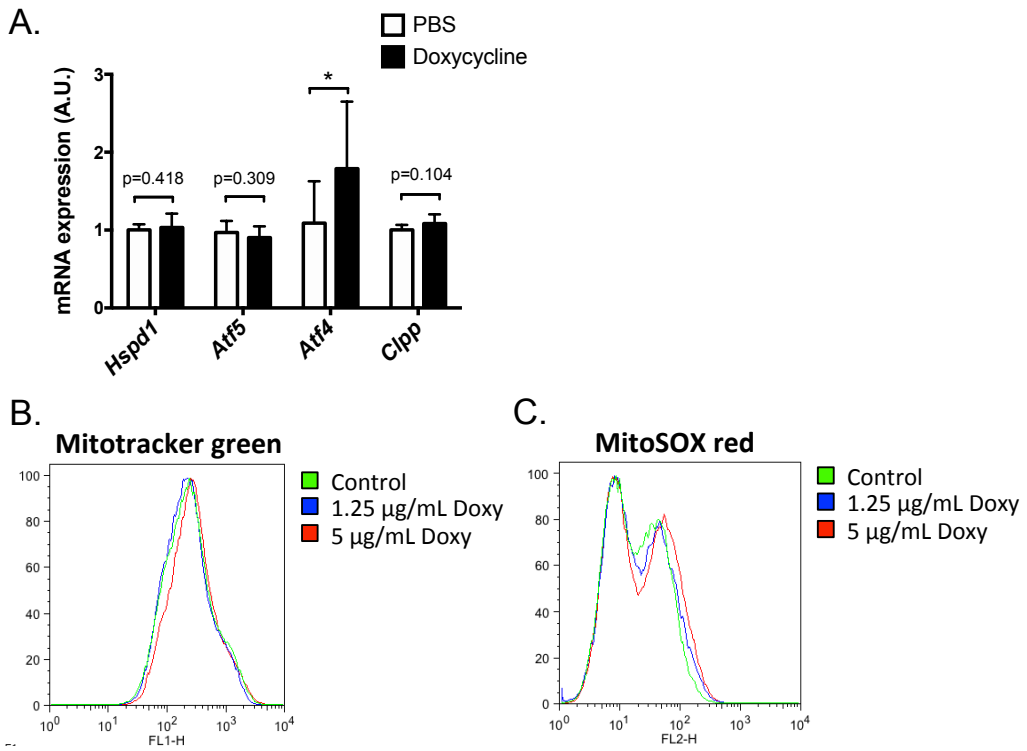

Figure 3-9 - Characterization of the effect of doxycycline treatment in mouse liver and human liver cells.

(A) Expression of the UPR^{mt} genes *Hspd1*, *Atf5*, *Atf4*, and *Clpp* by qPCR analysis in mouse liver collected 8h after injection of 1.75 µg/g body weight doxycycline. Data represent mean±SD of 5 mice/group assayed in triplicate. (B), (C) Mitochondrial content (Mitotracker green, B) and mitochondrial peroxide levels (MitoSOX red, C) in HepG2 cells after incubation for 24h with the indicated concentrations of doxycycline. Representative graphs of two independent experiments.

Taken together, our data indicate that low-dose doxycycline triggers changes in mitochondrial ETC function without compromising mitochondrial integrity, consequently activating disease tolerance mechanisms in a mouse model of bacterial sepsis.

3.5 Discussion

Many drugs have been repurposed for clinical uses that differ from their original application. Some antibiotics, in particular, have been proposed not only to target microorganisms but also to exert immunomodulatory effects of the host, through still poorly defined mechanisms[34]. Very recently, a study brought some mechanistic insight into this idea by showing that administration of aminoglycosides confers protection to viral infections in mice by inducing the expression of antiviral immunity genes[35].

Here, we report increased survival in a mouse model of sepsis upon treatment with doxycycline (Figure 3-1) and chloramphenicol (Figure 3-2), two antibiotics which, despite being structurally unrelated, exert their antimicrobial activity by binding the bacterial ribosome and inhibiting protein translation[36]. Mitochondrial ribosomes are structurally similar to their bacterial counterparts, as explained by the endosymbiont origin of mitochondria, which means that these antibiotics can impact mitochondrial translation and exert effects on the host. In fact, doxycycline and chloramphenicol treatment in *C. elegans* results in an imbalance of the nuclear and mitochondrial-encoded proteins of the ETC, which in turn triggers the UPR^{mt} and results in increased lifespan[16]. In mammals, the mechanistic details and physiological consequences of the administration of these antibiotics are much less clear. Incubation of mammalian cell lines with doxycycline has been shown to induce mitonuclear protein imbalance and up-regulation of UPR^{mt} and ISR markers[16,32,33,37]. In mice, long-term, high-dose doxycycline treatment also inhibits mitochondrial translation with concomitant decrease in oxygen consumption and ATP production[32] – whether these changes have an impact on host fitness has not been addressed so far.

As with other antibiotics, tetracyclines have been attributed anti-inflammatory effects for a long time[38]. These relate mostly with inhibition of metalloproteinase (MMP) activity, as tetracyclines chelate zinc ions essential for enzyme function. In animal models of ischemia-reperfusion, doxycycline-induced MMP inhibition has been associated with improved tissue function[39]. In human sepsis patients,

however, a study using a sub-antimicrobial dose of doxycycline (comparable to the one used in our study) revealed no effect in MMP activity[40]. While the drug was well tolerated in patients, the effect of this treatment in sepsis outcome was not reported[40].

In this study, we propose that tetracyclines, chloramphenicol, and possibly other drugs targeting mitochondrial function have protective roles that go beyond their previously described anti-inflammatory effects. In spite of the well-known role of mitochondria in defining pro- or anti-inflammatory programs in immune cells[41], we found no effect in cytokine secretion after treating BMDMs with doxycycline (Figure 3-5). Instead, doxycycline seems to act through mechanisms that preserve viability and function of parenchymal cells, as judged by the lower levels of damage in liver, lung, and kidney, despite similar pathogen loads (Figure 3-4).

In an attempt to link drug-induced mitochondrial perturbations and disease tolerance, we focused on the liver – due to its importance in metabolic control of disease progression – and carried out a series of *in vivo* and *in vitro* assays (Figures 3-8 and 3-9). Low-dose doxycycline treatment has no effect in mitochondrial morphology or proliferation within the first 24h of treatment, and causes a marginal increasing in ROS levels. We found little evidence for the induction of the transcriptional programs UPR^{mt} and ISR, supported only by a mild up-regulation of *Atf4*, in line with previous studies[37]. Nevertheless, we noticed a severe reduction in the activity of the ETC complexes III and IV in mouse liver. Inhibition of the ETC activity has been found beneficial in the context of type II diabetes and obesity, an effect attributed to an optimized fuel usage and changes in energy expenditure and thermoregulation[19,42]. These changes are likely advantageous in the context of sepsis, where mitochondrial dysfunction and metabolic reprogramming account for severe tissue damage[3]. More detailed molecular mechanisms are required, however, to fully understand how changes in mitochondrial function can be harnessed for therapeutic use in sepsis.

We have tested the effect of doxycycline in fungal and protozoan infection models and found no substantial improvement under the tested conditions (Figure 3-7). This is not completely surprising in light of the effects of the drug in mitochondrial function. Invasion by different pathogens requires different immune responses and result in a multitude of tissue damage patterns and metabolic changes. Considering the importance of mitochondria in determining infection outcomes, the protective effect of doxycycline is likely context-dependent. This is illustrated by a recent study reporting dramatic differences in glucose metabolism when comparing viral and bacterial infections[43]. In this study, a therapeutic intervention that optimizes glucose usage and confers disease tolerance to viral infections, results in decreased survival in bacterial infections[43].

In summary, our work suggests that mitoribosome-targeting drugs constitute a valuable tool to induce disease tolerance to bacterial infections, by minimizing tissue damage independently of the pathogen load. Not only does this open new therapeutic perspectives in promoting tissue function during sepsis, it also provides a window to explore the beneficial effects of mild perturbations in core cellular functions. Our laboratory has previously reported a protective effect of DNA damage responses in sepsis[6], which we now extend to mitochondrial stress responses. Other organelles and cellular functions can potentially be harnessed for similar therapeutic interventions, and while we could not find benefits in other organelle-targeting drugs (which can be attributed to inadequate dosage or unexpected side effects), our results encourage the pursuit of such benefits in future studies.

3.6 Acknowledgements

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Chapter 4

The role of liver metabolism in the pathophysiology and outcomes of sepsis

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Author contributions

I was responsible for planning, executing and analyzing data from all experiments in this chapter. André Barros and Elsa Seixas provided assistance in the *in vivo* work. Liver RNA-Seq was performed at Genecore (EMBL Genomics Core Facilities), under the supervision of Vladimir Benes. André Barros performed the analysis of RNA-Seq data. Liver metabolomics was executed at the Metabolomics Facility from the Vienna Biocenter Core Facilities under the supervision of Thomas Köcher. Hyon-Seung Yi and Minho Shong provided the *Crif1*$^{lox/lox}$ mouse strain. Luís Ferreira Moita supervised the project and reviewed this chapter.

4.1 Abstract

Sepsis is characterized by a dysregulated host response to infection, which encompasses excessive inflammation and perturbed metabolic function. The liver plays a key role in integrating several metabolic pathways and controlling organismal bioenergetics. However, the contribution of this organ for sepsis pathology is still poorly understood.

Here, we used a combination of transcriptomics, metabolomics, and pharmacological studies to better characterize liver function in a mouse model of bacterial sepsis. We found that both fatty acid oxidation and response to glucocorticoids are impaired in mouse liver from early time-points of infection. Maintenance of either pathway is essential but not sufficient for survival to infection.

We further demonstrate that doxycycline, a drug that impairs mitochondrial respiration, improves both lipid metabolism and glucocorticoid signaling. This result can be replicated by other mild perturbations in the electron transport chain, namely treatment with phenformin and partial depletion of the mitochondrial protein CRIF1.

4.2 Introduction

Infections inflict profound changes in host metabolism. In immune cells, induction of glycolysis provides both ATP and substrates to be used in anabolic pathways. These in turn support the synthesis of lipids, proteins, and nucleotides necessary for cell proliferation and effector immune responses[1]. Parenchymal cells, in contrast, undergo metabolic rewiring that favors catabolic, energy-saving programs[2].

Sepsis, in particular, is characterized by severe perturbations in cellular function and inter-organ communication, together with dramatic metabolic alterations in

all organs[3]. The action of stress hormones, such as catecholamines and glucocorticoids, as well as cytokines, such as TNFα, increases lipolysis in adipocytes, flooding the blood with energy-rich free fatty acids (FFA)[4,5]. In the skeletal muscle, increased protein catabolism provides amino acids to support immune cell proliferation, while providing an alternative source of energy[6].

The liver integrates several metabolic pathways and suffers dramatic functional changes during sepsis, which have been the focus of recent attention and controversy. In mice, sepsis progresses with severe hypoglycemia, posing an important threat to host survival. As a consequence, maintenance of hepatic gluconeogenesis has been deemed essential to avoid lethal hypoglycemia[7]. In parallel, production of ketone bodies in the liver provides a valuable source of energy when glucose is not readily available[8].

Fatty acid oxidation (FAO) in the mitochondria constitutes a major source of cellular energy, by providing substrates both for ketogenesis and oxidative phosphorylation. FAO is regulated by the transcription factor PPARα, which, upon binding of FFAs, translocates to the nucleus and induces the expression of genes involved in lipid transport and oxidation in the mitochondria[9]. The role of hepatic lipid metabolism in sepsis has only very recently begun to be addressed[8,10,11], and the molecular mechanisms that regulate metabolic adaptation during infection and prevent energetic failure remain elusive.

We have previously shown that doxycycline, a drug that induces an imbalance between nuclear and mitochondrial-encoded proteins of the mitochondrial ETC, increases survival in a mouse model of sepsis. Doxycycline-treated mice have lower levels of tissue damage, especially in the liver. This is accompanied by changes in ETC activity in the liver, thus raising the hypothesis that transient changes in mitochondrial function promote metabolic adaptation that induces tissue tolerance.

Herein, we used a combination of transcriptomics, metabolomics, and pharmacological studies to better understand liver pathophysiology during bacterial sepsis in mice. We found that deficient FAO and response to glucocorticoids are hallmarks of sepsis pathology and correlate with poor

infection outcomes. In addition, we observed improved FAO and glucocorticoid signaling in livers of doxycycline-treated mice. Remarkably, different strategies to reduce ETC activity, namely phenformin treatment and conditional, liver-specific knockdown of *Crif1* are also capable of inducing disease tolerance against bacterial sepsis.

4.3 Methods

4.3.1 Experimental Models

4.3.1.1 Mice

All animal studies were performed in accordance with Portuguese regulations and approved by the Instituto Gulbenkian de Ciência ethics committee (reference A002.2015) and DGAV. C57BL/6J mice were obtained from Instituto Gulbenkian de Ciência. Male mice, 8 to 12 weeks old were used, except if otherwise stated. *Crif1*$^{lox/lox}$ mice[12] were obtained from M. Shong (Chungnam National University School of Medicine, Daejeon, South Korea). Mice were maintained under specific pathogen-free (SPF) 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.

4.3.1.2 Bacterial cultures

See section 3.3.1.4.

4.3.2 Method details

4.3.2.1 *E. coli*-induced sepsis model and drug treatments

See section 3.3.2.1 for details on *E. coli* infection model and doxycycline treatment.

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 and at the indicated concentrations: phenformin (vehicle: PBS, dose 100 µg/g); etomoxir (vehicle: PBS, dose 15 µg/g); mifepristone (vehicle: 100% DMSO, dose 30 µg/g); CP868388 (vehicle: 7% DMSO, dose 3 µg/g), dexamethasone (vehicle: PBS, dose 5 µg/g). Octanoic acid was dispersed in 0.5% methylcellulose and supplemented by oral gavage (200 µL/mouse) at 2, 8, 24, and 48h post-infection.

4.3.2.2 Liver-specific gene editing with adeno-associated virus (AAV)

AAV serotype 8 constructs (details in the Appendix) 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.

4.3.2.3 Colony Forming Units assay

See section 3.3.2.3.

4.3.2.4 Biochemical assays in mouse serum

See section 3.3.2.4.

4.3.2.5 Histopathology

See section 3.3.2.5.

4.3.2.6 Immunoblotting

See section 3.3.2.7.

4.3.2.7 RNA extraction and qPCR

See section 3.3.2.8.

4.3.2.8 Liver 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[13]. Sequences were then aligned against the *Mus musculus* genome version 97, with the annotation file for the genome version 97, both obtained through the website of Ensembl. The alignment was performed using STAR[14], 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)[15]. Data from raw counts were normalized through a Regularized Log Transformation (rlog) to create the Principal Component Analysis plot and Heatmaps[15]. The log2FC provided by the standard DESeq2 model was shrunk using the 'ashr' algorithm[16]. 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.

4.3.2.9 Liver metabolomics

Sample preparation

Liver samples (30-80 mg) were weighed and homogenized in 500 µL ice-cold methanol:acetonitrile:H2O (2:2:1, v/v) using a TissueLyser II. Homogenates were incubated at -80 °C for 4h and centrifuged at 20000 x*g* 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 x*g* 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 x*g*, 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 x 2.1 mm; 5 µm) or an RP-column (Waters, ACQUITY UPLC HSS T3 150 x 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 21 min to 60% B followed by 5 min hold at 80% B in HILIC (25 mM ABC) and 90% B in RP (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). Ionization potential was set to +3.5/-3.0 kV, the sheet gas flow was set to 20, and an auxiliary gas flow of 5 was used. Samples were analyzed in a randomized fashion and QC samples were additionally measured in confirmation mode to obtain additional MS/MS spectra for identification. Obtained data sets were processed by compound discoverer 3.0 (Thermo Fisher Scientific). Compound annotation was conducted by searching the mzCloud database with a mass accuracy of 3 ppm for precursor masses and 10 ppm for fragment ion masses as well as ChemSpider with a mass accuracy of 3 ppm using BioCyc, Human Metabolome Database, KEGG, MassBank and MetaboLights as databases.

Targeted metabolomics

Each sample was injected onto a SeQuant ZIC-pHILIC HPLC column (Merck, 100 x 2.1 mm; 5 µm) operated with an Ultimate 3000 HPLC system (Dionex, Thermo Fisher Scientific) at a flow rate of 100 µL/min and directly coupled to a TSQ Quantiva mass spectrometer (Thermo Fisher Scientific).

For all transitions, the optimal collision energy was defined by analyzing pure metabolite standards. Chromatograms were manually interpreted using TraceFinder (Thermo Fisher Scientific), validating experimental retention times with the respective quality controls of the pure substances. In HILIC, a 15 min gradient (A: 95% ACN, 5% 10 mM aqueous ammonium acetate; B: 50% ACN 50% 10 mM aqueous ammonium acetate) was used for separation. The following transitions have been used for quantitation in the negative ion mode (2.8 kV): stearic acid 283.1 *m/z* → 265.1 *m/z* and palmitic acid 255.1 *m/z* → 227.1 and in the positive ion mode (3.2 kV): stearoylcarnitine 428.1 *m/z* → 85

m/z, palmitoylcarnitine 400.1 *m/z* → 85 *m/z*, myristoylcarnitine 372.1 *m/z* → 85 *m/z* and lauroylcarnitine 344.1 *m/z* → 85 *m/z*.

4.3.2.10 Electron transport chain (ETC) complex activity

See section 3.3.2.9.

4.3.2.11 Quantification and statistical analysis

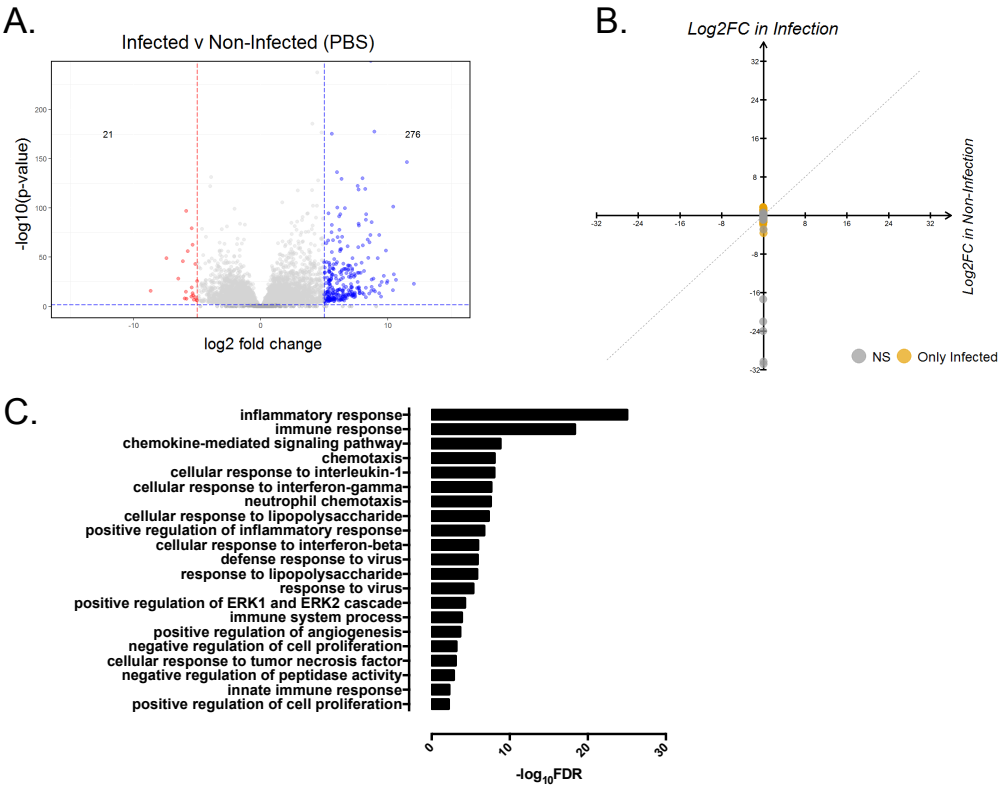
Mantel-Cox test was used for survival curve analysis. For infections with *E. coli*, mice with no changes in body temperature and weight within the first 24h (temperature >35ºC and body weight >95%) were excluded from the analysis. Mann-Whitney test was used for pairwise comparisons and two-way ANOVA 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: $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***); $p < 0.0001$ (****).

4.4 Results

4.4.1 Fatty acid oxidation and response to glucocorticoids are essential for sepsis outcomes

To begin the characterization of liver physiology during sepsis and assess possible liver-specific disease tolerance programs, we performed bulk RNA-Seq in mouse liver 8h after *E. coli* infection with and without doxycycline treatment. By comparing infected and non-infected, PBS-treated mice, we observed the

expected up-regulation of a high number of genes associated with an inflammatory response (Figure 4-1A, 4-1C).



D.

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

Figure 4-1 - Bulk RNA-Seq in mouse liver 8h after infection and doxycycline treatment. (A) Volcano plot with differential expression of genes in infected and non-infected, PBS-treated mice from RNA-Seq analysis in the liver. Numbers indicate genes with log2 fold change <-5 or >5 and $p<0.05$. (B) Scatter plot of genes affected by doxycycline treatment in infected versus non-infected groups. Yellow dots indicate genes differentially expressed in infected mice ($p<0.05$); gray dots indicate non-statistically significant genes ($p\geq0.05$). (C) Top GO_BP annotation of genes up-regulated during infection (PBS-treated mice), log2 fold change>5; $p<0.05$. (D) Gene functional clustering of down-regulated genes upon doxycycline treatment (infected groups), log2 fold change <-1, $p<0.05$.

Surprisingly, when comparing PBS and doxycycline treated mice in the absence of infection we found no statistically significant differential gene expression (Figure 4-1B). In *E. coli*-infected mice, doxycycline treatment results in a discrete number of up- and down-regulated genes compared to PBS-treated controls (Figure 4-1B). We then used the DAVID bioinformatics database[17,18] to perform a gene functional classification of differentially expressed genes in infected, doxycycline treated mice. We found a single significant cluster, which relates to decreased production of collagen upon doxycycline treatment (Figure 4-1D). Collagen is a well-known marker of liver fibrosis and changes in collagen metabolism have been associated with disease severity in sepsis[19]. These findings, together with serology and pathology data (described in Chapter 3) further support the notion that doxycycline helps limiting liver damage from very early time-points of infection.

As we did not find substantial doxycycline-induced transcriptional changes that could sufficiently explain the protective effects of the drug, we then investigated the liver metabolic profiles in the presence of doxycycline and infection, given the centrality of liver function and metabolic changes during sepsis. To this end, we used an untargeted metabolomics approach to study metabolic changes in the liver 8h after infection.

Analysis of the top up- and down-regulated metabolites showed a very pronounced accumulation of acylcarnitines and glucocorticoids in the liver of infected mice (Figure 4-2).

While glucocorticoids have an important role in modulating both inflammation and metabolism during infection, the abnormal acylcarnitine profile is indicative of a defective import and oxidation of fatty acids in mitochondria, which may be a cause of liver failure and metabolic collapse during sepsis.

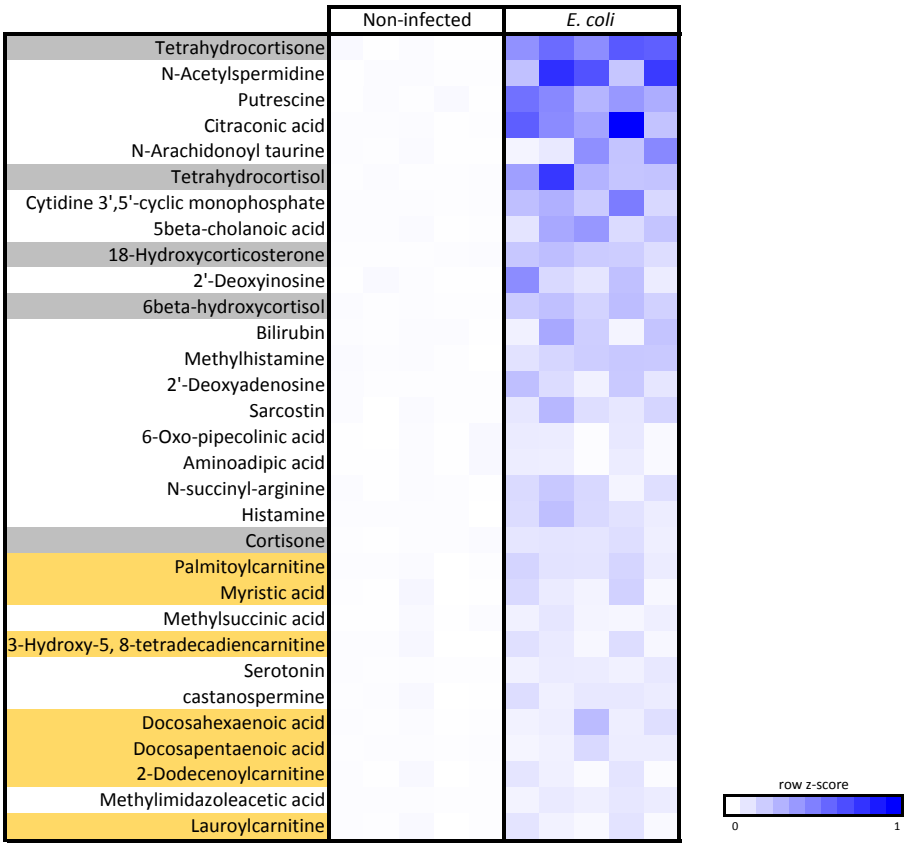


Figure 4-2 - Untargeted metabolomics analysis in mouse liver 8h after infection.
Top up-regulated metabolites in liver of infected mice ($p<0.05$). Glucocorticoids are highlighted in gray and metabolites involved in fatty acid oxidation are highlighted in yellow.

To further validate and investigate the identified FAO signature, we then measured mRNA levels of *Ppara*, a master regulator of fatty acid oxidation (FAO), and several of its transcriptional targets, including *Cpt1*, *Cpt2* and *Slc25a20*, responsible for free fatty acid (FFA) import into the mitochondria, at 8 and 24h after infection. We found a decreased expression of all of the analyzed targets upon infection, which was maintained at least for the first 24h (Figure 4-3A), thus highlighting lipid metabolic dysfunction as a hallmark of sepsis pathophysiology. To causally test the importance of FAO for sepsis progression and survival, we treated mice with etomoxir[20], a frequently used inhibitor of CPT1a – the enzyme that catalyzes the conversion of free fatty acids to

acylcarnitines before their import across the outer mitochondrial membrane. We observed a significant increase in mortality as compared to non-treated controls (Figure 4-3B).

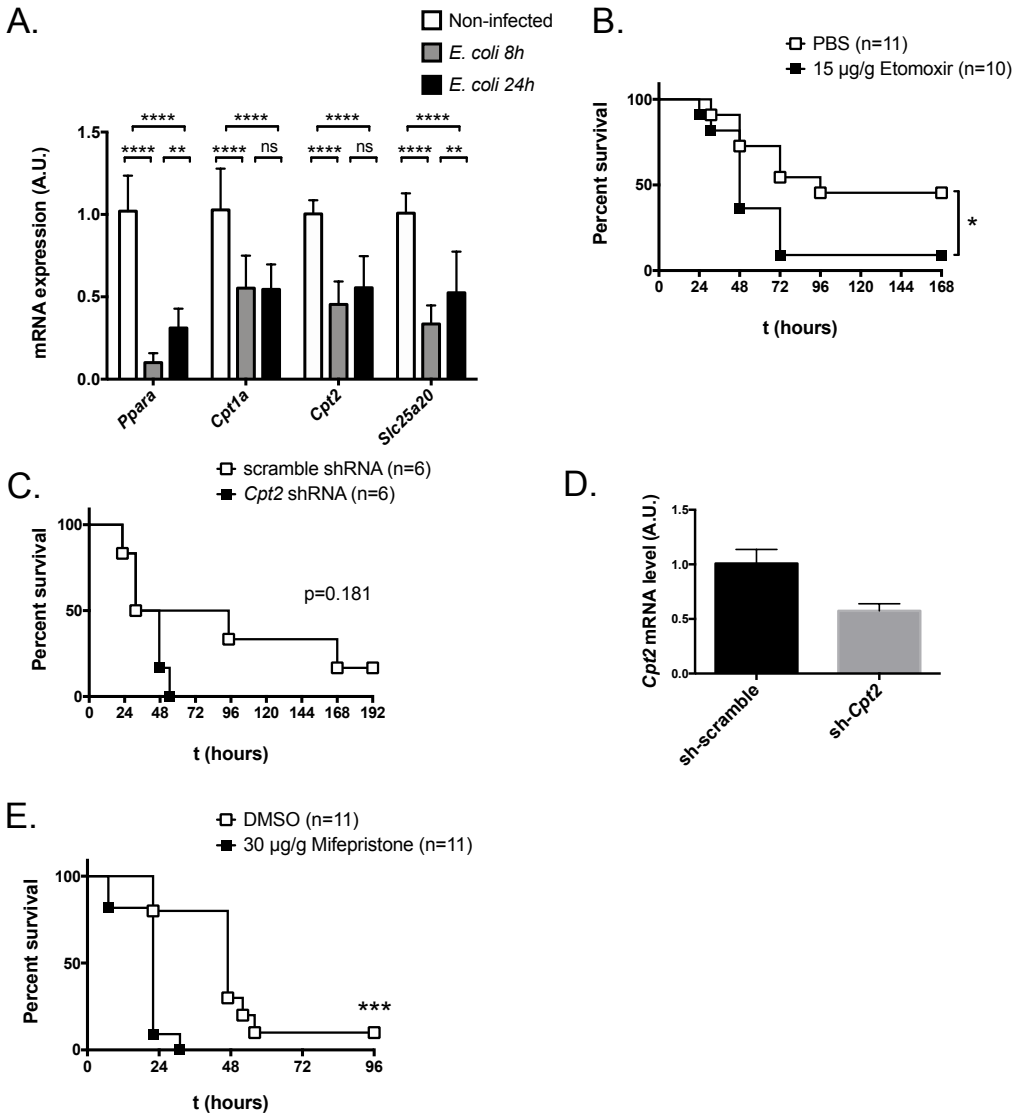


Figure 4-3 - Fatty acid oxidation and response to glucocorticoids are essential for survival to sepsis.

(A) Expression of *Ppara* and several of its targets by qPCR in mouse liver at 8h and 24h post-infection. Data represent mean±SD of 5 mice assayed in triplicate. (B) Survival after infection of C57BL/6J mice with $3x10^8$ CFU/mouse TetR CamR *E. coli* and treatment with 15 µg/g body

weight etomoxir. (C) Survival of *Cpt2*-depleted mice upon infection with *E. coli*. Mice were injected with AAV8 expressing *Cpt2* shRNA (or scramble shRNA as a control) 7 days before infection. (D) *Cpt2* mRNA levels in mouse liver 7 days after injection of AAV8 *Cpt2* shRNA. (E) Survival of *E. coli*-infected mice treated with 30 μg/g body weight mifepristone. (B) and (E) represent pooled data from two independent experiments. (C) and (D) represent a single experiment.

We also attempted to reduce the expression of *Cpt*2, another gene involved in the mitochondrial carnitine shuttle, by injecting a liver-specific adeno-associated virus (AAV) serotype 8 encoding *Cpt2* shRNA. We observed a slight, non-statistically significant increase in mortality (Figure 4-3C), which might be explained by the modest decrease in *Cpt2* mRNA levels following *Cpt2* shRNA treatment (Figure 4-3D).

Finally, we tested the contribution of glucocorticoid response to the outcomes of sepsis by treating mice with mifepristone, a glucocorticoid receptor (GR) antagonist. Mifepristone-treated mice succumbed to infection remarkably faster than the controls (Figure 4-3E), suggesting that glucocorticoid response is essential to maintain tissue function in face of the excessive inflammation and metabolic dysregulation that characterize the initial stages of sepsis.
Taken together, these results demonstrate that both FAO and response to glucocorticoids are necessary for recovery from sepsis, and that impairment of these pathways correlates with worse infection outcomes.

4.4.2 Doxycycline improves both FAO and response to glucocorticoids

Having identified FAO and response to glucocorticoids as necessary for survival in sepsis, we next investigated the role of doxycycline on these pathways. Considering the lack of transcriptional signatures in mouse liver upon doxycycline treatment (Figure 4-1B), we turned to an HPLC-MS analysis to identify several acylcarnitine and FFA species in mouse liver 8h after infection

with or without doxycycline treatment. We found that doxycycline partially corrects the accumulation of acylcarnitines and FFA upon infection (Figure 4-4), suggesting that the drug might be facilitating fatty acid transport into the mitochondria and/or boosting FAO. Levels of the ketone body β-hydroxybutyric acid are also slightly elevated in infected, doxycycline-treated mice, suggesting that improved FAO may result in higher production of ketone bodies to support energy generation.

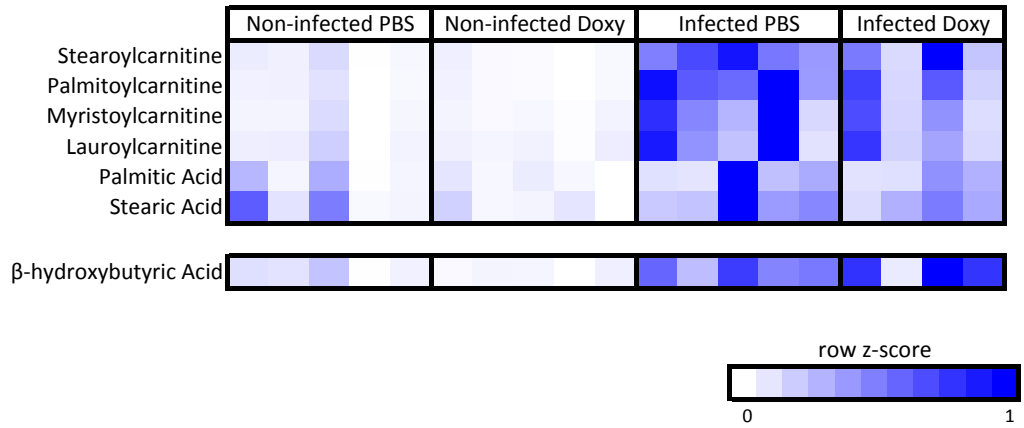


Figure 4-4 - Doxycycline improves fatty acid oxidation in the liver.
HPLC-MS analysis of FAO metabolites and β-hydroxybutyric acid in mouse liver 8h after infection and/or doxycycline treatment. Each square represents one mouse.

Remarkably, several attempts to correct mitochondrial FFA transport in liver by overexpressing several genes involved in this pathway, alone or in combination, using an AAV8 construct driven by liver thyroid hormone-binding globulin (TBG) promoter, conferred no survival advantage during infection (Figure 4-5A, 4-5B, 4-5C).

Additionally, an attempt to bypass the carnitine shuttle by orally supplementing mice with octanoic acid, a medium-chain FFA (C8:0) that freely diffuses into the mitochondria without the need for conjugation with carnitine, also did not consistently increase survival in mice (Figure 4-5D). Likewise, treatment with

the PPARα agonist CP868388 at the time of infection was not enough to improve survival (Figure 4-5E).

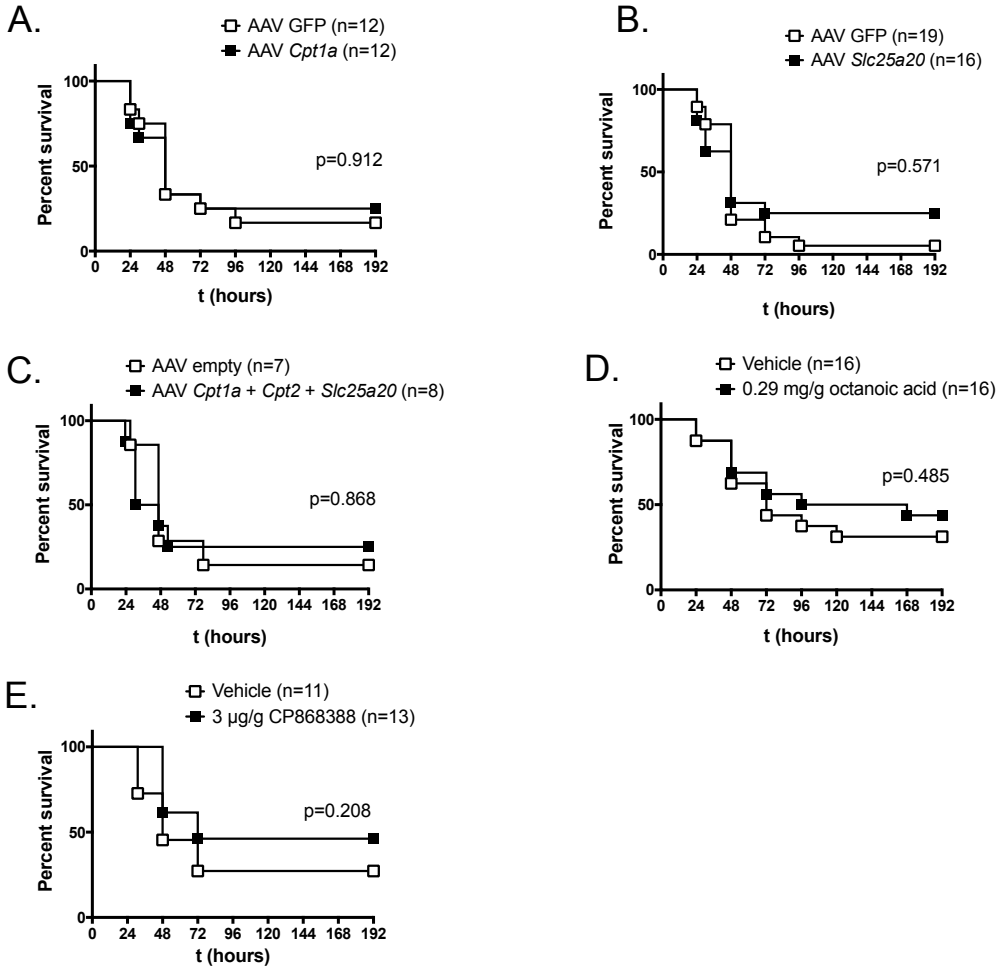


Figure 4-5 - Fatty acid oxidation gain-of-function experiments.
(A, B, C) Survival of C57BL/6J mice after infection with *E. coli*. Mice were injected with AAV8-TBG expressing SLC25A20 (A), CPT1a (B) or a combination of CPT1a+CPT2+SLC25A20 (1:1:1) (C) one week before infection. (D) Survival of mice after infection with *E. coli* and treatment with octanoic acid by oral gavage at 2, 8, 24, and 48h after infection. (E) Survival of mice after infection with *E. coli* and treatment with CP868388 (PPARα agonist) by IP injection at the time of infection. (A) and (D) represent pooled data from three independent experiments. (B) and (F) represent pooled data from two independent experiments. (C) represents data from a single experiment.

We then addressed the impact of doxycycline treatment in glucocorticoid response. Levels of glucocorticoids were sharply elevated in the liver in response to infection (Figure 4-6A). We found similar levels of the major glucocorticoids in the liver of PBS and doxycycline-treated mice, with marginal increase of some species (such as corticosterone and 18-hydroxycorticosterone) in doxycycline-treated groups (Figure 4-6A). Sepsis is characterized by resistance to glucocorticoids, which means that even high levels of these species, both endogenously produced and therapeutically administered, fail to produce anti-inflammatory and metabolic modulator effects[21]. In fact, pre-treatment of mice with the synthetic glucocorticoid dexamethasone before infection with *E. coli* failed to increase survival (Figure 4-6B).

To explore glucocorticoid signaling in this context, we probed liver extracts collected 8h after infection with or without doxycycline treatment for total glucocorticoid receptor (GR) and two markers of GR activation: Ser226 and Ser211. Infected, PBS-treated mice showed markedly reduced levels of all analyzed forms, supporting the idea of glucocorticoid resistance (Figure 4-6C). Interestingly, doxycycline treatment increases phospo-Ser226 and, to a lesser extent, phospho-Ser211, both in the presence and absence of infection, while total GR levels are also moderately increased in doxycycline-treated, *E. coli*-infected mice (Figure 4-6C). These observations indicate that doxycycline substantially increases the activation of the GR in response to glucocorticoids, which is normally blunted in sepsis[22].

Taken together, our data indicate that, while FAO and glucocorticoid signaling are necessary for survival in sepsis, independently rescuing these pathways is not sufficient to guarantee recovery from infection. Notably, doxycycline treatment is able to correct both FAO and glucocorticoid signaling, which may contribute substantially for tissue function maintenance and metabolism during sepsis.

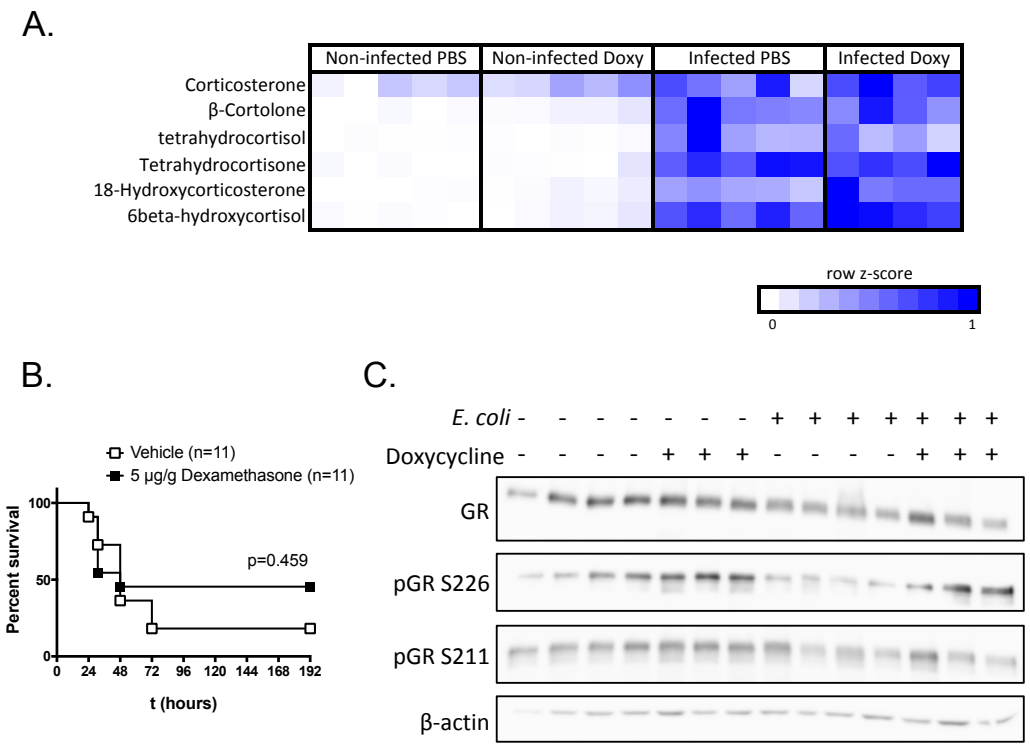


Figure 4-6 - Glucocorticoid signaling in sepsis pathology.
(A) HPLC-MS analysis of glucocorticoids in mouse liver 8h after infection and/or doxycycline treatment. Each square represents one mouse. (B) Survival of mice after infection with *E. coli* and treatment with dexamethasone (GR agonist). Pooled data from two independent experiments. (C) Protein levels of total and phosphorylated glucocorticoid receptor (GR) in mouse liver at 8h after infection or doxycycline treatment. Each lane represents one mouse.

4.4.3 Mild, transient perturbations in mitochondrial function are associated with increased survival in sepsis

As discussed in Chapter 3, doxycycline-induced disease tolerance is associated with a perturbation in mitochondrial function, namely decreased activity of mitochondrial ETC complexes III and IV. To address the question of whether the induction of disease tolerance by ETC required general or specific perturbations of ETC complexes, we next focused on the biguanide antidiabetic drug phenformin, previously reported to inhibit complex I of the ETC[23].

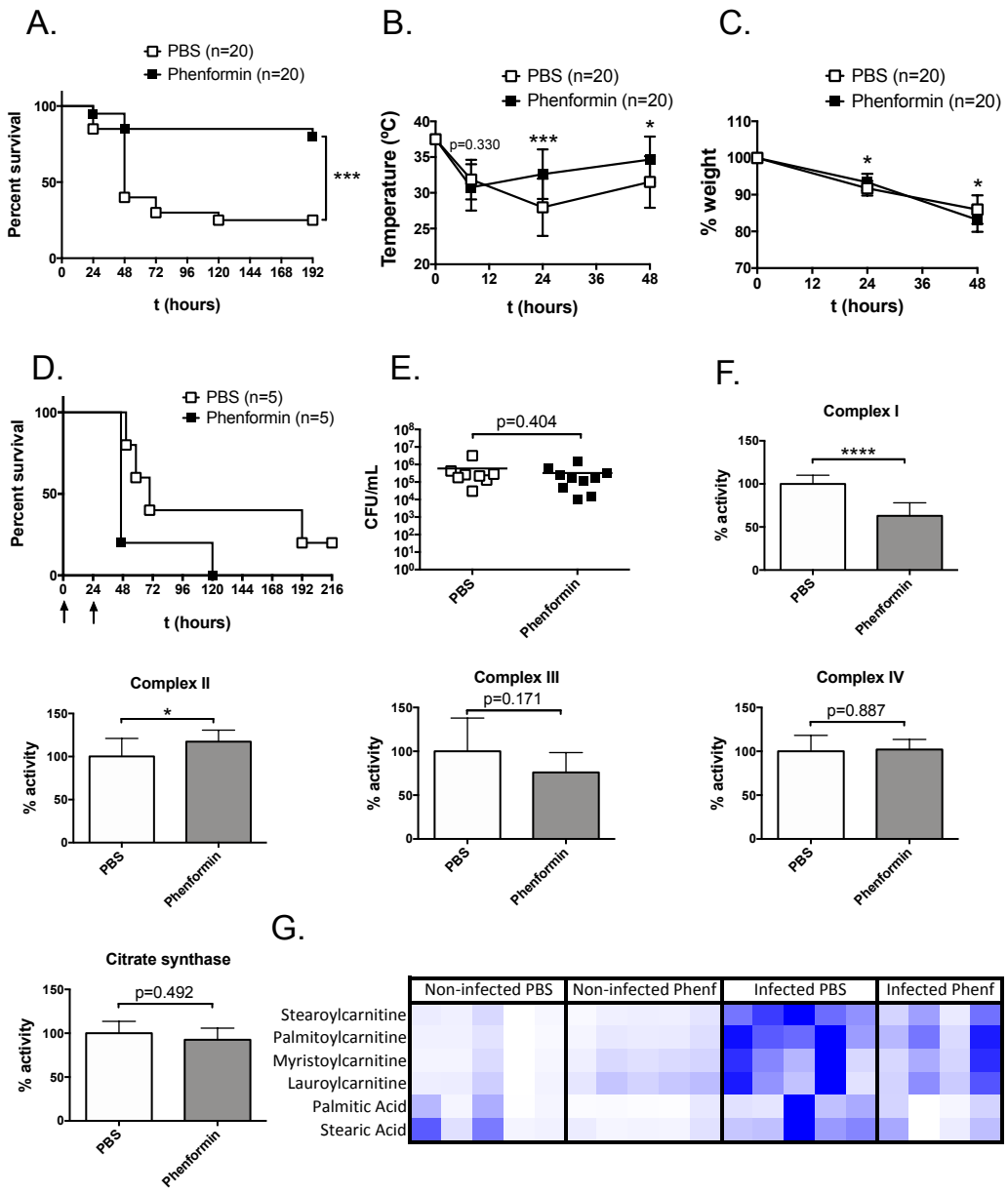


Figure 4-7 - Phenformin, a complex I inhibitor, induces disease tolerance in sepsis. Survival (A), rectal temperature (B) and % initial body weight (C) after infection of mice with *E. coli* and treatment with a single injection of 100 µg/g body weight phenformin at the time of infection. (D) Survival after infection of mice with *E. coli* and treatment with 100 µg/g body weight phenformin at 0 and 24 h (E) Bacterial loads in blood of PBS and phenformin-treated mice, 24 h after E. coli infection. (F) Enzymatic activity of the ETC complexes 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) HPLC-MS analysis of FAO metabolites in mouse liver 8h after infection and/or phenformin treatment. Each square represents one mouse. (A, B, C) represent pooled data from four independent experiments. (D) represents data from a single experiment. (E) represents pooled data from two independent experiments. Squares represent individual mice; bars indicate the mean. (F) represents mean±SD of 5 mice/group from a single experiment.

We found that injection of a single dose of phenformin at the time of *E. coli* infection resulted in remarkable survival advantage (Figure 4-7A) and improvement in body temperature control (Figure 4-7B), with modest effects in body weight (Figure 4-7C). Interestingly, administration of two doses of phenformin, separated by 24h, lead to increase mortality (Figure 4-7D) in striking contrast to a single administration.

Similarly to doxycycline, phenformin confers protection to sepsis by inducing disease tolerance mechanisms, as bacterial load in mouse blood collected 24h after infection shows no difference between PBS- and phenformin-treated mice (Figure 4-7E).

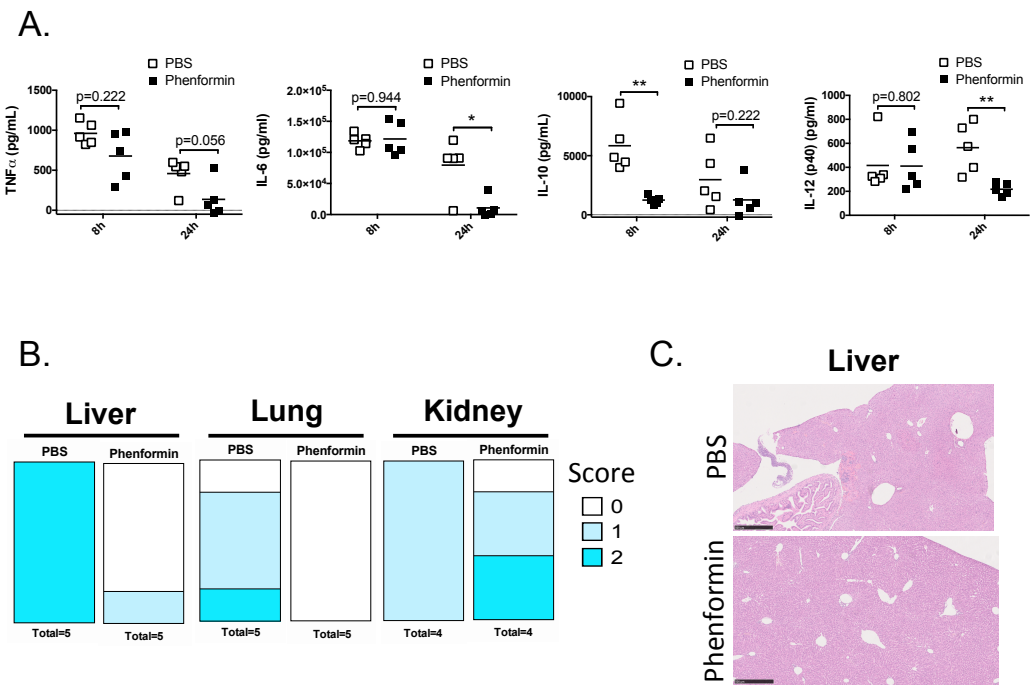


Figure 4-8 - Phenformin treatment reduces inflammation and tissue damage in sepsis.

(A) TNFα, IL-6, IL-10, and IL-12/IL23(p40) levels in mouse serum at the indicated time-points after infection. Squares represent individual mice and bars indicate the mean; data obtained from a single experiment. (B, C) Organ damage score (B) and representative images of Hematoxylin-Eosin stained slides (C) from tissues collected 24 h after infection. Score 0 = no lesions; 1 = very mild; 2 = mild lesions (n=4-5 mice/group).

We then evaluated the enzymatic activity of ETC complexes in mouse liver collected 12h after phenformin treatment and concluded that complex I activity is dramatically decreased (61% of the control), while complexes II, III and IV, as well as CS show little to no changes (Figure 4-7F). Phenformin treatment also results in decreased accumulation of acylcarnitines and FFAs in mouse liver 8h after infection (Figure 4-7G).

Additionally, phenformin-treated mice present lower levels of the major pro-inflammatory cytokines at 24h, but not at 8h post-infection; and decreased IL-10 levels at 8h (Figure 4-8A). Phenformin treatment also results in lower scores of tissue damage 24h after infection (Figure 4-8B, 4-8C).

As we observed a protective effect of phenformin treatment in a single injection, but not in repeated administrations, we hypothesized that mild, but not stringent, perturbations in mitochondrial function could be mechanistically linked to disease tolerance. To causally prove this hypothesis, we next used a genetic system to induce transient perturbations in mitochondrial function. CRIF1 is a mitoribosomal protein with an important role in the assembly and function of ETC complexes[24]. A previous study linked tissue-specific *Crif1* knockout with reduced ETC activity, resulting in systemic metabolic benefits[25]. Prompted by the doxycycline-induced improvement in liver pathology and metabolism during sepsis, we decided to test the impact of targeted liver deletion of CRIF1 in sepsis. To this end, we took advantage of the AAV8-TBG strategy to obtain fast and efficient gene editing in mouse liver. Intravenous injection of Cre-expressing AAV8 in homozygous *Crif1*$^{lox/lox}$ or heterozygous *Crif1*$^{lox/-}$ mice led to high protein levels of Cre recombinase after 7 days, accompanied by reduced CRIF1 protein and mRNA levels (Figure 4-9A). Mice homozygous for the flox allele suffered almost full deletion in mRNA (98%) and protein levels, whereas heterozygous

mice showed a 30% decrease in mRNA and only modest decrease in protein levels (Figure 4-9A).

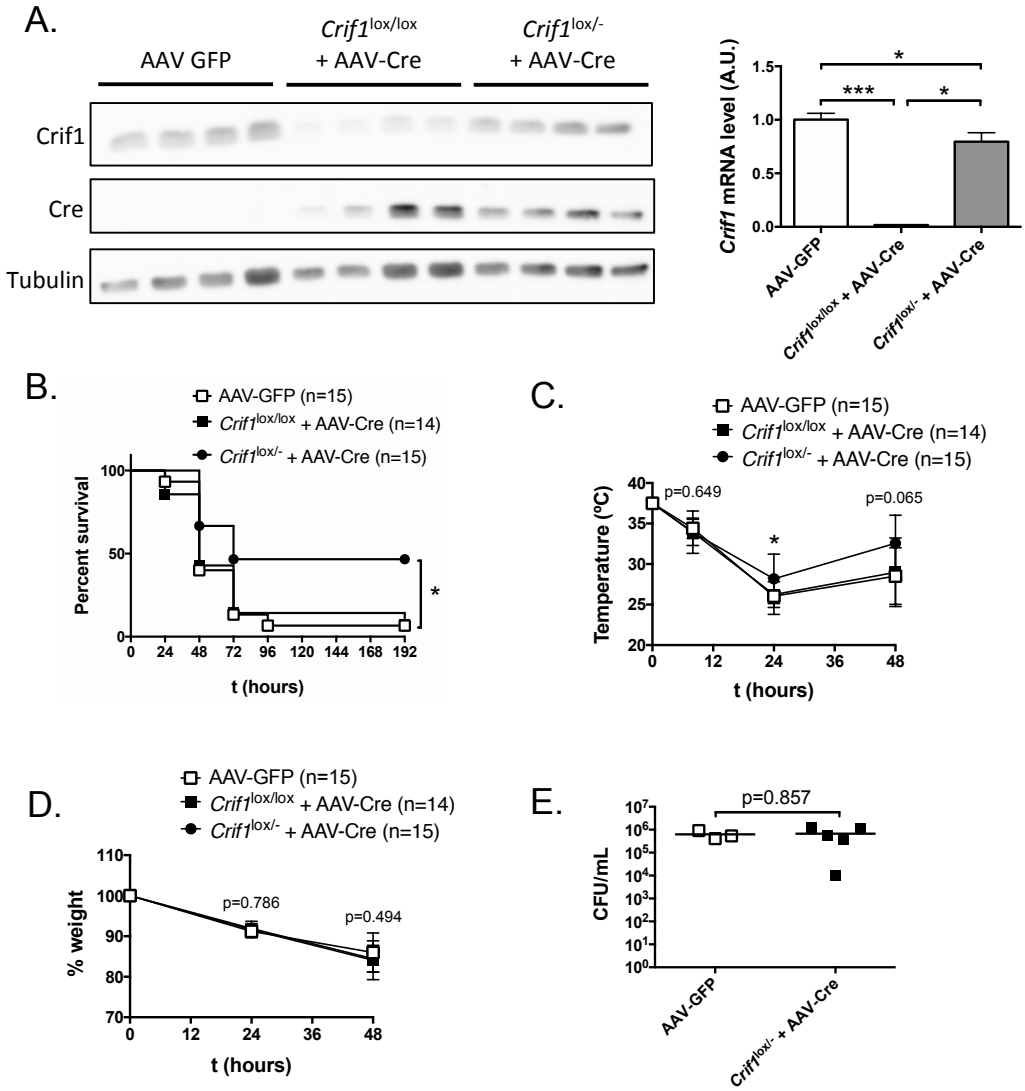


Figure 4-9 - Genetically induced mild perturbation in mitochondrial function is protective against sepsis.

(A) CRIF1 and CRE recombinase protein levels and *Crif1* mRNA levels in *Crif1*$^{lox/lox}$ or *Crif1*$^{lox/-}$ mice 7 days after injection of liver-specific AAV8-TBG expressing Cre recombinase (or GFP as a control). (B, C, D) Survival (B), rectal temperature (C), and % initial body weight (D) 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). (E) Bacterial loads in *Crif1*$^{lox/-}$ mouse blood 24h after infection. Data in (A) represents mean±SD of 4 mice/group assayed in

triplicate. (B, C, D) represent pooled data from three independent experiments. (E) represents data from a single experiment. Squares represent individual mice; bars indicate the mean.

Strikingly, *E. coli* infection performed 7 days after AAV-Cre injection resulted in increased survival of *Crif1*$^{lox/-}$ mice but not of *Crif1*$^{lox/lox}$ littermates (Figure 4-9B), thus supporting the notion of a beneficial role for mild, transient mitochondrial perturbations. *Crif1*$^{lox/-}$ mice showed less severe hypothermia (Figure 4-9C), but no differences in body weight when compared to *Crif1*$^{lox/lox}$ mice and mice injected with a control AAV-GFP vector (Figure 4-9D). Bacterial loads in blood of *Crif1* depleted mice were similar to the controls (Figure 4-9E), supporting the notion of disease tolerance induced my mild mitochondrial perturbations.

In summary, we have proven 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.

4.5 Discussion

Infected animals engage a dormancy state that includes sickness behaviors (anorexia, social isolation, loss of libido, among others) and metabolic changes that support the energetic costs of immunity. Infection-induced dormant states are largely beneficial and have been associated with disease tolerance and tissue protection programs[2]. In sepsis, however, metabolic responses to infection are exacerbated and result both in the accumulation of toxic species and in energetic failure[3].

In this study, we focused on the role of liver pathophysiology in sepsis and found two major pathways that are impaired from early stages of infection: fatty acid metabolism and response to glucocorticoids.

Very recently, two different studies addressed the role of fatty acid oxidation and PPARα during sepsis, with somewhat contradictory results[10,11]. Paumelle et al.

reported increased *Ppara* expression in *E. coli*-infected mouse liver and decreased survival and ketogenesis in liver-specific *Ppara*-knockout mice[10]. In critically ill patients, however, *Ppara* levels were found reduced, when compared to healthy controls[10]. A different study, by Ganeshan et al., reported impaired PPARα signaling and FAO upon LPS injection in mice, and claimed that ketogenesis is primarily sustained by amino acid catabolism[11]. Our results show decreased expression of *Ppara* and several of its targets, together with accumulation of FFAs and acylcarnitines in the liver of infected mice, in accordance with the latter study[11]. Moreover, we demonstrated that FAO is essential for survival to sepsis, as shown by the fact that treatment with the CPT1 inhibitor etomoxir decreases survival. These findings are corroborated by clinical studies showing that high circulating levels of acylcarnitines[26] and low expression of *Ppara*[27] correlate with poor sepsis prognosis.

Furthermore, we attempted to correct the deficient transport of fatty acids to the mitochondria. Overexpression of genes of the carnitine shuttle conferred no survival advantage, nor did supplementation with medium chain fatty acids. Even though these FFAs can freely diffuse into the mitochondria without the need for a specialized transport, they will most likely accumulate due to defects in downstream steps of FAO. This is problematic not only from a bioenergetics viewpoint, but also because high levels of lipids may cause steatosis, which perturbs tissue function[28].

Another promising approach to correct defects on lipid metabolism in sepsis is the stimulation of its master regulator PPARα. In a previous study, the clinically available PPARα agonist fenofibrate was reported to increase survival in a mouse model of sepsis induced by *Salmonella typhimurium*[29]. However, this protective effect was attributed to an increased recruitment of neutrophils to the site of infection, while the effects of fenofibrate in systemic lipid metabolism were not analyzed. In our study, we focused instead on the more potent and specific PPARα agonist CP868388[30] and found no survival advantage under the tested conditions. Together, our data suggest that, although FAO is essential for

survival to sepsis, strategies that specifically aim to correct lipid metabolism are not sufficient to guarantee recovery from infection.

A different aspect of liver pathophysiology of sepsis arising from our study is an impaired glucocorticoid signaling. Infection induces the release of high levels of glucocorticoids from the adrenal glands, which dampen the immune response and alter metabolism by increasing gluconeogenesis, lipolysis and FAO. Upon binding of its ligands, GR undergoes a complex signaling pathway that involves dimerization, post-translational modifications, and translocation to the nucleus, where it acts as a transcription factor[31,32]. We noticed decreased protein levels of total and phosphorylated GR in the liver, despite extremely high levels of glucocorticoids, supporting the idea that glucocorticoid resistance contributes to sepsis pathology[21]. Similarly to the results obtained in FAO, treatment with a GR antagonist resulted in worsened infection outcomes, but no improvement was found upon treatment with a GR agonist. Interestingly, PPARα has been shown to block GR activation[33], which may help explaining why therapies that individually target one of these pathways are not sufficient to increase survival.

Interestingly, both FAO and GR signaling were found improved during infection in doxycycline-treated mice. We have previously discussed that doxycycline-induced disease tolerance was associated with inhibition of ETC activity in mouse liver. These results suggest that perturbations in ETC activity trigger a stress response that induces both GR signaling and a metabolic shift from glucose oxidation to FAO. In the context of sepsis, improved oxidation of lipid species is likely advantageous, as it supports ketone body synthesis, which can then be used as a source of energy by a variety of tissues. It is noteworthy, however, that oxidation of ketone bodies or acetyl-CoA generated in β-oxidation still requires a functional ETC, which partly explains why mild, but not stringent ETC perturbations result in tissue protection.
The link between ETC inhibition and metabolic benefits, namely increased FAO, has been established in previous studies, most notably in the context of

obesity[25,34,35]. The exact mechanisms that explain this metabolic shift remain elusive, but they are believed to involve the secretion of mitochondrial stress response molecules (known as mitokines), such as GDF15[25] or FGF21[36]. While our transcriptomics analysis has not identified an up-regulation of any of such known molecules in the liver of doxycycline-treated mice, this should be more carefully addressed in future studies. In fact, we cannot rule out the presence of still unknown mitokines, or the secretion of known factors, including GDF15 and FGF21, by other organs, such as the skeletal muscle.

Finally, we provided evidence that mild ETC perturbations, both pharmacological and genetically induced, are sufficient to confer disease tolerance to bacterial sepsis. Phenformin, an antidiabetic drug with no reported antibiotic effect, shows a remarkable protective effect when administered in a single dose, which largely overlaps with doxycycline in all analyzed aspects of mouse pathology and metabolism. The fact that phenformin and doxycycline target different complexes of the ETC – phenformin exclusively inhibits complex I activity, whereas doxycycline targets complexes III and IV – suggests that induction of disease tolerance is a consequence of the overall output of ETC activity rather than the activity of specific complexes.

The comparison between phenformin and other drugs with a similar mechanism of action provides further evidence for the link between ETC inhibition and disease tolerance. When compared to metformin, the most widely used member of the biguanide family, phenformin shows a much more potent inhibitory effect of complex I of the ETC[23]. Interestingly, metformin treatment resulted in no survival advantage (Chapter 3), whereas a similar dose of phenformin is able to rescue ~80% of infected mice.

The results obtained with liver-specific CRIF1 knockdown provide not only a causal link between mitochondrial stress and disease tolerance, but also insights into tissue-specificity, magnitude and duration of the underlying perturbations. As previously discussed, protective perturbations in ETC function need to be mild, so as not to completely compromise energy generation – this is

likely the case of the full CRIF1 knockout, which showed no survival advantage. Partial CRIF1 knockdown, obtained in heterozygous mice, resulted in ~50% survival, a more modest effect compared to the one obtained with drug treatments. Reasons for this may include the need for systemic, rather than liver-specific, perturbations in mitochondrial function – the contribution of other organs was not considered in this study. Furthermore, the temporal activation of mitochondrial stress responses should be considered. While drug treatments provide a transient perturbation of mitochondrial function induced at the time of infection, genetic manipulation results in gradual decrease of CRIF1 levels over the 7 days that preceded infection – this more prolonged stress condition may have effects on organismal physiology that are hard to control[37].

Taken together, our results prove that hepatic metabolism – in particular, a functional mitochondrial FAO – and stress responses – such as glucocorticoid signaling – are essential for survival to sepsis and can be harnessed for therapeutic purposes by mild perturbations in ETC activity.

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Chapter 5

Doxycycline-induced lung repair

Parts of this chapter have been submitted for publication:

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Author contributions

I was responsible for planning, executing and analyzing data from all experiments in this chapter. André Barros and Elsa Seixas provided assistance in the *in vivo* work. Lung RNA-Seq was performed at Genecore (EMBL Genomics Core Facilities), under the supervision of Vladimir Benes. André Barros performed the analysis of RNA-Seq data. Luís Ferreira Moita supervised the project and reviewed this chapter.

5.1 Abstract

Lung dysfunction is not only highly prevalent in sepsis but also a major cause of long-term morbidity. Doxycycline treatment increases survival in a mouse model of bacterial sepsis, which is accompanied by reduced lung damage. In this study, we set out to identify lung-specific tissue protective mechanisms of doxycycline. We report that intra-tracheal administration is sufficient to reproduce the protective effect of the drug. Doxycycline accelerates infection resolution in a model of Influenza, which relies specifically in lung damage. Transcriptomics analysis of doxycycline-treated mouse lung shows an infection-independent reduction in the expression of lung stem cell genes. Our data suggests that doxycycline triggers stem cell differentiation and lung remodeling, which may help sustain tissue architecture and function during infection. Future studies providing more detailed mechanisms on doxycycline-induced lung protection may help shed light upon the complex dynamics of progenitor lineage expansion and proliferation in the lung in the context of infection.

5.2 Introduction

Impaired lung function is one of the most prominent consequences of sepsis. Endothelial leakage and compromised alveolar barriers lead to accumulation of fluid in the alveoli, which in turn impairs gaseous exchanges and decreases lung compliance[1]. This set of symptoms, also known as acute respiratory distress syndrome (ARDS), can be further aggravated by mechanical ventilation of hospital-admitted patients and constitutes a major cause of multi-organ failure and long-term sequelae of sepsis[2,3].

Following the initial inflammatory insult, the respiratory epithelium engages an anti-inflammatory and resolving program involving a number of lipid-derived molecules, such as resolvins[4]. In parallel, regeneration of tissue architecture

involves cellular expansion and differentiation, in which the lung stem cells – or basal cells – play a key role[5].

The airway epithelium is a highly complex and heterogeneous population comprising over 40 different cell types[6]. These cells populate niches that differ dramatically from each other. Proximal regions have abundant ciliated and secretory (club) cells, while distal alveoli are mainly composed of surfactant producing (type I) and gas exchanging (type II) pneumocytes[6]. In the mouse respiratory tract, basal cells are thought to occupy mainly the trachea and proximal bronchi, although residual populations of distal stem cells have also been described[7]. In recent years, a few studies addressed the role of basal cells in lung remodeling upon chemical injury or viral infection, and found that these cells rapidly proliferate and differentiate into ciliated and club cells in proximal regions[8,9], or alveolar cells in the distal respiratory tract[7].

In this study, we aimed to investigate the effect of doxycycline treatment in mouse lung physiology during infection. We have previously described that systemic doxycycline treatment induces disease tolerance in a model of bacterial sepsis, accompanied by reduced lung damage. Here, we show that local administration of doxycycline to the lung recapitulates this protective effect. Moreover, transcriptional analysis of doxycycline-treated mouse lung shows remarkable changes in markers of epithelium differentiation independently of infection.

Our data suggests an unexpected role for doxycycline in promoting lung regeneration through a complex dynamics of cell reprogramming, which encourages more detailed mechanistic studies in the future.

5.3 Methods

5.3.1 Experimental models

5.3.1.1 Mice

All animal studies were performed in accordance with Portuguese regulations and approved by the Instituto Gulbenkian de Ciência ethics committee (reference A002.2015) and DGAV. C57BL/6J mice were obtained from Instituto Gulbenkian de Ciência. Male mice, 8 to 12 weeks old were used, except if otherwise stated. Krt6a-DTR mice[7] were obtained from W. Xian and F. McKeon (Harvard Medical School, Boston, MA, USA). Mice were maintained under specific pathogen-free (SPF) 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.

5.3.1.2 Bacterial cultures

See section 3.3.1.4.

5.3.2 Method details

5.3.2.1 *E. coli*-induced sepsis model and drug treatments

See section 3.3.2.1 for details on *E. coli* infection model.
Doxycycline hyclate was dissolved in PBS and injected intraperitoneally (200 µL/mouse) at 1.75 µg/g body weight 0, 24 and 48h after infection.
Intra-tracheal administration of doxycycline was performed as previously described[10]. 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 2h later.

5.3.2.2 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[11] 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.

5.3.2.3 Immunofluorescence

Mouse lungs were collected at the indicated time-points after doxycycline administration and immediately inflated and immersed in 10% buffered formalin. Samples were then embedded in paraffin and 3-µm longitudinal sections were made, such that the trachea and both lungs were included in a single section. Antigen retrieval was performed in 10 mM sodium citrate + 0.5% Tween 20, pH 6.0 for 5 min at 95ºC. Samples were then blocked in PBS containing 3% BSA + 2% normal goat serum and 0.025% Triton X100 for 1h at RT. To reduce unspecific binding to endogenous mouse antibodies, samples were incubated with Goat F(ab) polyclonal Secondary Antibody to Mouse IgG 1:1000 for 1h at RT. Sections were then stained with a mouse cytokeratin 6A antibody 1:100, overnight at 4ºC followed by Andy Fluor 647 Goat Anti-Mouse IgG 1:1000 for 1h at RT. Nuclear staining was made with DAPI. Samples were finally mounted on glass coverslips using Fluoromount-G. Tile-scan images of the whole lung were acquired using a Nikon High Content Screening microscope based on Nikon Ti, equipped with a 20x 0.75 NA objective, Quad DAPI and Quad Cy5 filtersets,

coupled with an Andor Zyla 4.2 sCMOS 4.2Mpx camera and controlled through Nikon NIS Elements.

5.3.2.4 Ablation of KRT6a^{+} cells

Mice hemizygous for the human diphtheria toxin receptor inserted in the *Krt6a* locus (Krt6a-DTR) and wild-type littermates were given an intra-tracheal administration of 12 ng/g diphtheria toxin (DT) according to the procedure described in 5.3.2.1. Experiments were performed 4 days after DT administration.

5.3.2.5 RNA extraction

For lung RNA-Seq, both lungs were harvested, cleaned from fat and bronchi and homogenized in 1 mL Trizol using a TissueLyser II. Homogenates were centrifuged at 20000 x*g* for 3 min at 4°C and 500 µL supernatant were used for RNA extraction, according to the procedure described in section 3.3.2.8.

5.3.2.6 Lung RNA-Seq

See section 4.3.2.8.

5.3.2.7 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 24h (temperature >35ºC and body weight >95%) were excluded from the analysis.
Mann-Whitney test was used for pairwise 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 (****).

5.4 Results

5.4.1 Doxycycline improves lung pathology during infection

As discussed in Chapter 3, doxycycline induces disease tolerance in a mouse model of bacterial sepsis, resulting in decreased damage of several tissues, most notably the lung and the liver. Guided by the improvement of lung pathology upon doxycycline treatment, we decided to explore the effect of local administration of the drug to the lung. We found that a single intra-tracheal administration of doxycycline 2h before *E. coli* infection results in significant increase in survival (Figure 5-1A), supporting a central role of the lung in sepsis outcome.

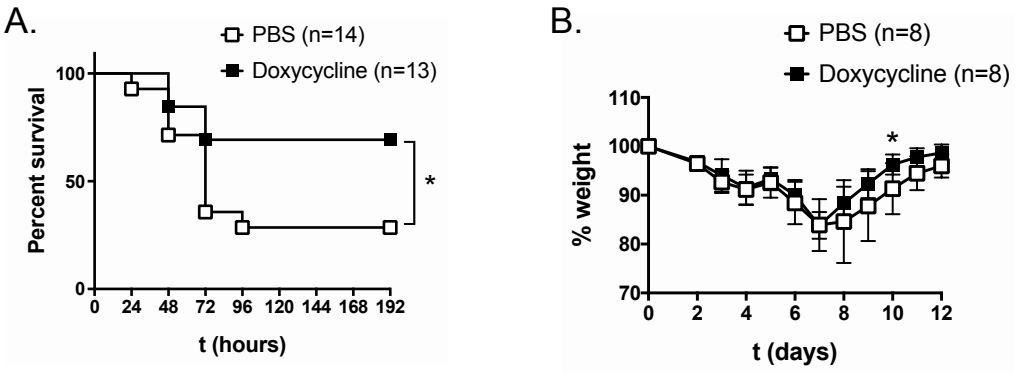

Figure 5-1 - Doxycycline improves lung pathology.
(A) Survival of mice after intra-tracheal delivery of 1.75 µg/g body weight doxycycline (or PBS as a control) followed by infection with *E. coli* 2h later. Pooled data from two independent experiments. (B) Percentage of initial weight in mice infected with a sublethal (100 pfu/mouse) dose of Influenza A PR/8 and treated with 1.75 µg/g body weight doxycycline (or PBS as a control) at days 4, 5, and 6 post-infection. Data is representative from two independent experiments.

To further explore the protective role of doxycycline in the lung, we used a model of Influenza infection, which causes lung damage and inflammation. To that end, mice were intranasally challenged with a sublethal dose (100 pfu/mouse) of Influenza PR/8 and doxycycline was injected intraperitoneally at days 4, 5, and 6 post-infection, the period at which viral loads are higher and lung lesions become apparent[9].

We observed that doxycycline-treated mice lose body weight at a similar pace to the controls, but recover faster from day 5 post-infection onwards, with a significant difference in body weight at day 10 post-infection (Figure 5-1B). These results suggest that doxycycline might be triggering tissue repair mechanisms at the level of the lung, which result in faster recovery.

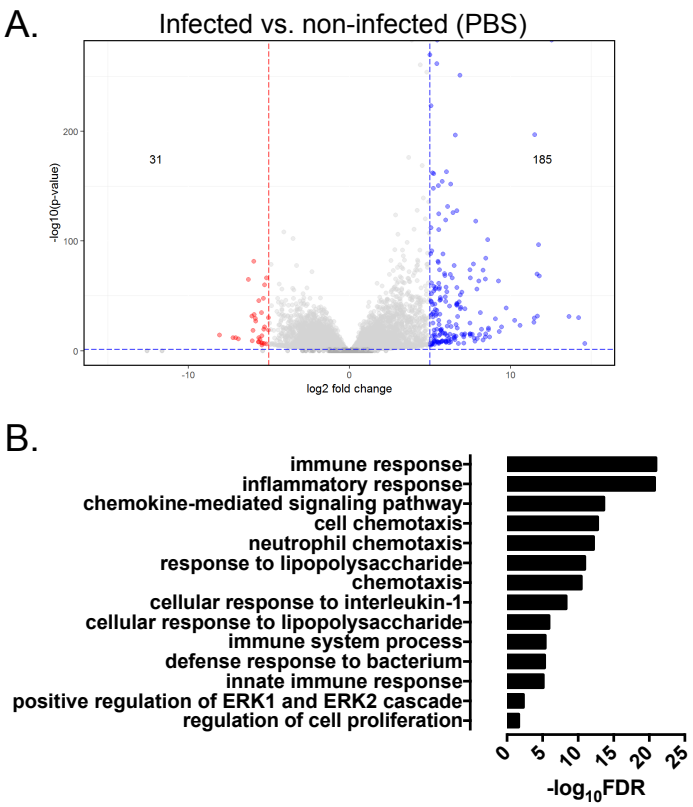

Figure 5-2 - Bulk RNA-Seq in lungs of *E. coli*-infected mice.

(A) Volcano plot with differential expression of genes in *E. coli*-infected and non-infected, PBS-treated mice from bulk RNA-Seq analysis in the lung at 12 h. Numbers indicate genes with log2 fold change <-5 or >5 and $p<0.05$. (B) Top GO_BP annotation of genes up-regulated during infection (PBS-treated mice), log2 fold change>5; $p<0.05$.

5.4.2 Doxycycline reprograms lung basal cells

To further investigate possible lung-specific mechanisms leading to epithelial repair, we performed bulk RNA-Seq in mouse lung 12h after infection with *E. coli* and intraperitoneal injection of doxycycline, and compared with non-infected and PBS-treated controls. As expected, a high number of genes were found up-regulated upon infection (Figure 5-2A), mostly related to an acute inflammatory response (Figure 5-2B).

Doxycycline treatment did not change the expression of the majority of these genes, but instead resulted in the strongly reduced expression of a group of genes in both infected and non-infected groups (Figure 5-3A, 5-3B), suggesting that drug-induced changes are independent of the infection. Functional clustering analysis[12,13] in non-infected, doxycycline-treated mice showed a remarkable similarity in the function of down-regulated genes, with 60% of the genes clustering in pathways related to keratinization and epithelium differentiation (Figure 5-3C, 5-3D).
In particular, the basal cell markers *Krt6a* and *Krt6b* are severely down-regulated (Figure 5-3D), suggesting that doxycycline might be driving differentiation of lung progenitor cells[14], leading to a more effective repair of the lung epithelium.

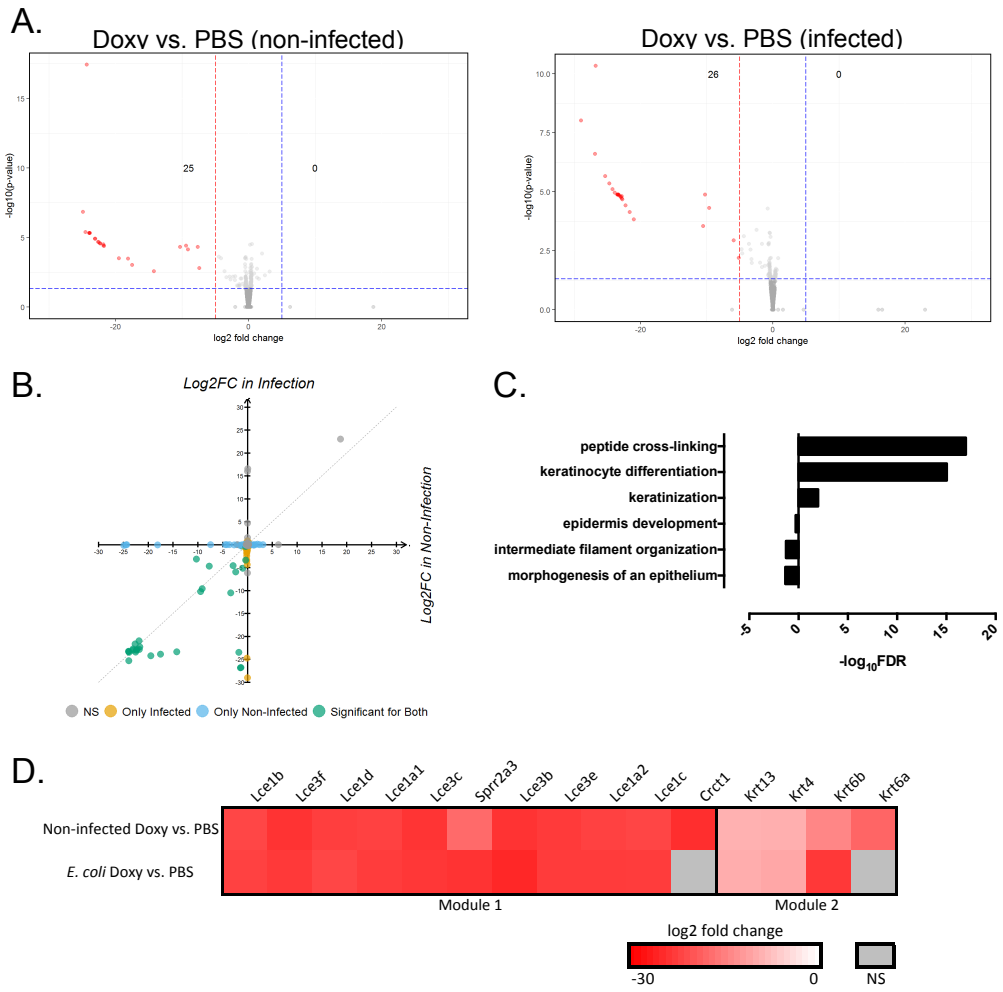

Figure 5-3 - Doxycycline reprograms lung basal cells.
(A) Volcano plots of doxycycline versus PBS-treated mice in the absence or presence of infection. Numbers indicate genes with log2 fold change <-5 or >5 and p<0.05. (B) Scatter plot of genes affected by doxycycline treatment in infected versus non-infected groups. Yellow dots indicate genes differentially expressed in infected mice (p<0.05); blue dots indicate genes differentially expressed in non-infected mice (p<0.05); green dots indicate genes differentially expressed in both conditions (p<0.05); gray dots indicate non-statistically significant genes (p≥0.05). (C) Top GO_BP annotation of genes down-regulated with doxycycline treatment (non-infected mice), log2 fold change<-5; p<0.05. (D) Heat maps of genes affected by doxycycline treatment in non-infected mice (log2 fold change <-5; p<0.05), after clustering with DAVID 'Gene Functional Classification'.

To validate and further investigate the effect of doxycycline in basal cell marker expression, we performed qPCR in the lung and trachea of non-infected,

doxycycline-treated mice. We observed reduced *Krt6a* and *Krt6b* expression in the trachea at 12h, but not 24h after drug treatment (Figure 5-4A).

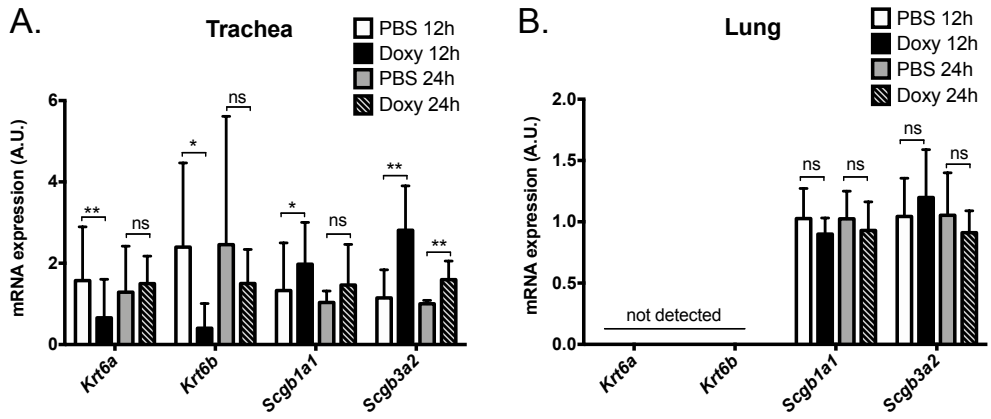

Figure 5-4 - Basal cells in doxycycline-treated mouse lung.
Expression of basal and secretory cell markers by qPCR in mouse trachea (A) and lung (B) at the indicated time-points after doxycycline administration. Data represent mean±SD of 3-6 mice assayed in duplicate.

In the lung, however, mRNA levels of these genes were below detection limit in all analyzed samples (Figure 5-4B). We also measured mRNA levels of the club cell markers *Scgb1a1* and *Scgb3a2* and found slightly increased expression in the trachea but not in the lung (Figure 5-4A, B).

Immunofluorescence analysis of whole-lung sections revealed abundant KRT6a expression in the upper respiratory tract, most notably in the trachea and bronchi (Figure 5-5A). Consistent with mRNA levels (Figure 5-4), KRT6a$^+$ cells were rare in the lower respiratory tract, showing only discrete clusters in the distal areas of lung parenchyma (Figure 5-5A). Abundance and distribution of KRT6a$^+$ cells was not significantly altered within 48h of doxycycline treatment (Figure 5-5B, C).

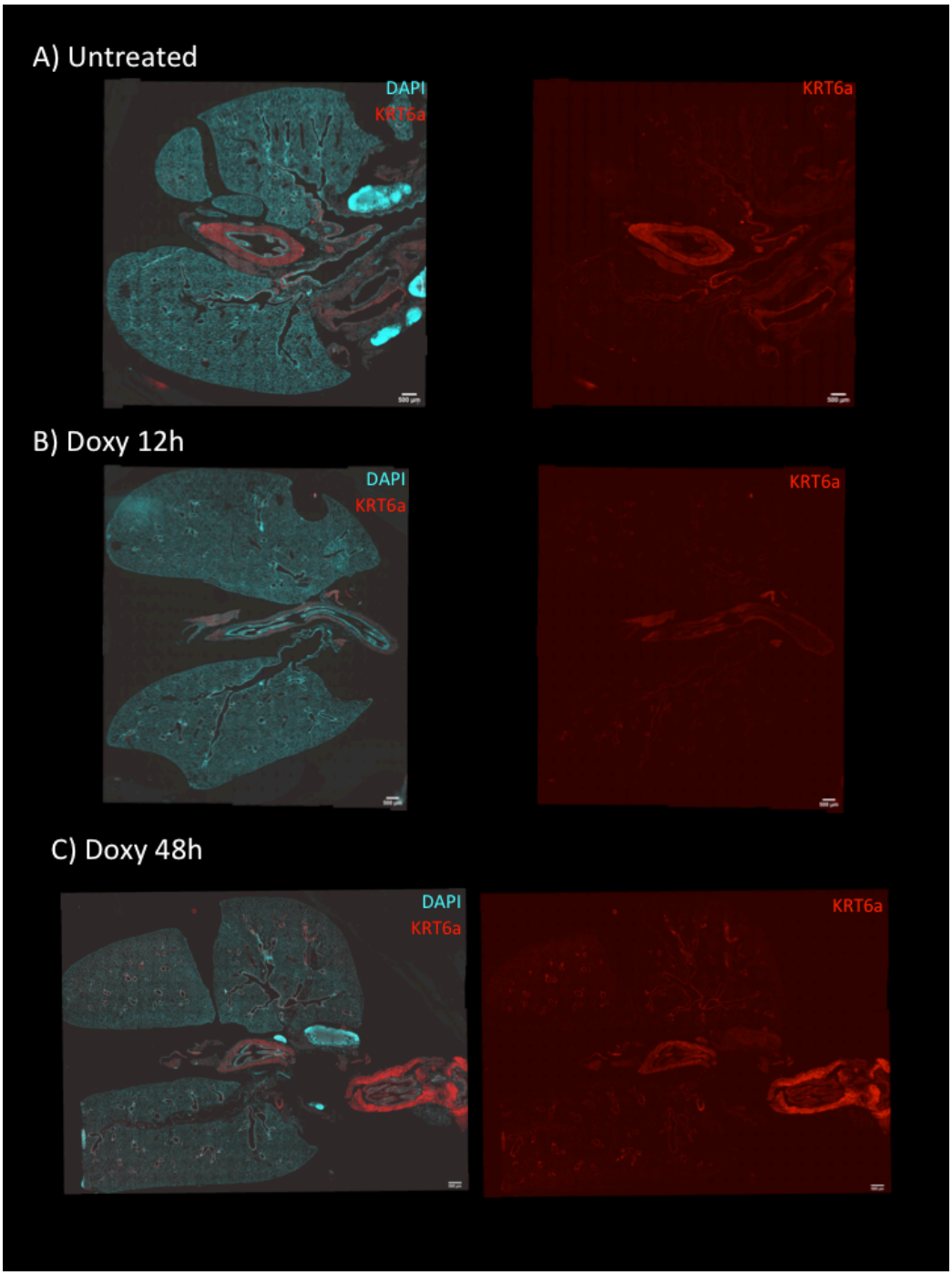

Figure 5-5 Immunofluorescence of KRT6^{+} basal cells in mouse lung
Longitudinal sections of lung and trachea stained for KRT6a in mice treated with vehicle (A) or doxycycline (B, C). Right panels show KRT6 staining and left panels show the merge between KRT6 and nuclear counterstaining (DAPI). Scale bar = 500 μm.

Finally, we used mice in which the human diphtheria toxin receptor was introduced into the *Krt6a* locus to specifically ablate KRT6^{+} cells[7]. Four days after intra-tracheal administration of diphtheria toxin, mice were infected with *E. coli* and treated with doxycycline. Preliminary results show that KRT6-depleted mice still present increased survival when treated with doxycycline (Figure 5-6).

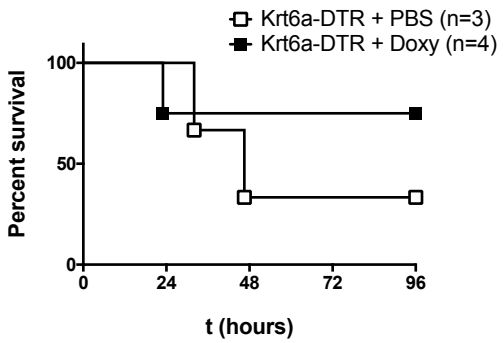

Figure 5- 6 Effect of KRT6 depletion in sepsis
Survival of KRT6a-depleted mice upon infection with *E. coli* and treatment with doxycycline. Krt6a-DTR mice were treated with 12 ng/g diphtheria toxin by intra-tracheal administration 4 days before infection. Data represents a single experiment

Taken together, our results show that doxycycline induces changes in cell populations in the lung. While this suggests an increased potential for reprogramming lung architecture, basal cells are the unlikely single target of the drug. Therefore, enhanced lung repair capacity induced by doxycycline likely depends on the combination of its effect on multiple cell lineages, rather than basal cells alone.

5.5 Discussion

Recovery from lung injury is essential in sepsis and other infectious conditions, and remains poorly understood. Inspired by the protective effect of doxycycline in sepsis, we began this study by addressing putative lung-specific mechanisms of action of this drug. Indeed, a single intra-tracheal administration of doxycycline is sufficient to increase survival to infection, although systemic effects derived from absorption of the drug from alveolar capillaries cannot be ruled out.

These tissue-specific effects were then confirmed in an unbiased transcriptional approach, which revealed that doxycycline treatment results in a dramatically different signature in the lung compared to the liver (Chapter 4). Doxycycline treatment has no effect on lung inflammatory and resolving programs during infection. Instead, we found surprising changes in lung cell populations, which are independent of infection. In particular, we noticed a down-regulation of genes of the late cornified envelope (*Lce*) and keratin (*Krt*) families, both of which are associated with peptide cross-linking in the epithelium and keratinocyte differentiation.

Interestingly, there is abundant evidence in the literature showing that cell differentiation programs are highly influenced by mitochondrial function[15,16]. In the intestine, activation of the UPR^{mt} is associated with loss of cell stemness and paracrine secretion of WNT[17]. Specifically in the lung, mitochondrial ROS have been associated with degradation of keratin filaments, although the consequences for tissue function and structure have not been addressed[18]. In the hematopoietic compartment, a regulatory branch of UPR^{mt} dependent on SIRT7 has been found to determine stem cell aging and differentiation[19]. Remarkably, doxycycline has been reported to increase hematopoietic stem cell survival and self-renewal, through mechanisms still not completely understood[20]. Given the previously discussed effect of doxycycline in mitochondrial function (Chapter 3), it is tempting to speculate that the observed drug-induced changes in lung cell populations are mediated by the mitochondria, although a specific link between the two has not been investigated in this study.

Following the RNA-Seq results, we focused specifically on basal cells, a population of stem cells of the respiratory tract. Our analysis of mRNA and protein levels indicates that in steady state conditions basal cells are most abundant in the upper respiratory tract, in accordance with previous studies[8,9]. In the trachea, down-regulation of *Krt6* mRNA levels upon doxycycline treatment is accompanied by increased expression of secretory (club) cell markers[21], indicating a drug-induced reprogramming which may help maintaining the epithelial architecture and function under stress conditions, such as infection. In distal areas of the lung, the picture is much less clear and was complicated by low levels of expression of basal cell markers and high heterogeneity between mice. While $KRT6^+$ cells have been deemed essential for alveolar regeneration upon infection[7], this process is still poorly understood and may involve a number of different progenitor lineages[22]. Examples include alveolar progenitor cells expressing integrin receptors[23], or the recently identified *Wnt*-secreting type II alveolar cells[24].

Moreover, the observed drug-induced changes in gene expression seem to be transient, and are dampened or absent at 24h after drug treatment. While basal cells are widely known to differentiate into other cell types, is has also been reported that mature cells are able to de-differentiate and acquire stem cell properties under stress conditions[25]. This may partly explain why doxycycline-treated mice show reduced levels of *Krt6* at 12h but not at 24h.

Finally, preliminary results obtained with depletion of $KRT6^+$ cells suggest that these cells are dispensable for the protective effect of doxycycline in sepsis. While these results need further validation, namely with respect to the efficiency of cell depletion, they help to support the notion of intricate spatial and temporal patterns of tissue remodeling involving multiple cell populations. These should be investigated in more detail, for example by resorting to lineage tracing methods, single-cell transcriptomics, or *ex-vivo* cultures of specific cell lineages.

In addition to models of sepsis, we employed a lung-specific viral infection in this study. Influenza has been widely used as a model in which acute lung injury is followed by active proliferation of stem cells to repopulate depleted niches and restore tissue function[7,9]. Here, we used body weight as an indirect measure of recovery from infection and found that doxycycline treatment improves infection outcome. Further studies should be conducted – in particular the analysis of lung pathology at different time-points of infection – to better understand the role of doxycycline in stem cell reprogramming and lung regeneration. The influenza model might actually be more advantageous to achieve these goals in future studies when compared to sepsis models, as it specifically relies on lung damage. Given the broad range of effects of doxycycline in host physiology, as discussed in previous chapters, a lung-specific infection model would facilitate the study of tissue-specific mechanisms and avoid confounding factors.

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Chapter 6

General discussion and perspectives

All living organisms have evolved mechanisms to detect external insults and correct deviations in homeostasis. The activation of stress responses has a multifaceted impact on organismal function, integrating immunity, nutrition, and behavior. Some of the most elegant examples of this crosstalk have emerged from work in nematodes. For example, inactivation of *C. elegans* core cellular genes involved in translation, protein turnover, or respiration have been shown to induce aversion behavior towards non-pathogenic bacteria and induction of detoxification and immunity programs[1]. Inspired by this framework of *cellular surveillance-activated detoxification and defenses (cSADDs)*[1], this thesis explored the idea of cytoprotective stress responses in the context of complex host-microbe interactions. We started by gathering evidence from the literature that pathogen-induced perturbations in homeostasis can be used by the host to detect pathogen invasion and kick-start the appropriate immune responses. Then, we used sepsis models in mice as an example of extreme deviation in homeostasis to understand whether chemically induced cellular stress responses, in particular those originated at the mitochondria, could be used to promote compensatory effects that increase survival.

For many decades, defects in mitochondrial function were unequivocally regarded as detrimental for health, as numerous mutations in genes coding for the mitochondrial proteome are associated with embryonic lethality or severe illness[2]. Moreover, some of the most potent known poisons, such as cyanide and rotenone, act by inhibiting the ETC activity. Only at the beginning of the 21st century this idea was challenged by the surprising observation that mild defects in the ETC activity prolong lifespan in nematodes. Exposure of *C. elega*ns larvae to RNA interference (RNAi) targeting nuclear encoded subunits of all ETC complexes resulted in a decrease in body size and ATP production, accompanied by increased lifespan[3]. An independent RNAi screen confirmed the association of attenuated mitochondrial function with increased longevity,

while uncoupling this effect from ROS production[4]. A deeper mechanistic insight was brought when knockdown of the complex IV subunit *cco-1* was found to induce expression of HSP60[5]. This induction of UPR^{mt} is required to sustain the effect of *cco-1* RNAi in longevity, thus establishing a causal link between mitochondrial stress responses and longevity[6]. Surprisingly, tissue-specific ETC defects can be communicated to distal cells to induce cell-non-autonomous protective responses mediated by Wnt signaling[6,7].

One important aspect that regulates beneficial mitochondrial stress responses is the magnitude and duration of stress. Early studies with RNAi targeting ETC function in nematodes demonstrated that lifespan extension is only achieved when the perturbation is triggered in larval stages and not in adults[3,8]. Moreover, the correlation between the levels of depletion of ETC components and longevity is not linear – for certain genes, high levels of depletion seriously compromise mitochondrial function and have a negative impact in longevity[8]. This is in line with the long recognized concept of 'mitochondrial threshold effect', according to which mitochondria are able to tolerate a certain limit of respiratory deficiency before cell viability is compromised[9]. In fact, mitochondria have a remarkable capacity to withstand stress, as shown by the fact that in rat muscle mitochondria treated with cyanide, 75% inhibition of complex IV activity resulted in only 20% decrease in the respiratory capacity[10]. This 'safe zone' of mitochondrial dysfunction can thus be harnessed to trigger protective phenotypes without risking organismal fitness – an idea that aligns with the previously discussed concept of hormesis[11].

In flies, muscle-specific ETC inhibition activates UPR^{mt} and increases lifespan though a mechanism that involves systemic insulin signaling – thus highlighting the cell-non-autonomous effects of mitochondrial stress[12]. In worms and mice, defects in mitochondrial translation induced by knockdown of the mitochondrial ribosomal protein S5 (*Mrps5*) result in mitonuclear protein imbalance that triggers UPR^{mt} and prolongs lifespan[13]. Notably, treatment of worms with

doxycycline and chloramphenicol, two drugs that bind to the mitoribosome and inhibit protein synthesis, has a similar beneficial effect[13]. Other long-lived mouse models of mitochondrial dysfunction include the deletion of *Surf1* (a complex IV assembly protein)[14], as well as mice heterozygous for *Mclk-1* (an essential enzyme for ubiquinone biosynthesis)[15].

The field of protective mitochondrial stress responses has been gradually expanding beyond longevity and providing evidence that these adaptive programs can be beneficial in a number of conditions. One paradigmatic example is how mitochondrial stress regulates immune responses. In *C. elegans*, the UPR^{mt} regulator ATFS-1 regulates not only the expression of stress responsive genes but also innate immune genes[16]. Exposure of worms to pathogens and bacterial toxins activates UPR^{mt} together with detoxification and immune gene expression, which suggests that mitochondria play a role in surveying the environment and integrating different defense programs[17–19]. In mammals, mild defects in mitochondrial DNA were found to trigger an antiviral response based on the expression of interferon-responsive genes[20].

A growing body of evidence is highlighting the importance of mild mitochondrial stress in improving metabolic dysfunctions. Mice with liver and muscle-specific knockout of the mitochondrial flavoprotein AIF2 present disturbed ETC function and concomitant improvement in insulin sensitivity in a model of type 2 diabetes[21]. Similarly, mitochondrial proteome imbalance in brown adipose tissue (BAT) confers protection against diet-induced obesity in mice[22]. In a mouse model of fatty liver disease, UPR^{mt} induced by supplementation of a NAD^+ precursor increases liver β-oxidation and improves disease outcomes[23]. Another curious example of whole-body beneficial effects of mitochondrial stress was found in mice carrying a muscle-specific deletion of *Crif1*, a mitoribosomal protein essential for the correct assembly of ETC complexes. These mice are protected against diet-induced obesity and present systemic metabolic benefits mediated by GDF15[24].

Together with GDF15, other mitokines – molecules secreted from cells undergoing mitochondrial stress that are released into circulation and promote distal beneficial effects – have been recently identified. These include FGF21[25] and mitochondrial-encoded peptides such as MOTS-c[26], both of which have been associated with anti-obesity effects.

Our study opens new perspectives way beyond the scope of sepsis, spanning to areas of research related to inflammation, metabolism, and stress responses. Since the early 1990's, the link between metabolic alterations – namely insulin signaling[27], mitochondrial respiration[3], or mTORC activity[28] – and longevity generated vast interest. To this day, the search of an 'anti-aging pill' has been centered in drugs that link metabolic reprogramming with cytoprotection, such as metformin or rapamycin[29].

Likewise, research in obesity has been gathering increasing interest, particularly in what relates to the crosstalk of inflammation and metabolism[30]. Our findings largely overlap with the previously proposed idea that ETC inhibition results in metabolic benefits. Furthermore, we propose additional mechanisms of tissue protection, such as changes in glucocorticoid response and tissue regeneration mechanisms, which are likely to play a role in many inflammatory conditions.

A more complete understanding of mechanisms regulating whole-body homeostasis is highly desirable as it paves the way for preventive and/or therapeutic strategies in conditions characterized by inflammation, metabolic dysregulation, and loss of organ function, which afflict modern societies with increasing social and economic impact.

Recovery from infection requires the combination of resistance mechanisms – which have been extensively studied for decades – and disease tolerance – for which the molecular mechanisms have only recently began to be addressed[31]. The need for strategies that limit tissue damage has become particularly evident in the case of sepsis, a heterogeneous and multifactorial syndrome for which no

specific therapies have been found so far. In particular, the role of metabolic regulation has been increasingly appreciated in sepsis pathophysiology and therapeutics[32]. Following a very recent stream of articles linking metabolic reprogramming and disease tolerance in sepsis[33–35], our work uncovers several drugs which, by perturbing electron transport chain activity, promote metabolic changes and limit tissue damage during infection. Unexpectedly, treatment with doxycycline has broader effects on mouse physiology, namely changes in glucocorticoid signaling in the liver and epithelial cell reprogramming in the lung. Our work highlights complex, tissue-specific mechanisms that link mitochondrial stress responses and disease tolerance, and encourages more detailed mechanistic studies. Of particular interest is the fact the local perturbations in mitochondrial function, such as lung-specific administration of doxycycline, or liver-specific CRIF1 depletion result in systemic benefits and increased survival. While the mechanisms of inter-organ communication were not addressed in this work, these results are reminiscent of previously reported cell-non-autonomous effects of mitochondrial stress in flies and nematodes[6,12].

Extensive characterization of the action of doxycycline in mouse liver and lung allowed for the identification of three major axes that contribute to survival to severe infection: metabolic reprogramming, glucocorticoid signaling, and lung regeneration. However, the major outstanding question is which of these effects are mechanistically linked to perturbations in mitochondrial function. In future studies, it would be advantageous to have a more complete picture of these pathways in: 1) phenformin or chloramphenicol-treated mice, as well as CRIF1-depleted mice, to better understand which characteristics are mechanistically linked to ETC perturbations, and which are induced by side effects of doxycycline; and 2) other target organs (brain, adipose tissue, and adrenal glands are particularly attractive).

Changes in mitochondrial function have dramatic effects in cell fate and tissue function. In the next sections, I will speculate on how doxycycline-induced mitochondrial stress can be linked to each of the observed phenotypes.

Metabolism and bioenergetics. We have uncovered one important aspect of sepsis pathophysiology related to impaired FAO and accumulation of lipid species in the liver, which are partly corrected by doxycycline. The effect of doxycycline is advantageous from a bioenergetics viewpoint. Most likely, perturbation of ETC activity triggers a cellular adaptation that optimizes fuel utilization, in this case by increasing lipid metabolism. Our results indicate that the end products of β-oxidation are then diverted to ketone body synthesis, which can be used to generate ATP in several organs, most importantly the brain, in accordance with previous studies[34,35]. However, the mechanistic links between ETC inhibition and increased FAO are still missing. In future studies, this question should be approached in a tissue-specific manner, to avoid the confounding effect of inter-organ communication. The use of primary hepatocyte cultures, for example, would allow for extracellular flux analysis, carbon tracing, and ATP measurements upon ETC inhibition. This would provide a more complete idea of the liver-specific energetic adaptations resulting from perturbations in the ETC.

Metabolite signaling. Beyond bioenergetics, lipid species play a number of signaling and biosynthetic roles in the mitochondria[36]. Examples include the role of cardiolipin in mitophagy[37], ceramide in stress responses and apoptosis[38,39], or long chain fatty acids in ADP/ATP trafficking[40]. In this thesis, we have shown that hepatic mitochondrial transport and oxidation of fatty acids are impaired during sepsis and proved that β-oxidation is essential for survival. However, supplementation with medium chain fatty acids failed to produce any protective phenotype. This result underscores the need for a fully functional lipid import, trafficking, and metabolic machinery to maintain cellular viability. Supplementation with a single lipid species, even if it is enough to support

energy generation through β-oxidation, is likely insufficient to maintain the complex network of lipid-mediated cellular functions. Therefore, our results encourage a more careful examination of lipid contents in mitochondria during infection, and a complete understanding of the pathways affected. Having this information, we can then design more rational approaches for nutritional support or pharmacological targeting of the affected transport and signaling pathways.

Glucocorticoid signaling. Mitochondrial function and glucocorticoids are tightly connected. In fact, the first step in the conversion of cholesterol to steroid hormones through the action of cytochrome P450scc takes place at the IMM[41]. Interestingly, cholesterol trafficking to the mitochondria is dependent on the steroidogenic acute regulator protein (StAR), whose levels are regulated by mitochondrial proteases that respond to mitochondrial stress[42]. This raises the intriguing possibility that perturbations in mitochondrial function, namely doxycycline treatment, may indirectly regulate steroid synthesis and transport – a possibility that should be more carefully addressed by analyzing the effect of doxycycline in steroidogenic tissues, such as the adrenal glands.

Lung regeneration. Remodeling of lung architecture is a complex and dynamic process that relies on multiple cell lineages, some of which are rare and poorly characterized[43,44]. Mitochondrial function has been associated with changes in cell fate and stem cell function[45], raising the possibility that changes in mitochondrial bioenergetics and signaling might trigger differentiation of lung stem cells. In fact, doxycycline has been proposed to induce self-renewal of pluripotent stem cells[46]. A more detailed metabolic and transcriptional characterization of different lung cell lineages would provide more evidence on the exact trigger for doxycycline-induced lung regeneration.

In general, future studies trying to pursue more detailed molecular mechanisms of disease tolerance will be challenged by the high inter-individual variability associated with mouse models of sepsis, as well as the pleiotropic effects of

drugs. While the latter can be partially solved by the use of CRIF1-depleted mice as a genetic tool to induce tissue-specific perturbations in mitochondrial function, the former is an intrinsic challenge of this topic of research. As suggested above, many of the tissue-specific mechanisms can be addressed by the use of cell cultures, organoids, or purified cell lineages. These studies may provide more detailed molecular mechanisms, which can later be confirmed *in vivo*. It is important to keep in mind, however, that sepsis is a multi-organ, multi-factorial process in which a change in a given cell population can have unpredictable effects on organismal physiology.

Another major question that emerges from this study is the clinical applicability of the treatments described here. Therapeutic strategies that promote disease tolerance present a valuable complement to the currently used antimicrobial therapies and organ support measures in critically ill patients[47]. However, the success of a drug in pre-clinical models of sepsis is only occasionally replicated in the clinical setting. Mouse models of sepsis fail to completely reproduce the pathophysiology of human sepsis, which is often complicated by co-morbidities and secondary infections[48,49]. In addition, patient treatment is only possible after the onset of severe symptoms, a phase at which systemic inflammation and metabolic dysfunction are difficult to revert. In contrast, the experimental setup used in this study involves administration of drugs at the time of infection, allowing the protective effects to occur from very early stages of the disease. Therefore, future attempts to translate mitochondrial-targeting drugs into the clinic need to address and succeed in a series of experimental setups of increasing complexity. From the tested drugs, doxycycline is best positioned for these studies, as it is routinely used in clinical practice and well tolerated, including in sepsis patients[50]. Future studies in mice should focus on different mouse strains, or even outbred mice, and a more diverse panel of bacterial species (the use of polymicrobial models of infection would be preferred, although it would be hard to control for the antibiotic effect of the drug). Finally,

encouraging results in mouse models of sepsis should be validated in larger mammals, for example pigs, in which the pathophysiology of sepsis more closely resembles human patients.

As discussed above, cellular surveillance systems are key for restoring homeostasis under stress conditions. From the organelle-specific stress responses initially addressed in the work, mitochondria rapidly emerged as the most promising targets. This is hardly surprising in light of the multifaceted roles of mitochondria in cellular energetics, signaling and cell fate decision. Moreover, given their endosymbiont origin, mitochondria have evolved complex communication strategies with other cellular structures. More surprisingly, mitochondria are emerging as central players in host-microbe interactions, as they can rapidly perceive and respond to pathogen attack[16,39]. For example, sensing of live, but not heat-killed bacteria by macrophages has been shown to reduce complex I assembly and activity, while increasing complex II activity[51]. Conversely, some defense programs are orchestrated by the mitochondria in response to infection, such as the recently described mitochondrial-derived vesicles carrying antimicrobial compounds[52]. The fact that the perturbations in mitochondrial function described in this thesis show a protective effect against bacterial infections, suggests that still-unknown crosstalks between bacteria and mitochondria might be favoring host fitness specifically in this context. 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 that inevitably will result both from the presence of the pathogen and from the collateral damage from the effector resistance mechanisms. 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.

The natural implication of this hypothesis is that an intact mitochondrial structure and function are essential for pathogen elimination and recovery from infection. In fact, a compromised activity of ETC complexes has been described as a hallmark of sepsis pathology across several models[53]. Additionally, sepsis patients with increased mitochondrial complex IV activity have been associated with higher chances of survival[54]. To reconcile these findings with the observations in this thesis, one must note that protective perturbations in ETC need to be mild and transient. These perturbations should not severely compromise the overall capacity of generating ATP, and should allow for the necessary compensatory responses to take place before infection-associated mitochondrial dysfunction appears.

Around 500 years ago, Paracelsus stated that "all things are poison, and nothing is without poison; only the dose permits something not to be poisonous". The need for a right balance between stress and homeostasis remains a challenge, as became apparent throughout this thesis. A better understanding of the interactions between host physiology and the multiple surrounding stimuli will certainly help us in dealing with modern challenges of biology and medicine.

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Appendix

Table of reagents and resources used in this thesis

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
beta Actin antibody [SP124]	Abcam	Cat# ab115777, RRID:AB_10899528
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_2801538
Recombinant Anti-MTCO1 antibody [EPR19628]	Abcam	Cat# ab203912, RRID:AB_2801537
Recombinant Anti-ATP5A antibody [EPR13030(B)]	Abcam	Cat# ab176569, RRID:AB_2801536
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
Cytokeratin 6A Monoclonal Antibody (LHK6B)	ThermoFisher Scientific	Cat# MA5-14127
Goat F(ab) polyclonal Secondary Antibody to Mouse IgG - H&L	Abcam	Cat# ab6668
Andy Fluor 647 Goat Anti-Mouse IgG (H+L) Antibody	Tebu-bio	Cat# L125A
Bacterial and Virus Strains		

Escherichia coli MG1655 TetR CamR	This thesis	
Influenza A/Puerto Rico/8/34 (H1N1)	Maria João Amorim (Instituto Gulbenkian de Ciência)	Wit, E. de *et al.* Efficient generation and growth of influenza virus A/PR/8/34 from eight cDNA fragments. *Virus Res.* **103**, 155–161 (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
Chemicals, Peptides, and Recombinant Proteins		
Doxycycline hyclate	Sigma-Aldrich	Cat# D9891
Phenformin Hydrochloride	Sigma-Aldrich	Cat# P7045
Chloramphenicol	Sigma-Aldrich	Cat# C0378
Metformin hydrochloride	Sigma-Aldrich	Cat# PHR1084
Menadione sodium bisulfite	Sigma-Aldrich	Cat# M5750
Trifluoperazine dihydrochloride	Sigma-Aldrich	Cat# T8516
Bortezomib	Tebu-bio	Cat# 21910-2120
Nicotinamide adenine dinucleotide	Cayman	Cat# 17118
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
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# O4126
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
RNeasy Mini Kit	Qiagen	Cat# 50974106
RNase-Free DNase Set	Qiagen	Cat# 50979254
cOmplete, Mini, EDTA-free	Roche	Cat# 11836170001
PHOSSTOP	Roche	Cat# 4906837001
(+)-Etomoxir sodium salt hydrate	Sigma-Aldrich	Cat# E1905
CP-868388	Sigma-Aldrich	Cat# PZ0149
Mifepristone	Sigma-Aldrich	Cat# M8046
Dexamethasone	Merck Sharp & Dohme	Oradexon 5 mg/mL
Octnoic acid	Sigma-Aldrich	Cat# C2875

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
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) Berkhout SC5314	Salomé LeibundGut-Landmann (University of Zurich, Switzeland)	Gillum, A. M. *et al*. Isolation of the Candida albicans gene for orotidine-5'-phosphate decarboxylase by complementation of *S. cerevisiae* ura3 and *E. coli* pyrF mutations. *Mol. Gen. Genet.* **198**, 179–82 (1984).
C57BL/6J *Mus musculus*	Instituto Gulbenkian de Ciência	IMSR Cat# JAX:000664, RRID:IMSR_JAX:000664

Crif1$^{lox/lox}$ *Mus musculus*	Minho Shong (Chungnam National University, Daejeon, South Korea)	Kwon, M. *et al.* Crif1 is a novel transcriptional coactivator of STAT3. *EMBO J.* **27**, 642–653 (2008).
Krt6a-DTR *Mus musculus*	Wa Xian and Frank McKeon (Harvard Medical School, Boston, MA, USA)	Zuo, W. *et al.* P63 + Krt5 + distal airway stem cells are essential for lung regeneration. *Nature* **517**, 616–620 (2015).
Plasmodium berghei ANKA	Miguel Soares (Instituto Gulbenkian de Ciência)	Pamplona, A. *et al.* Heme oxygenase-1 and carbon monoxide suppress the pathogenesis of experimental cerebral malaria. *Nat. Med.* **13**, 703–710 (2007).
Oligonucleotides		
Mouse *Gapdh* qPCR primers	N/A	Fw: AACTTTGGCATTGTGGAAGG Rv: ACACATTGGGGGTAGGAACA
Mouse *Cpt1a* qPCR primers	N/A	Fw: CTCCGCCTGAGCCATGAAG Rv: CACCAGTGATGATGCCATTCT
Mouse *Cpt2* qPCR primers	N/A	Fw: CAAAAGACTCATCCGCTTTGTTC Rv: CATCACGACTGGGTTTGGGTA
Mouse *Slc25a20* qPCR primers	N/A	Fw: GCGCCCATCATTGGAGTCA Rv: CACACCAGATAACATCCCAGC
Mouse *Crif1* qPCR primers	N/A	Fw: TGCTCGCTTCCAGGAACTATT Rv: CATAGCAGCAATTCGTGCCT
Mouse *Actb* qPCR primers	N/A	Fw: GGCTGTATTCCCCTCCATCG Rv: CCAGTTGGTAACAATGCCATGT
Mouse *Tufm* qPCR primers	N/A	Fw: GCAGCCACTCTATTGCGAG Rv: CCGACCTTGCAGAAATGGG
Software and Algorithms		
Prism 6	GraphPad	N/A
R v3.5.3	The R Foundation	N/A
TraceFinder	Thermo Fisher Scientific	N/A
ImageJ v1.52	NIH	N/A
Nikon NIS Elements	Nikon	N/A

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