Universidade de Lisboa
Faculdade de Medicina de Lisboa

SEVERE SEPSIS

Protective Role of Epirubicin

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To my wife Isa,

To my daughters Sofia and Maria.

Preface

I am a medical doctor. My work consists of attempting to restore the physiology of my patients back to homeostasis. To accomplish this as a surgeon, I often have to invade the outer boundary of the organism and operate in cavities, mostly the abdomen. Surgery is, by itself, a (controlled) aggression to the organism. The real art of Surgery is doing what we must while causing the least possible harm.

General and Digestive Surgery, my specialty, is essentially called upon to excise lesions (neoplastic and others), to control a focus of infection or to stop an otherwise lethal hemorrhage. Whenever possible, restoration of intestinal continuity and bodily functions are undertaken. This simplistic description serves to illustrate that when all goes as expected, the patient recovers from surgery and after a period of convalescence he is able to go home or to continued care.

Two groups of patients have always concerned me: those that do not follow this path and those that come to the hospital in an emergency setting with primary severe abdominal infections.

The former group usually develops secondary peritonitis following restorative surgery and despite not frequent, it is a reality even in modern surgery. The later includes all the patients that we encounter in emergency wards with acute abdomen, and those that we are called upon to observe due to suspected peritonitis.

In both groups, we must make an early diagnosis and initiate fast therapeutic intervention in order to control the source of infection. It is the only way to protect them from systemic repercussions and to put them in the appropriate path to health. When treatment is delayed, both groups progress to a potentially lethal syndrome known as Sepsis.

A great deal of progress has been made in the field of General and Digestive Surgery: new materials, better operating room conditions and above all better knowledge to assess which patients benefit from conventional surgery or from damage control surgery. This has been of paramount importance, as we now know that a limited group of patients is better treated with aggressive control of the "contamination" source, temporary closure and quick return to the Intensive Care Unit (ICU) to restore homeostasis. Later, definitive surgery can be performed in safer and better physiological conditions.

We also have new technologies that allow us to perform less invasive operations, with less "surgical trauma" to the patient. Laparoscopy, "single-access surgery" and robotic assisted surgery can be performed safely, reproducibly and with less disruption to the, sometimes fragile, homeostatic balance of our patients.

Additionally, in the last two centuries there have been huge scientific developments to control infection. We now have broader spectrum and more effective antibiotics. State-of-the-art ICUs allow for patients to be invasively monitored continuously and administer advanced organ support measures such as invasive mechanical ventilation, inotropic and chronotropic vaso-active

drugs, hemodialysis, continuous veno-venous hemodiafiltration, total parenteral nutrition that can sustain almost indefinitely human life.

Nevertheless, Sepsis mortality remains high.

Early in my residency, I chose Intensive Care Medicine as one of the voluntary rotations. During this period I faced both sides of the problem. I became aware of the efforts that we, as surgeons, put in operating the septic patient and at the same time could witness the tireless work of intensivists trying to keep our patients alive. Unfortunately, we still lose patients that have no visible contamination source, no evidence of bacteria in body fluids or cavities, and were given all organ support measures. It seems that despite the best surgery, the best antibiotic available to control infection, and the best ICU support that we can offer, organ dysfunction can occur that irreversibly leads to organ failure and death.

For me, this is one of the most puzzling and distressful events a surgeon comes across.

To tackle the problem of Severe Abdominal Sepsis has been my objective since then.

During my 5th year of residency, with the support of Prof. Paulo Costa, I applied to the Gulbenkian Program for Advanced Medical Education, coordinated by Prof. Leonor Parreira. The aim of this program was to make possible for interested M.D.s to combine their clinical activity with research, and to acquire solid scientific bases to further develop quality research projects based on their clinical problems, in their specific areas of interest.

I was accepted and started the Program in October 2008. During the initial faculty lectures, I met Prof. Luis Moita and we started to discuss the problem I wanted to address. His laboratory, the "Cell Biology of the Immune System Unit", already had an interest in the study of innate immunity. So I can say there was an immediate connection and the project started in April 2009. Prof. Luís Moita and Prof. Paulo Costa agreed to be co-supervisors of my PhD thesis, for which I submitted the project to Faculdade de Medicina da Universidade de Lisboa.

We have chosen to start this project with our clinical questions in mind, translating them to simple questions that could be addressed in the laboratory. From our starting hypothesis, the results obtained raised new questions and experiments, whose results we hope could in a near future be transferred back to the clinical practice.

From 2009, I attempted to reconcile the regular activity of an attending surgeon with my scientific research. I finished my residency in 2011 and continued to operate and assist patients, as I maintained my activity in the Lab. For this endeavor, I had not only the unconditional support of my Surgery Director - Prof. Paulo Costa - and Laboratory Director - Prof. Luis Moita, but a lot of encouragement and help from my clinical and laboratory colleagues. Dra. Helena Lopes da Silva, my Chief of Surgery, was a key character in this journey, as she not only supported but also sometimes forced me to continue.

Later on, I was invited by Prof. António Parreira to join and start the Digestive Surgery Program in the Comprehensive Cancer Center at Champalimaud Foundation, Lisbon. I began this exciting new endeavor in

January 2012. During this last period I maintained my research and clinical activities, a fruitful collaborative effort which I hope to continue...

This manuscript represents the path of a surgeon actively attending to his patients in the Clinic and an investigator in Prof. Moita's Laboratory, who besides being my supervisor, naturally became my Mentor and Friend.

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Abbreviations

αANP	α-Atrial Natriuretic Peptide
ACCP	American College of Chest Physicians
ADHD	Attention-Deficit Hyperactivity Disorder
AIDS	Acquired Immunodeficiency Syndrome
ALI	Acute Lung Injury
ALT	Alanine Transaminase
APACHE II	Acute Physiology and Chronic Health Evaluation II
aPTT	activated Partial Thromboplastin Time
ARDS	Acute Respiratory Distress Syndrome
ARF	Acute Renal Failure
ATGs	Autophagy-Related Proteins
ATM	Ataxia Telangiectasia Mutated
ATR	Ataxia Telangiectasia and Rad3 Related
ATS	American Thoracic Society
CARDs	Caspase Recruitment Domains
CARS	Compensatory Anti-Inflammatory Response Syndrome
CHEK1	Checkpoint Kinase 1
CK	Creatine Kinase
CLP	Cecal Ligation and Puncture
CLRs	C-type Lectin Receptors
CNS	Central Nervous System
COX-2	Cyclooxygenase-2
CVVHDF	Continuous Venovenous Hemodiafiltration
DAMPs	Damage Associated Molecular Patterns
DAS-ELISA	Double Antibody-Sandwich Enzyme-Linked Immunosorbent Assay
DAT	Dopamine Active Transporter

DC	Dendritic Cells
DDR	DNA Damage Response
DHPS	Dihydropteroate Synthetase
DIC	Disseminated Intravascular Coagulation
DNA	Deoxyribonucleic Acid
dsRNA	Double Stranded Ribonucleic Acid
EC	Endothelial Cell
ER	Endoplasmic Reticulum
ESICM	European Society of Intensive Care Medicine
FA	Fanconi Anemia
FACS	Fluorescence-Activated Cell Sorting
FancD2	Fanconi Anemia Group D2 Protein
H&E	Hematoxylin and Eosin Stain
HMGB 1	High-Mobility Group Box 1
HO-1	Heme-Oxygenase 1
HR	Homologous Recombination
HSPs	Heat Shock Proteins
ICL	DNA Interstrand Cross-Links
ICU	Intensive Care Unit
iE-DAP	γ-D-glutamyl-meso-Diaminopimelic Acid
IFNγ	Interferon γ
IL	Interleukin
iNOS	inducible NO Synthase
IPS-1	IFN-β Promoter Stimulator-1; MAVS; VISA; Cardif
IRF	IFN-Regulatory Factor
Keap1	Kelch Like-ECH-Associated Protein 1
KO	Knockout
LDH	Lactate Dehydrogenase
LPS	Lipopolysaccharide
LRR	Leucine-Rich Repeats
MCP-1	Monocyte Chemotactic Protein
MDA-5	Melanoma Differentiatiation-Associated Protein-5

MDF	Myocardial Depressant Factor
MDP	Muramyl Dipeptide
MHC	Major Histocompatibility Complex
MIF	Macrophage Migration Inhibitory Factor
miR	MicroRNA
MIT	Massachusetts Institute of Technology
MODS	Multiple Organ Dysfunction Syndrome
MOI	Multiplicity Of Infection
MyD88	Myeloid Differentiation Primary Response Gene 88
NAIPs	NLR Family Apoptosis Inhibitory Proteins
NALPs	NACHT-, LRR-, and Pyrin Domain-Containing Proteins
NET	Norepinephrine Transporter
NF-κB	Factor Nuclear Kappa B
NLR	Nucleotide-Binding Oligomerization Domain-Like (NOD-Like) Receptors
NMDA	N-Methyl-D-Aspartate
NMRI	Naval Medical Research Institute
NO	Nitric Oxide
NOD	Nucleotide-Binding Oligomerization Domain
NOS	Nitric Oxide Synthase
Nrf2	Nuclear Factor (Erythroid-Derived 2)-Related Factor-2
NSAID	Non-Steroidal Anti-Inflammatory Drug
PAF	Platelet-Activating Factor
PAMPS	Pathogen Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PEEP	Positive End-Expiratory Pressure
PFU	Plaque-Forming Units
PIRO	Predispotion, Insult, Host Response, Organ failure
PMA	Phorbol 12-Myristate 13-Acetate
PRR	Pattern Recognition Receptor
PT	Prothrombin Time
Puma	P53 Upregulated Modulator of Apoptosis

RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
RIG-1	Retinoic Acid-Inducible Gene I
RIPK1	Receptor-Interacting Protein Kinase 1
RLRs	RIG-I-Like Receptors
RNA	Ribonucleic Acid
RNAi	Ribonucleic Acid Interference
RNS	Radical Nitrogen Species
ROS	Radical Oxygen Species
SAD	Sepsis Associated Delirium
SAPS II	New Simplified Acute Physiology Score
SCCM	Society of Critical Care Medicine
SERT	Serotonine Transporter
shRNA	short hairpin Ribonucleic Acid
SIRS	Systemic Inflammatory Response Syndrome
SIS	Surgical Infection Society
SLPI	Secretory Leukocyte Protease Inhibitor
SOFA	Sequential Organ Failure Assessment
ssRNA	single strand Ribonucleic Acid
STING	Stimulator of IFN Genes; MPYS; MITA; ERIS
SvO2	Venous O2 saturation
TBK1	TANK-Binding Kinase 1
TGFβ	Transforming Growth Factor-β
THP-1	Human Acute Monocytic Leukemia Cell Line
TIR	Toll/IL-1R Romology
TLR	Toll-like Receptor
TLS	Translesion DNA Synthesis
TNFα	Tumor Necrosis Factor α
TRC	The RNAi Consortium
TRIF	Toll/IL-1 Receptor Domain-containing Adaptor Inducing IFN-β
UTI	Urinary Tract Infection

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Acknowledgments

First I would like to express my recognition and deepest gratitude to Prof. Luís Moita, whom I had the pleasure to cross paths five years ago and accepted to supervise my PhD project. As a scientist, his exceptional knowledge and thoroughness combined with his M.D. background have provided me exceptional guidance, critical advice and an example to follow. I wish to thank him for believing in this project of physician-scientist and for his unconditional and continuous support. It is an honor to have you as a friend.

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We always stand in the shoulders of giants...

I thank my parents, Maria Elisabete e José Figueiredo, for all their love, for their sacrifice in giving their sons the best education and for being my role models. To my brother João and all my family, my gratitude for their unstoppable and steadfast support and loving care.

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Abstract

Sepsis remains a poorly understood systemic inflammatory condition with high mortality rates and limited therapeutic options in addition to organ support measures. Most often, this syndrome is triggered by a bacterial infection that causes excessive production of pro-inflammatory mediators, including the initial critical tumor necrosis factor (TNF) and interleukin 1β (IL-1β), leading to the activation of spiraling signaling cascades ultimately causing multi-organ failure and death.

To find compounds that modulate the systemic inflammatory response syndrome, we performed an *in vitro* drug screen aiming at identifying pharmacologic agents that could simultaneously inhibit the secretion of two key initiators of sepsis: TNF and IL-1 β .

Our attention was directed to the clinically approved group of anthracyclines, because in the top 20 of selected candidates, we found 3 representative agents of this group: epirubicin, doxorubicin and daunorubicin.

This drug screen has also identified additional anti-inflammatory drugs that have been further studied by our collaborators. For instance, gambogic acid and celastrol are currently being tested as treatment adjuncts in auto-immune arthritis models.

In vivo, we have observed that anthracyclines confer protection against severe sepsis induced by cecal ligation and puncture (CLP), a murine model of peritonitis.

The protective effect of epirubicin decreases tissue damage in the target organs (liver, lung and kidney) and dampens the circulating pro-inflammatory mediators such as TNF, IL-1 β and IL-6. This is also true for late inflammatory mediators like HMGB1, which is lower in the plasma and in the tissues.

Epirubicin is effective in other inflammatory models such as endotoxemia (induced by intraperitoneal LPS injection) and monostrain pulmonary infection (elicited by intra-nasal inhalation of bacteria).

Anthracyclines induce disease tolerance to infection (an increased capacity to cope with the same pathogen burden), as demonstrated by similar CFU counts in blood, spleen and target organs between the treated and non-treated groups. Additionally, this is supported by the fact that broad-spectrum antibiotic treatment delays mortality in CLP but does not preclude it, compared to epirubicin treatment.

Using an shRNA-based screen we identified the Ataxia Telangiectasia Mutated (ATM) as a mediator of the protective effect of anthracyclines. ATM deficient ($Atm^{-/-}$) mice are refractory to this protective effect succumbing to severe sepsis with similar kinetics to the non-treated wild-type mice. This protective effect relies on the activation of a DNA damage response and the autophagy pathway specifically in the lung, as demonstrated by deletion of the ATM or the autophagy-related protein 7 (Atg7) specifically in this organ.

Importantly, epirubicin specifically induces LC3b lipidation (a marker for autophagy activation) in the lung, a response that is low or absent in this organ in the absence of anthracycline treatment, but occurs spontaneously and transiently in the liver and also in the kidney, by sepsis induction alone.

According to the above mentioned, we propose that lung protection is an early priority in sepsis management.

These results support the hypothesis that ATM acts in mediating the induction of autophagy by epirubicin, which is critical for the protection conferred by this drug in the septic model that we investigated.

Our work has identified several original pharmacologic actions of anthracyclines, and particularly epirubicin: dampen inflammation; scavenge ROS; induce organ protection through the autophagy pathway. Together, these features might confer a useful addition to the standard of care treatments for septic patients.

We have further investigated the immune-suppressive phase that ensues sepsis and found that epirubicin treated mice are not immune-depressed with the used doses. This observation supports epirubicin use in the clinical setting of septic syndromes.

Our research suggests that epirubicin has a useful therapeutic window in sepsis.

If anthracyclines are administered in combination with a broad-spectrum antibiotic such as meropenem, they protect from CLP mortality even

if they are given only 24 hours after the initial procedure, provided meropenem is administered within the initial 12 hours after CLP.

The 24-hour therapeutic window that we tested might be sufficient to make this drug useful in the clinical setting. Especially to reduce organ dysfunction (and probably mortality) from sepsis in most patients who are either in the hospital or seek medical attention within the first few hours of symptoms initiation.

Under conditions that reflect the human standard of care of this pathology, epirubicin and more generally the group of anthracyclines may be effective at preventing mortality due to sepsis.

An additional important result of our work is the identification of ATM as a novel negative regulator of the inflammatory response and a critical determinant for inducing tolerance to the burden of infection, making it an attractive molecular target for novel therapies for inflammation-driven conditions.

Resumo

A sépsis é uma doença inflamatória sistémica cujos mecanismos moleculares ainda se encontram mal compreendidos. A sua elevada mortalidade é condicionada pelas poucas opções terapêuticas disponíveis, que não vão além de medidas de suporte de órgão em ambiente de unidade de cuidados intensivos.

A sépsis é frequentemente iniciada por uma infecção bacteriana, que por sua vez origina uma produção excessiva de mediadores pró-inflamatórios. No conjunto das citocinas pró-inflamatórias, o Factor de Necrose Tumoral (TNF) e a Interleucina 1β (IL-1β) são consideradas os mediadores críticos, nas primeiras horas deste processo. A libertação destas citocinas, pelas células efectoras do sistema imunitário, leva à activação descontrolada de vias de sinalização que podem ser responsáveis pela disfunção de vários órgãos, condicionando em última análise a morte do doente.

Iniciámos o nosso projecto de investigação por um "rastreio" farmacológico *in vitro*, com o objectivo de identificar compostos que inibissem simultaneamente os mediadores-chave neste síndrome: o TNF e a IL-1β. Deste modo, tentámos seleccionar drogas que modulassem o síndrome de resposta inflamatória sistémica. Um dos nossos melhores candidatos foi o grupo farmacológico das antraciclinas. Estes fármacos encontram-se aprovados, para uso clínico em doentes oncológicos, há já vários anos. Nos melhores 20

candidatos, observámos 3 compostos representantes deste grupo: a epirrubicina, a doxorrubicina e daunorrubicina.

O nosso "rastreio" farmacológico também identificou outros compostos anti-inflamatórios que estão actualmente a ser estudados por outros investigadores deste laboratório. A título de exemplo, o ácido gambógico e o celastrol estão actualmente a ser testados como agentes terapêuticos em modelos animais de artrite autoimune, com resultados muito positivos.

In vivo, observámos que as antraciclinas possuem um efeito protector no modelo de peritonite grave em ratinho: a laqueação e perfuração do cego (CLP).

A protecção conferida pela epirrubicina diminui a lesão tecidual nos órgãos mais afectados pela sépsis (fígado, pulmão e rim) e diminui os mediadores inflamatórios circulantes (TNF, IL-1β e IL-6). Este efeito verifica-se também nos mediadores pró-inflamatórios de libertação tardia, como demonstrado pela diminuição do HMGB1, quer em circulação, quer nos tecidos.

A epirrubicina é também protectora noutros modelos inflamatórios, como o da endotoxémia (induzido pela injecção intraperitoneal de LPS) e o de pneumonia bacteriana (provocado pela inalação de uma estirpe bacteriana).

As antraciclinas induzem tolerância à infecção (uma capacidade superior para suportar a mesma dose de patogéneos), conforme se pode demonstrar pelas contagens semelhantes de bactérias (CFUs) em circulação, no baço e nos órgãos alvo (fígado e rins), em animais sujeitos a CLP, entre os grupos tratados com epirrubicina e os não tratados. Além do atrás exposto,

verifica-se que o tratamento com antibiótico de largo espectro, comparativamente ao tratamento com epirrubicina, atrasa a mortalidade na sépsis grave mas não a impede.

Através de um novo "rastreio" genético, *in vitro,* utilizando tecnologia com shRNA, identificámos o gene Ataxia Telangiectasia Mutated (ATM) como sendo o mediador deste efeito protector das antraciclinas. Os ratinhos deficientes neste gene ($Atm^{-/-}$) são resistentes a este efeito protector e morrem com uma cinética semelhante aos ratinhos controlo não tratados com epirrubicina. Ratinhos $Atm^{-/-}$, tratados com epirrubicina, apresentam lesão de órgão e mediadores pró-inflamatórios semelhantes aos não tratados.

Este efeito protector das antraciclinas parece depender da activação de uma resposta reparadora de lesões do DNA e da via da autofagia. Este mecanismo é sobretudo importante no pulmão, como demonstramos pela remoção selectiva e específica do gene ATM, ou da proteína envolvida no processo de autofagia ATG7, no tecido pulmonar de ratinhos.

A epirrubicina induz especificamente a lipidação da proteína LC3b (marcador de activação da via da autofagia) no pulmão. Na ausência de qualquer tratamento com antraciclinas, esta resposta não se verifica neste órgão, mas ocorre espontaneamente e transitoriamente no fígado e rins de animais apenas submetidos a sépsis grave.

As nossas observações levam-nos a propor que a protecção do pulmão e da função respiratória são uma prioridade fulcral no tratamento da sépsis grave e do choque séptico.

No modelo animal de sépsis grave e choque séptico, os nossos resultados suportam a hipótese de que a indução da autofagia, mediada pelo ATM, é um dos mecanismos críticos do efeito protector da epirrubicina.

Os resultados do nosso trabalho evidenciaram novos efeitos farmacológicos das antraciclinas, e particularmente da epirrubicina: composto anti-inflamatório; captador e quelante de radicais livres de oxigénio; protector da lesão de órgão pela indução da via da autofagia. Em conjunto, estas características podem adicionar alguma vantagem, à melhor terapêutica instituída, no contexto do tratamento de doentes sépticos.

Investigámos ainda a fase de imunossupressão que se segue à fase pró-inflamatória na sépsis. Observámos que a epirrubicina, nas doses utilizadas, não agrava a imunodepressão característica deste período. Este resultado suporta o seu potencial uso clínico nos síndromes sépticos.

A nossa investigação no modelo animal sugere que a epirrubicina pode actuar numa janela terapêutica. As antraciclinas têm ainda um efeito protector, mesmo que só administradas 24 horas depois do início do quadro séptico, desde que associadas a um antibiótico de largo espectro (como o meropenem).

A janela terapêutica que testámos (24 horas) poderá ser suficiente para tornar esta droga útil no contexto clínico. Especialmente quando utilizada com o objectivo de reduzir lesão de órgão (ou mesmo a mortalidade), em doentes que se encontram internados no hospital, ou que procuram assistência médica nas primeiras horas após o início dos sintomas.

Em humanos, a epirrubicina poderá ser eficaz na prevenção da mortalidade induzida pela sépsis, quando associada à melhor terapêutica disponível, habitualmente administrada no tratamento desta patologia.

Os nossos resultados identificaram ainda o gene ATM como um novo regulador negativo da resposta inflamatória e um determinante crítico na indução da tolerância à infecção. Estas características tornam-no num potencial alvo de terapêutica molecular em situações clínicas de causa inflamatória.

Chapter I - Introduction

Sepsis Definition

Sepsis is derived from the Greek word "σηψιζ" that means putrefaction or decomposition. It appears in literature even before Hippocrates [1]. Leeuwenhoek described the so-called "animalcules", in the 1680s. But it was only after the works of Koch, Pasteur, Semmelweiss and Lister that bacteria were related to infection, and Microbiology was born. In the field of Sepsis, Schottmueller reported in 1914 that the existence of pathogenic germs in the blood was responsible for systemic signs, defining the term septicemia as "... a state of microbial invasion from a portal of entry into the blood stream which causes sign of illness..." [2].

The terms sepsis, septicemia and septic shock have been used interchangeably for the last 50 years. It was only in 1991, that a consensus conference held by the Society of Critical Care Medicine (SCCM) and the American College of Chest Physicians (ACCP), tried to define the concept of sepsis [3].

The problem of not existing a unified definition for sepsis created complications in patient selection and stratification regarding clinical trials and research. The term Systemic Inflammatory Response Syndrome (SIRS) was introduced to define the response that was also observed in patients subjected to trauma, burns, pancreatitis or ischemia, but with no infection.

Sepsis includes a continuum of conditions ranging from systemic inflammatory syndrome (SIRS) to septic shock [4].

Table I - Definition of Sepsis and Sepsis Syndromes

SIRS	**(at least 2 present)**	**1) Heart rate > 90 beats/minute** **2) Respiratory rate >20 breaths/min** **3) Body temperature >38°C or <36°C** **4) Leukocytes >12.000/mm^3 or <4.000/mm^3**
Sepsis	**SIRS +**	**Documented or strongly suspected infection**
Severe Sepsis	**Sepsis +**	**Any organ dysfunction or tissue hypoperfusion**
Septic Shock	**Severe Sepsis +**	**Persistent hypotension despite adequate fluid resuscitation**

The mortality risk increases from sepsis to septic shock, as organ dysfunctions are added.

These definitions remain operational after 20 years and still allow a standard for clinical trials [4].

Throughout these years a doubt has persisted and in present days it is still not answered if sepsis and septic shock are individual manifestations of a single syndrome, or if they represent a spectrum of syndromes in which they are the limit points.

An international consensus conference was held in 2001 by the SCCM, the ACCP, the American Thoracic Society (ATS), the European Society of Intensive Care Medicine (ESICM), and the Surgical Infection Society (SIS), and tried to address some complementary questions.

Experts in the field tried to improve the definitions so that they could reflect the current understanding of the pathophysiology of these syndromes [5]. While the 1991 definitions are very useful to clinicians and researchers, they are insufficient to stratify or prognosticate the individual response to insult. SIRS definition is very practical, but excessively sensitive and non-specific. This is more evident in surgical patients, because most of them exhibit postoperative SIRS, making it very difficult to distinguish from secondary sepsis.

The consensus proposed a change from the short four-point definition of SIRS to a broader list of signs that would diagnose sepsis.

Table II - Diagnostic criteria for sepsis

DIAGNOSTIC CRITERIA FOR SEPSIS (adapted from Levy et al., 2003) Presence of infection, documented or suspected, and:	
General Signs and Symptoms	**Fever; Hypothermia** **Tachypnea; Respiratory alkalosis**
Inflammatory Reaction	**Leukocytosis or leukopenia** **Increased C reactive protein, Procalcitonin, IL-6**
Hemodynamic and Tissue Perfusion Alterations	**Hypotension** **Tachycardia** **Increased cardiac output; Wide pulse-pressure; Low systemic vascular resistance; High SvO2**
Signs of Organ Dysfunction	**Hypoxemia; Acute lung injury (ALI)** **Altered mental state** **Altered renal function; Oliguria; Positive fluid balance; Edema** **Unexplained hyperglycemia** **Thrombocytopenia; Disseminated intravascular coagulation (DIC)** **Altered liver tests (hyperbilirubinemia)** **Intolerance to feeding (ileus)**

This conference opened the way to establish a new concept in staging sepsis, reproducing what is well established in Oncology with the TNM staging system. This staging model for risk stratification in severe sepsis was named

PIRO and stands for Predisposition of the host, Insult/infection, Response, and Organ dysfunction (PIRO).[6] It is also a method devised to reduce the heterogeneity of this disease process.

PIRO Concept

The PIRO concept is born from the attempt to create a staging model for risk stratification in severe sepsis. The goal was to discriminate distinct levels of mortality risk within each of the four categories (P, I, R, and O). In each category, points were to be attributed and risk levels would be able to predict mortality, independent of the risk already calculated in other categories [6].

Predisposing Factors

The age of the host is obviously important to dictate the course of infection. Patients with co-morbidities that lead to an immune-depressed state or receiving immune-suppressive therapy have different features. Genetic traits that progress with increased susceptibility to infection can be: complement deficiencies [7], neutrophil defects, mutations in PRR molecules [8 9], and differences in cytokine expression [10 11].

Infection

The type of microorganism that causes the infection and the site of infection are key determinants of the outcome. Urinary tract infections (UTIs) have considerable less mortality rates than pulmonary or abdominal sepsis [12].

The volume of inoculum and the virulence of the pathogen are also important to consider.

Response

The reaction to infection is different between patients and temporally fluctuates in the same patient. An assessment of the inflammatory and immune response of the host can be evaluated indirectly by elevation in leukocytes, levels of C reactive protein and procalcitonin. In a near future, improvements in genomics and proteomics may allow a correct evaluation of a patient's immune response status [13].

Organ Dysfunction

Sepsis mortality is tightly correlated with the degree and number of failing organs. There are several scoring systems, but the most commonly used in ICUs is Sequential Organ Failure Assessment (SOFA). This is a continued care score as it is used to track a patient's status, based on the sum of six different scores, for the respiratory, cardiovascular, hepatic, coagulation, renal and neurological systems. Other scores evaluate the severity of global pathophysiologic changes and are able to assess the mortality risk. The Acute Physiology and Chronic Health Evaluation II (APACHE II) [14] and the New Simplified Acute Physiology Score (SAPS II) [15] are frequently used examples.

Briefly, in spite of the impressive scientific advances and biomedical research in this field, clinicians continue to depend on a nonspecific blend of clinical signs and biochemical anomalies to diagnose sepsis and sepsis syndromes.

Epidemiology of Sepsis Syndromes

Epidemiology studies in sepsis have one major caveat. It was only very recently that a broad unifying definition of sepsis and sepsis syndromes was achieved and spread in the literature. As epidemiology relies mainly on retrospective analysis, almost all major studies are hindered by this limitation. Nevertheless, very important conclusions can still be drawn and new consistent and uniform evidence keeps building up every year. Epidemiology is of paramount importance to determine health care policies, the allocation of health care resources, and the distribution of funding for sepsis research.

Sepsis affects over 18 million patients worldwide. It is the leading cause of death in intensive care units (ICU), excluding neurotrauma, and the third most important cause of death in the hospital [16].

There are no portuguese data publicly available to assess national incidence, the most common pathogens involved and national mortality of this disease. We rely in international data for imperfect extrapolation.

Incidence

The annual incidence of sepsis reported in the literature has worldwide variations, but regularly stands between 50 and 100 cases per 100,000 inhabitants in industrialized countries. The degree of discrepancy depends on

the study design and varies in terms of inclusion/exclusion criteria, timing of inclusion, diagnosis criteria of sepsis and even bed availability and ICU admission policy.

In European countries, the estimated annual incidence of severe sepsis fluctuates from 51 cases per 100,000 population (England and Wales) to 95 cases per 100,000 population (France) [17, 18]. In 2004, the data from Australian and New Zealand intensive care units documented 77 cases of severe sepsis per 100,000 inhabitants [19]. When severe sepsis is defined as a diagnosis of sepsis and acute organ dysfunction, the incidence of severe sepsis in the United States, during 1997-2002 was 91 cases per 100,000 population [20].

The incidence has been increasing steadily 1% each year. In the next decades, as life expectancy increases, it is projected to increase 1.5% each year[21]. The frequency of cases and deaths related to severe sepsis exceeds the numbers of persons with breast cancer and AIDS.

Economic Issues

Each case spends an average of $22,100, with total costs of $17 billion/year in the United States. Costs are typically higher in children, intensive care unit patients, mortality cases, surgical patients, and individuals with organ dysfunction [21]. However, these figures relate only to direct costs of treatment in the hospital, which contribute to about 20-30% of the total costs involved in Severe Sepsis. When the indirect costs are accounted, mainly related to loss of productivity and need for prolonged continued care, the bill rises [22].

Causative Agents

Microbiologic cultures of septic patients are usually positive in only 46%-58% of the patients.

Abdominal infections are typically polymicrobial, a consequence of the saprophytic flora of the gut. Gram negatives account for 26,5% of isolated agents in abdominal sepsis, with *Escherichia coli* (15,4%) and *Pseudomonas spp* (11,7%) being the major representatives. In patients with abdominal infections, the most commonly isolated Gram-positive organisms are *Streptococcus spp* (24,1%) and *Staphylococcus spp (*22,2%*)*. *Candida spp* is also present in 19,2% of these patients [23].

The occurrence of secondary infections is also greater in patients admitted in ICUs with abdominal infections (43%) than in infections of other source. Secondary respiratory, skin or wound-related and bloodstream infections are common. In this population, the development of secondary infections significantly increases severity of illness and ICU length of stay.

Mortality

Hospital mortality from sepsis has ranged from 25% to 80% over the last few decades.

Mortality rates are correlated with the number of organs failing and ranges from less than 10% in systemic inflammatory response syndrome (SIRS), 30% in sepsis, 45% in severe sepsis to almost 70% in septic shock. Organ

dysfunction has a cumulative effect: 15% mortality without organ failure versus 70%, with three or more organs failing [24].

Considering organ dysfunction in severe sepsis, lungs fail in 18% percent of patients, kidneys in 15%; and less frequently occurs cardiovascular failure (7%), hematologic failure (6%), metabolic failure (4%) and neurologic failure (2%).

The French EPISEPSIS group reported an overall mortality of 35% at 30 days, rising to 41.9% by 2 months, and by that time 11.4% of patients remained hospitalized.

Among other variables, mortality is also dependent on age, sex and co-morbidities. In children it starts from 10%, reaching 38% in patients over 85 years old. Women present with lower age-specific incidence and mortality. [21]

The site of infection influences mortality, with respiratory, gastrointestinal, urinary tract and primary bloodstream infections representing 80% of all infections admitted in ICUs. Sepsis from an abdominal or respiratory origin has the highest morbidity and associated mortality.

Patients with abdominal sepsis present frequently with septic shock, coagulation failure and renal dysfunction, comparing to respiratory sepsis that is more common to have neurologic symptoms and signs. Although median length of ICU stay is comparable in the two groups, the median length of hospital stay is considerably longer in the abdominal sepsis population [23].

For the survivors, sepsis worsens underlying co-morbidities and significantly reduces quality of life.

Inflammation, Immunology and Pathophysiology

Inflammation can be defined as an evolutionary conserved response to harmful stimuli. Its main purpose is to mount an adaptive reaction to limit tissue damage and at the same time restore homeostasis [25].

Traditional stages of an inflammatory response comprise:

1) Recognition of infection or tissue damage;
2) Recruitment of cells to the site of injury;
3) Elimination of the causative agent;
4) Resolution of inflammation and return to homeostasis.

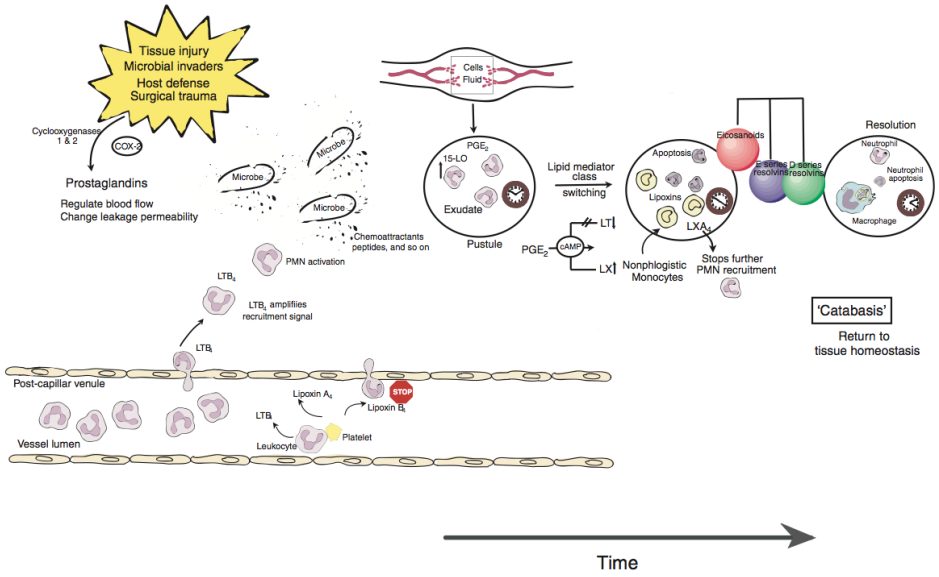

Figure 1 - Evolving inflammation: initiation and resolution. (Adapted from Serhan and Savill, 2005)

Inflammation has diverse triggers. Infection, tissue injury (trauma, burns, ischemia-reperfusion lesion) and even chronic tissue stress can elicit a local inflammatory response by tissue-resident macrophages and mast cells.

Classic manifestations of inflammation are redness, swelling, heat, pain and conceivably loss of tissue function, which reflect increased vascular permeability allowing for extravasation of serum components and immune cells.

Recognition

To control infection, complex innate immune pathways that recognize the invading microorganisms elicit an inflammatory response. They work interdependently and synchronously.

In innate immunity, the molecular biology of this recognition step is different from the detection of microbial components by antigen receptors of T and B lymphocytes [26]. Innate immune cells cannot recognize each individual pathogen and react specifically to it. Instead they recognize general molecular patterns, microbial “motifs” rather than exact features of each pathogen [27]. These microbial features, pathogen-associated molecular patterns (PAMPs) are molecular structures that are found in microorganisms but not in the host. PAMPs are structures indispensable for pathogen survival and aggressive behavior, so that microorganisms cannot “erase” them and escape immune recognition.

Innate immune receptors are germline encoded and recognize these patterns leading to the activation the effector cells [28]. Toll-like receptor 4

(TLR4) was the first pattern recognition receptor (PRR) to be identified [29]. It detects lipopolysaccharide (LPS) of Gram-negative bacteria. Several families of these gatekeepers of inflammation have been identified (Figure 2).

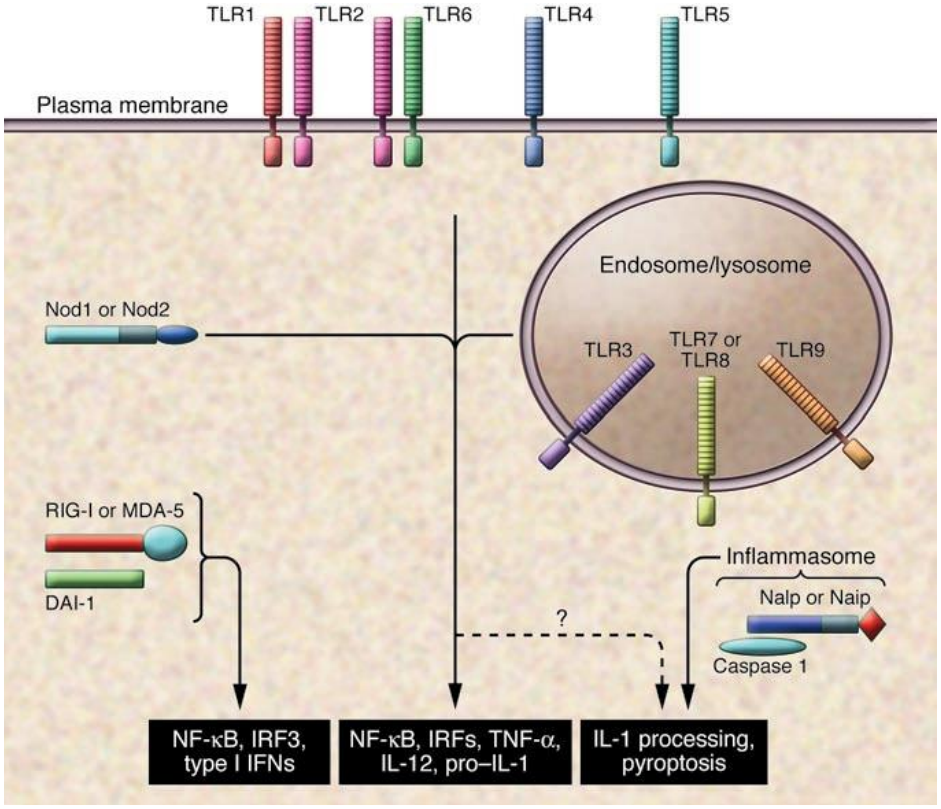

Figure 2 - Pattern recognition receptors and inflammation. (Adapted from Barton, 2008)

Toll-like Receptors (TLRs)

TLRs are an ancient family of transmembrane receptors present in phagocytic cells (macrophages, neutrophils and dendritic cells - DCs) and recognize conserved components of bacteria, viruses, fungi and protozoa to activate these cells. TLRs are constituted by a N-terminal leucine-rich repeats

(LRRs), a transmembrane region and a cytoplasmic Toll/IL-1R homology (TIR) domain. To this day, 10 TLRs have been described in humans and 12 in mice.

The signaling cascade activated by Toll-like receptors culminates in the activation of NF-κB and IFN-regulatory factor (IRF) transcription factors [30]. These transcription factors not only drive the expression of pro-inflammatory genes (TNFα and IL-1) but also induce the cascade of signals that activate adaptive immunity. The localization of TLRs in the cell reflects their function in ligand recognition. TLRs that detect self-nucleotides are compartmentalized to prevent unnecessary activation, and possibly induce constant autoimmunity responses. TLRs 1,2,4,5 and 6 are located on the plasma membrane and TLRs 3,7 and 9 are located in endosomal compartments (Figure 3).

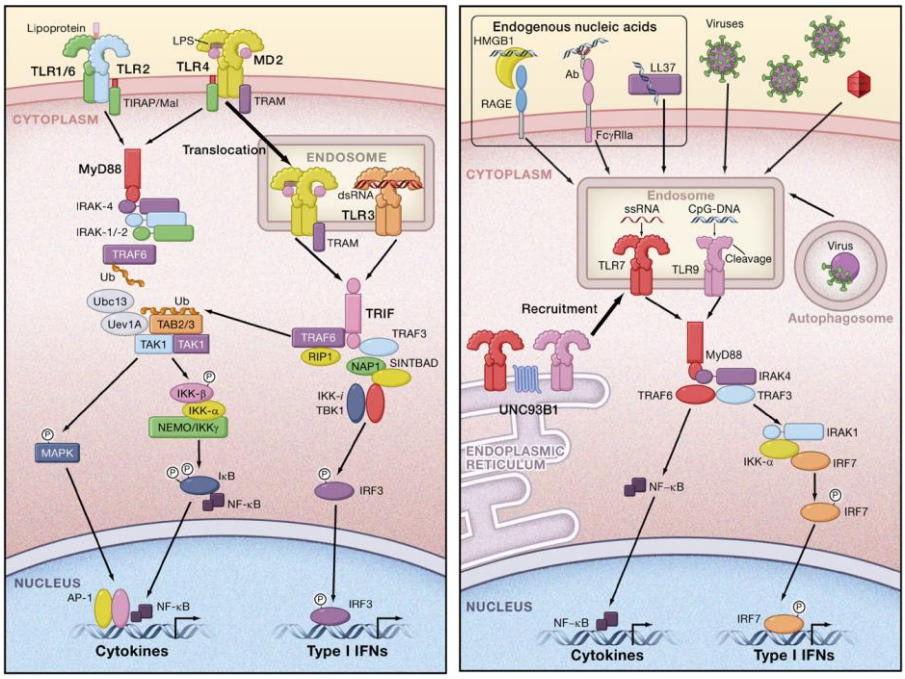

Figure 3 - Toll-like receptor signaling pathways: Bacterial components and Nucleic Acids sensing by TLRs. (Adapted from Takeuchi and Akira, 2010)

PAMP sensing by TLRs activates transcriptional induction of different genes, depending on the TLR and cell type that is affected, activating distinct signaling pathways. The MyD88-dependent signaling pathway and the TRIF-dependent signaling pathway are key players in activating NF-κB and Type I IFN response genes.

Several endogenous proteins act as chaperons in delivering ligands to TLRs located in endosomes and lysosomes. For instance, the nuclear DNA-binding protein high-mobility group box 1 (HMGB1) has been associated in TLR recognition and signaling pathways. HMGB1 can be passively released by necrotic cells or actively secreted by macrophages and monocytes [31]. It can bind, stabilize and present genomic DNA released from necrotic cells to TLR9, acting as an amplifier of the inflammatory reaction during sepsis and sepsis-like syndromes by stimulating TLR9.

NOD-like Receptors (NLRs)

A large family of cytosolic PRRs is involved in the detection of pathogens that are capable of penetrating host cells, called nucleotide-binding oligomerization domain-like (NOD-like) receptors (NLRs). They also activate NF-κB and prompt the expression of pro-inflammatory cytokines [32].

A subclass of NLR family, which includes the NLR family apoptosis inhibitory proteins (NAIPs) and NACHT-, LRR-, and pyrin domain-containing proteins (NALPs), regulates activation of the Inflammasome, a multiprotein complex involved in activating Caspase-1, a protease that processes pro-IL-1 into the mature active form that is then actively secreted [33]. NAIPs and NALPs recognize bacteria, bacterial RNA, uric acid crystals, bacterial toxins and

flagellin that are present in the cytosolic compartment after bacterial penetration or protein injection across the cellular membrane.

NLR and TLR pathways are interrelated and apparently seem to reinforce each other in multiple hubs. It is known that NLRs can synergize with TLRs to enhance cytokine production and TLR activation regulates the activity of the inflammasome[34].

RIG-I-like receptors (RLRs)

The cytosolic PRRs class of RIG-I-like receptors (RLRs) is involved in identification of nucleic acids. The name derives from the founding member of this family: retinoic acid-inducible gene I (RIG-I) [35]. RLRs have 2 N-terminal caspase recruitment domains (CARDs), a central DEAD box helicase/ATPase domain and a C-terminal regulatory domain.

RIG-I and melanoma differentiatiation-associated protein-5 (MDA-5) sense foreign RNA in the cytosol through their RNA helicase domains. The innate immune system recognizes foreign DNA in the cytosol through a group of proteins called DNA-dependent activator of IFN-regulatory factors (DAI) [36].

Unlike the relationship of TLRs and NLRs, there is not yet evidence that RLR pathways contribute to inflammasome activation, nor that there is interconnection between TLRs and RLRs.

C-type lectin receptors (CLRs)

This family of transmembrane receptors sense carbohydrate motifs in viruses, bacteria and fungi. C-type lectin receptors (CLRs) perform their

signaling transmission by activating expression of pro-inflammatory cytokines or by inhibiting TLR-mediated immune complexes.

Damage-associated receptors

Tissue damage and cell death can occur without infective organisms, as in the event of trauma, ischemia and ischemia reperfusion. The inflammatory syndrome associated with these injuries can be as lethal as in infection driven inflammation, leading to circulatory collapse and multiple organ dysfunction [37]. TLRs and other PRRs seem to recognize mediators of this sterile injury. Although surrounded by a certain degree of controversy, it appears that endogenous ligands like heat shock proteins (HSPs), defensins, uric acid crystals among other biomolecules can stimulate PRRs without the presence of microorganisms [38, 39].

Recruitment of Immune Cells / Elimination of Causative Agent

Tissue-resident macrophages and DCs detect and phagocyte the invading microorganism. When PRRs of these immune cells sense PAMPs they initiate inflammatory signaling pathways and start secreting cytokines, chemokines and preformed secondary mediators. These molecules act as danger signals to other immune cells in order to promote the clearance of infectious agents by recruitment of other cells.

Tissue damage, caused by any injury, is itself recognized by the immune system. Endogenous damage-associated molecular patterns (DAMPs) are “self” danger signals released by injured tissues [40].

Primarily, the secretion of cytokines, chemokines, vasoactive amines and proteolytic enzymes transform the infected site to an inflamed site. Mainly TNFα, IL-1 and lipid mediators alter the endothelium of capillaries and venules allowing for plasma proteins and leukocytes to extravasate locally, while preventing erythrocyte exit.

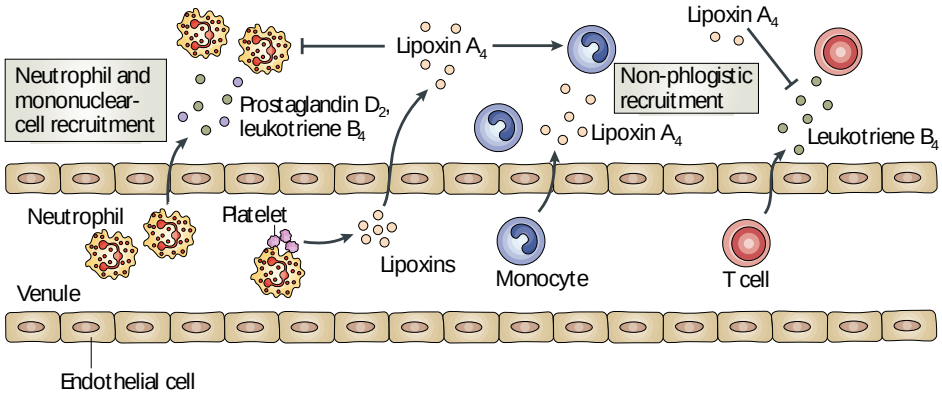

Figure 4 - Neutrophil recruitment is mediated by endogenous and exogenous chemoattractant gradients. Leukotrienes amplify the process. (Adapted from Serhan et al., 2008)

Neutrophils arrive within minutes to hours, followed by a posterior influx of monocytes [41]. After activation, neutrophils start to attack the "invaders" with the contents of their granules: radical oxygen species (ROS), radical nitrogen species (RNS), proteinases, cathepsins and elastases. The later serine proteases are able to breakdown components of the extracellular matrix and destroy host cells leading to tissue destruction. Neutrophil granule

contents do not discriminate between microbial and "self" targets, so a great deal of collateral damage and mayhem ensues [42].

The organism mounts this somewhat counterintuitive toxic response in an attempt to contain the infection at a critical initial phase, before a full immune response has been established. Afterwards, neutrophils enter in apoptosis and are then cleared by macrophages.

Macrophages arrive at the site of injury, attracted by the same signals as neutrophils, besides phagocytosis of apoptotic neutrophils they also contribute to the killing of pathogens. They engulf and degrade microorganisms using proteases, antimicrobial peptides, ROS and RNS.

Resolution and Tissue Repair

As the immune cells start to deal with the infection, a tissue-repair response simultaneously begins and as the aggression is controlled, a resolution phase commences. A class switch of arachidonic acid-derived eicosanoids from pro-inflammatory prostaglandins and leukotrienes to anti-inflammatory lipoxins mainly orchestrates this resolution stage. This step is also dependent of resolvins, protectins and growth factors (especially transforming growth factor-β: TGFβ).

Lipoxins act as pro-resolution agents by blocking the neutrophil influx, recruiting monocytes that clear the cellular and microbial debris, and beginning tissue remodeling [43].

Macrophages, neutrophils and epithelial cells produce secretory leukocyte protease inhibitor (SLPI), which inactivates the granulocyte serine proteases and additionally drives the inflammatory response towards resolution [44].

Autophagy - Regulator of Inflammation

The cellular machinery involved in protein degradation has always been connected to the control of innate immune responses [45, 46]. The selective protein degradation complex of Ubiquitin-Proteasome has been studied for a long time, but recent interest has fallen upon another clearance system - Autophagy [47].

This system is responsible for bulk degradation of proteins and delivers cytoplasmic contents to lysosomes. This system reuses intracellular constituents, providing an amino acid pool during starvation periods [48]. Mice that are deficient for autophagy-related protein (Atg) 3, Atg5, or Atg7 die within 1 day of birth, implying the importance of autophagy in the starvation period that ensues the neonatal period [49, 50].

Autophagy clears old and damaged organelles, degrades protein aggregates and lipid vesicles, contributing this way to cellular homeostasis, control cell death/survival cycles and lipid metabolism.

ATGs have been identified by yeast genetic screening, they compose a complex autophagic machinery, and are highly conserved proteins that act in host defense by inducing pathogen degradation and generate acquired

immunity [51]. Mammalian counterparts have been recently identified: ULK1 (Atg1); Atg3-5; beclin (Atg6); Atg7; LC3 (Atg8); Atg9a; Atg10; Atg12; Atg13L; Atg14L; Atg16L; FIP200 (Atg17); and WIPI-1 (Atg18) [52, 53, 54, 55, 56, 57, 58].

A coordinated action of Atgs is necessary to mediate membrane trafficking and autophagossome formation (Figure 5).

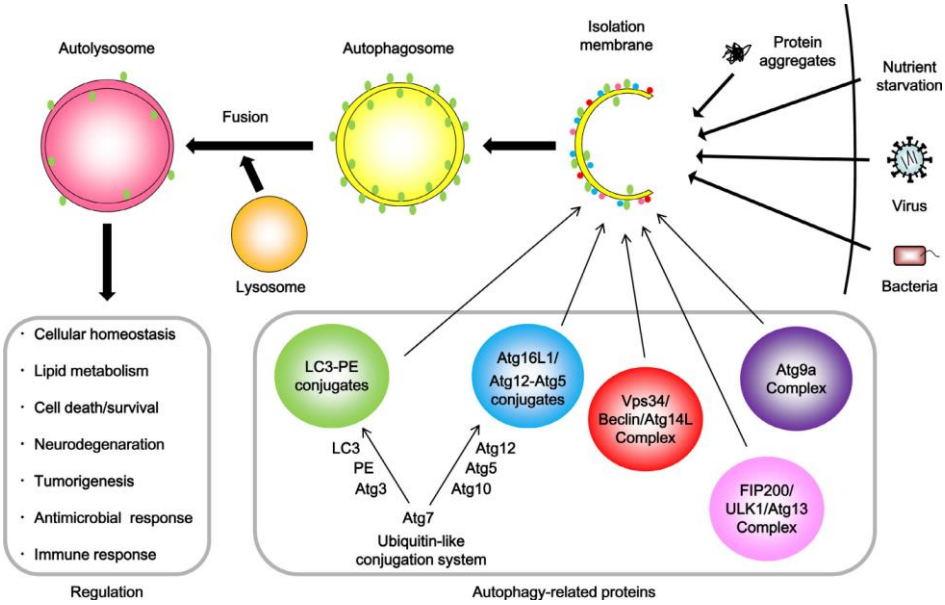

Figure 5 - Autophagy has a central role in regulating inflammation and immune mediated responses to infection. (Adapted from Saitoh and Akira, 2010)

More than 18 ATGs have been identified in mammals, with increasing functional roles being discovered [59].

The autophagic machinery is involved in:

1. ***Direct elimination of infectious agents:***
    a. Autophagosome formation - Sequestosome 1 (SQSTM1)/p62 and LC3 are recruited to bacteria-containing ubiquitinated vacuoles upon infection and promote the killing of invading pathogens [60, 61]
    b. Intracellular trafficking of anti-bacterial proteins (Irga6), from the ER-Golgi to the microbe-containing vacuoles, promoting the elimination of intracellular pathogens like *Toxoplasma gondii* [62]

2. ***Antigen presentation to antigen-specific T cells:***
    a. MHC class II molecules are located on autophagosomes and the autophagic complex facilitates presentation of viral and self-antigens by MHC class II molecules to $CD4^+$ T cells [63, 64]
    b. Autophagy regulates MHC class I-dependent presentation of viral antigens to $CD8^+$ T cells, as in the case of human herpes virus 1 (HHV1) infection [65]

3. ***Innate immune responses elicited by engagement of PRRs and pathogen components***

Autophagy and Inflammation

Genome-wide association studies identified Atg16L1 as a candidate gene responsible for susceptibility to inflammatory bowel disease - Crohn's disease [66]. When intestinal epithelial cells are damaged, commensal bacteria permeate this protective layer and can activate PRRs, inducing intestinal inflammation.

Atg16L1 is necessary to control endotoxin-induced inflammatory responses, especially pro-inflammatory cytokine secretion [67]. Macrophages derived from Atg16L1 KOs secrete higher amounts of IL-1β and IL-18 in response to LPS. Atg7-deficient macrophages also show enhanced production of IL-1β.

Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF), an adaptor molecule involved in TLR3/4 signaling pathways, is also mediating the synthesis of IL-1β in Atg16L1 deficient macrophages (Fig. 8). ATP, uric acid crystals, silica and asbestos can produce the synthesis of ROS, promoting the activation of caspase-1 [68, 69] (Figure 6).

Loss of autophagy can result in increased ROS in immune-competent cells due to mitochondrial turnover disruption [70].

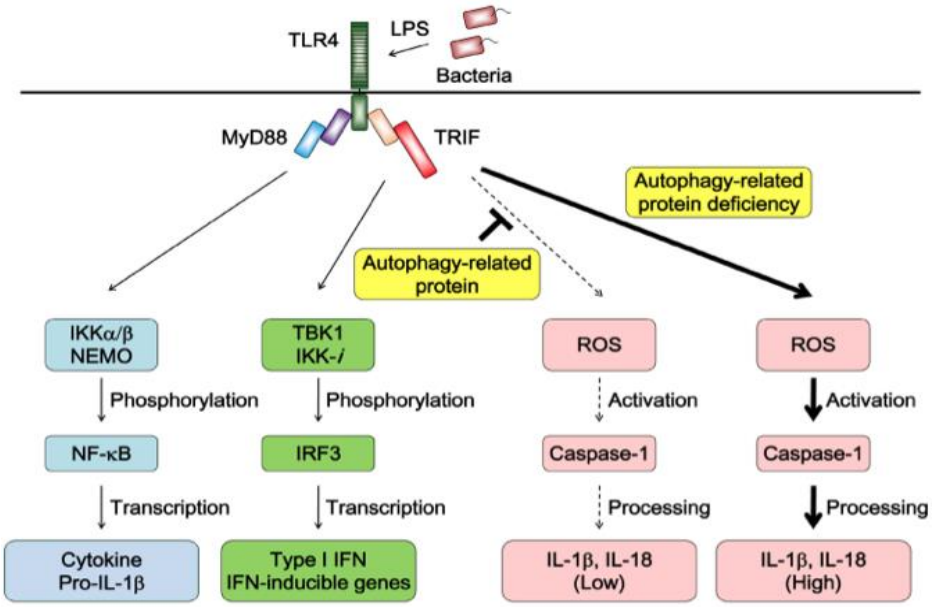

Figure 6 - Atgs regulate LPS-induced IL-1 production. TLR4 triggers both MyD88- and TRIF-dependent signaling pathways after activation by LPS. The IKK-α - IKK-β - NEMO complex activates transcription factor NF-κB, which induces the transcription of pro-inflammatory cytokines, namely pro–IL-1. The TBK1–IKK-ι complex mediates the activation of the transcription factor IRF3, which promotes transcription of type I IFNs and IFN-inducible genes. In autophagy-deficient cells, high levels of ROS are generated, which mediate TRIF-dependent caspase-1 activation, resulting in increased processing of IL-1. However, in wild-type macrophages, LPS induces limited amounts of IL-1 as the result of a lack of ROS generation. (Adapted from Saitoh and Akira, 2010)

Autophagy and TLR signaling pathways

TLR activation prompts phagosome maturation after bacterial exposure and promotes MHC class II-dependent bacterial antigen presentation [71, 72].

In macrophages, certain components of fungi cell wall stimulate TLR2 and induce the fusion of LC3-positive phagosomes with lysosomes. Interestingly, MyD88 is not necessary for the recruitment of LC3 to phagosomes, suggesting

an alternative pathway for TLR2-dependent maturation of phagosomes[73]. However, TLR2 signaling is necessary but not sufficient for the induction of phagosome maturation and the mechanism by which ATGs promote the fusion of phagosomes with lysosomes is not well understood.

Activation of TLR7 induces autophagy and the elimination of *Bacillus Calmette-Guerin* with the formation of autolysosomes [74]. After TLR7 stimulation, Atg5 and beclin are necessary to induce autophagy in macrophages. Although MyD88 is not necessary for the formation of phagolysosomes after zymosan activation, it is involved in the complex of autolysosomes [73].

In macrophage cell lines, mycobacteria elimination is also reliant on TLR4 activation and PI3K-dependent formation of LC3 positive autophagosomes [75]. There is evidence that LPS stimulation increases the number of autophagosomes in human monocytes and that TRIF, RIPK1 and p38 signal transducers are required for TLR4-induced formation of LC3 positive autolysosomes.

Autophagy and Anti-bacterial response

Nod1 and Nod2, acting as intracellular sensors, recognize specific bacterial components as γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP), inducing the antibacterial defense with antimicrobial peptides and cytokine production. It has been described a strong association between Nod2 mutations and Crohn's Disease [76].

Nod1 has also been implicated in the same inflammatory bowel disease, as an increased susceptibility gene in Crohn's [77]. The immune response

to bacterial polypeptides and microbial infection are impaired in patients harboring Nod2 mutations, and Nod1 and Nod2 deficiency results in enhanced intestinal inflammation upon bacterial infection[78]. Interestingly, DCs expressing Crohn's disease-associated Nod2 or ATG16L1 variants fail to induce autophagosome formation and antigen presentation in response to MDP.

NLRs can trigger autophagy in macrophages and lymphoblasts, and Nod1 and Nod2 are specifically involved in the formation of bacterial autophagosomes [79] (Figure 7).

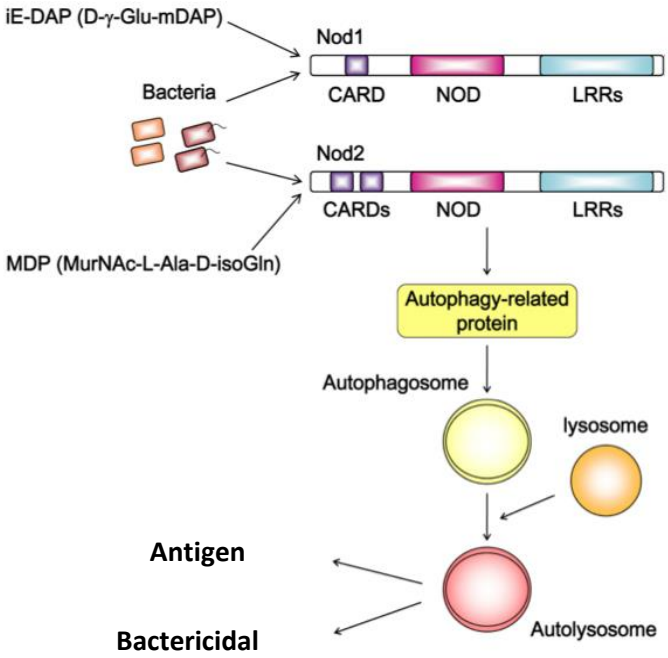

Figure 7 - Autophagosome formation triggered by Nod1 and Nod2 activation. Leucine-rich repeats of Nod1 and Nod2 detect iE-DAP and MDP, respectively. After sensing these ligands, Nod1 and Nod2 induce the formation of autophagosomes, leading to the promotion of antigen presentation and enhancement of bactericidal responses. Atg5, Atg7, and Atg16L1 are involved in Nod1- and Nod2-mediated formation of autophagosomes. (Adapted from Saitoh and Akira, 2010)

Nod1 and Nod2 are not necessary to form autophagosomes induced by nutrient starvation and rapamycin treatment, hinting that they are specifically involved in the formation of bacterial autophagosomes and antibacterial response processes. Moreover, both Nod1 and Nod2 associate with Atg16L1 and recruit it to bacterial entry sites after infection.

Autophagy and Anti-viral response

In host defense against RNA viral infection, type I IFNs (IFN-α and IFN-β) initiate the expression of antiviral factors and establish an antiviral response state [45].

Autophagy is necessary for TLR-7-dependent production of type I IFNs and cytokines to cope with RNA viruses' infection [80]. DCs detect ssRNA of RNA viruses via TLR7, expressed in lysosomes. TLR7 activates IRF7, which elicits IFN stimulation-responsive element-dependent transcription mediated by MyD88 and starts producing type I IFNs.

RLRs sense the cytoplasmic dsRNA of RNA viruses and transfer signals to IPS-1 (CARD-containing mitochondrial protein), which activates IRF3 and NF-κB, leading to the production of type I IFN and the expression of IFN-inducible genes.

It has been documented that a cellular deficiency in autophagy, mediated by a change in RLR-IPS-1 signaling pathway, induces a disturbance in cellular homeostasis [81]. When there is Atg5 deficiency and loss of autophagy, old and damaged mitochondria accumulate and IPS-1 is overexpressed. RLR activation induces an excess of ROS by the accumulated mitochondria, consequently over-activating IPS-1-dependent innate immune responses.

Atg9 and double-stranded DNA-induced immune responses

Bacterial DNA induces the expression of a type I IFN response and the secretion of pro-inflammatory cytokines, activating a strong innate immune response [46].

The receptor for single-stranded DNA - TLR9 - is involved in the protection of DNA virus infections and the ligands for TLR9 can efficiently induce an acquired immune response after vaccination.

Meanwhile, dsDNA derived from bacteria and DNA viruses, and host genomic DNA from dying cells, can activate a type I IFN response and induce the expression of IFN-inducible genes through a TLR-independent manner [82, 83].

The specific dsDNA sensors are still to uncover, but there is evidence that TANK-binding kinase 1 (TBK1) and stimulator of IFN genes (STING), function as mediators in dsDNA-induced immnune responses. TBK1 and STING are also required for the efficient assembly of an acquired immune response to the DNA-based vaccines, and exert an important role in host defense against the DNA virus HSV1.

Upon stimulation with dsDNA, STING moves from the ER to the Golgi apparatus, assembling with TBK1 in the cytoplasmic punctate structures. A membrane trafficking system mediates this dynamic process of STING, promoting an efficient induction of innate immune responses to dsDNA.

When dsDNA stimulation occurs, STING co-localizes with Atg9a in the Golgi and with LC3 in cytoplasmic punctate structures, but not with other Atgs, suggesting a unique function of Atg9a or LC3 in controlling innate immune responses [84].

In summary, the study of Atgs and their role in innate immunity has clearly documented the importance of intracellular traffic mechanisms in the host defense against invading pathogens. Moreover, recent studies in the field of autophagy have documented that Atgs play a key role in regulating inflammation. It has also been discovered that a deficiency in autophagy regulators leads to the development of immune-related diseases like Crohn's.

Hence, heightening the autophagic activity can be a potential target to protect the host against infectious and inflammatory diseases.

Unresolving / Pathological Inflammation

The inflammatory reaction initiates a dual response. At the same time intruding pathogens are being eliminated, there is concomitant induction of tissue repair. Exacerbated pro-inflammatory mediators lead to loss of homeostasis, with systemic inflammation overcoming anti-inflammatory local counter-regulation. There is vascular leakage, tissue damage with consequent multiple organ dysfunction and ultimately death.

If the inflammatory response is not able to control infection, or if it fails to repair the injured tissue or adapt to stress and return to homeostasis, then pathological inflammation arises. Inflammation needs to be effectively terminated after removal of the original trigger and after repair of damaged tissue. In the susceptible host, over-production of inflammatory mediators or an exaggerated response to their presence can lead to septic shock, tissue

destruction, permanent loss of function, immunodeficiency or autoimmunity (Figure 8).

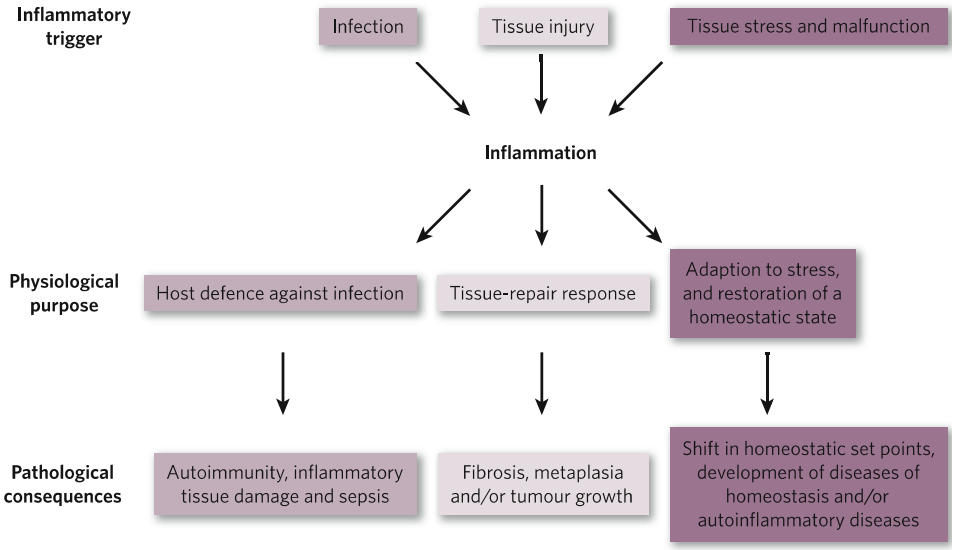

Figure 8 - Inflammatory response, physiological purpose and pathological consequences. (Adapted from Medzhitov, 2008)

The inflammatory signaling pathway, independently if it is triggered by exogenous or endogenous factors, always progresses in the same generic manner:

Summarizing, multiple triggers of inflammation induce the secretion of countless secondary mediators, which in turn compromise the functionality and viability of many tissues and target organs.

Sepsis is a heterogeneous and dynamic syndrome caused by immune dysfunction that might assume different aspects temporally and in different components of the immune system.

Sepsis is a paradigm of an uncontrolled or unresolved inflammatory cascade in response to microbial invasion (inducer), activates PRRs (receptors & sensors) that act through the release of a "cytokine storm" (mediators) that if not dampened, will ultimately lead to lung, kidney, liver, hematologic and CNS injury (end targets).

Autoimmune chronic inflammation and secondary inflammatory tissue damage are also good examples. Tuberculosis induced inflammation can persist years after the effective elimination of *Mycobacterium tuberculosis*. The sequels, parenchymal destruction and diminished respiratory capacity, can last for decades.

Deficient healing with vicious scars, joint fibrosis and even tumor growth can be harmful consequences of exaggerated inflammation [25]. Fibrosis is an important constraint in organ function. We have plenty cases in the clinic: arterial inflammation induced by cholesterol deposits; hepatitis from viruses, alcohol, toxins or parasites; pneumonitis or bronchiolitis induced by asthma or non-ionizing radiation; and even chronic inflammatory bowel diseases (Crohn's). They all represent models of pathological implications of unresolved inflammatory stimulus that in the end will compromise organ function [85].

Tolerance and Resistance to infection

In the last century, we have learned from evolutionary biologists and plant biologists that organisms rely on two methods to fight infections: increasing resistance to pathogen load and increasing host tolerance to pathogen burden [86]. They both comprise the defense mechanism by which our immune system deals with invading pathogens.

Resistance can be defined as a measure of the capacity of a host to control a certain amount of pathogen burden and thereby maintain health. Tolerance, on the other hand, is a measure of the ability of the host to limit the health impact of a given pathogen burden and thus survive infection [87].

From an evolutionary perspective, resistance and tolerance have distinctive consequences on the pathogen and on the host.

Microorganisms that we call pathogens have competitive advantage in host-pathogen battles. This is especially true because they have much shorter generation cycles than their hosts and also because killing infectious agents has substantial self-inflicted collateral damages and loss of energy resources.

Resistance immune-related mechanisms act by directly limiting the pathogen burden, so if a host develops resistance to a certain pathogen, the microorganism will be forced to evolve a method to overcome the resistance. In a microorganism population, this drives the selection of more aggressive strains and those who are able to combat the resistant host. This concept is somewhat similar to the increasing antibiotic resistance that unfortunately we see everyday in the clinical setting.

On the contrary, immune mechanisms that augment tolerance do not necessarily lead to a selection of highly resistant pathogens, as tolerance eases disease severity to a given pathogen and does not act upon the microorganism. It does not elicit an antagonistic counter-adaptation by pathogens, because it does not attack their fitness.

Tolerance has a neutral evolutionary drive [88].

From a medical point of view, increasing the knowledge of tolerance mechanisms can offer better and more effective therapies, as pathogens are not predictable to develop resistance and they can remain longer in our clinical armamentarium.

In Sepsis, tolerance properties of an organism refer to how he controls the mechanisms of synthesis, of repair and how he avoids the damage byproducts that accumulate during severe infection.

Here too, we must shift our attention from the specific and limited pathogen control to a broader "damage control" perspective.

Animal Models of Sepsis and Septic Shock

In Sepsis, as in any other medical area, all new treatment approaches or experimental devices ultimately require validation through well-designed controlled clinical trials. However, these are virtually always preceded by experiments *in vitro* and in animal models [89].

Despite bearing in mind the 3Rs (Refinement, Reduction and Replacement) of Russell and Burch, in "The Principles of Humane Experimental Technique", we maintain the necessity of using specific animal models. These can be used to address invasive monitoring questions and procedures, test prototype drugs in large scale and acquire useful pathophysiologic data. Meaningful clinical controlled studies are difficult because sepsis, as a clinical entity, is a very heterogeneous syndrome with huge confounding variables: diversity of disease, age, coexisting morbidities, diversity of infective pathogens, different supportive therapies, and different treatment guidelines in each center. These confounders are easier to control in well-designed animal model studies, which are less expensive, less time consuming and allow acquisition of reproducible data with smaller samples.

Animal models of sepsis and septic shock are not optimal, some do not mimic human sepsis, and all have potential advantages and limitations. First of all, we cannot lose sight that we are modeling in another species. For instance, rodents are quite resistant to endotoxin, have distinct hemodynamic profiles and limited blood volume, comparing to humans. The natural history of severe sepsis in animal models is different from human sepsis, starting fast with a

hypodynamic circulatory failure and a rapid resolution or decline to mortality. In the clinical setting, we usually witness an insidious development of multiple organ dysfunction, conditioning the mortality days/weeks after the initial causative injury [90].

The animal models most extensively used to study SIRS and sepsis syndromes can be divided in endotoxicosis models and sepsis models.

Endotoxicosis Models

Gram-negative bacteria contain in their wall a macromolecular glycolipid - lipopolysaccharide (LPS). LPS has two components: O-specific chain (polymer of oligosaccharides, accounts for antigenic variability) and the core (oligosaccharide covalently bound to lipid A) [89]. When administered to human volunteers, endotoxin can mimic many of the features described in sepsis.

Although there are several similarities between sepsis and endotoxemia, some differences in pathophysiologic parameters and hemodynamic variables make the results obtained in the endotoxicosis model difficult to extrapolate to human sepsis [91, 89]:

- Humans with "compensated sepsis" have characteristically elevated cardiac output and low systemic vascular resistance - *Hyperdynamic State*. Bolus injections of LPS induce low cardiac output and normal/high systemic vascular resistance
- In endotoxicosis models, gluconeogenesis is suppressed and hypoglycemia occurs, whereas the opposite is observed in sepsis

- Controversy arises as to the correlation of circulating endotoxin levels and clinical severity, outcome or microbiological status.
- Occasionally, elevated levels of endotoxin can be detected in patients with meningococcemia which have started antibiotic therapy, translating a large amount of bacteria being killed and their components released into circulation [92]
- Human volunteers that are rendered tolerant to LPS still manifest signs of SIRS during infection with viable Gram-negative organisms
- Endotoxin is released by Gram-negative bacteria inducing sepsis, but is not released by Gram-positive, which have equal mortality sepsis rates [93, 94]
- Some of the inbred mice strains are very hyporesponsive to LPS, while at the same time manifest increased susceptibility and mortality in response to infection by Gram-negative pathogens
- Use of corticosteroids and anti-TNFα antibodies have been very effective in endotoxemia models, but have failed to demonstrate efficacy in clinical trials [95].

However, LPS remains pathophysiologically important in the development of human sepsis and one of the first mechanisms described [96].

The main endotoxicosis models are:

1) LPS administration intravenous, in small (sub-lethal) doses - Large LPS doses induce a hypodynamic pattern, whereas small doses elicit a hyperdynamic picture[93].

2) LPS administration with aggressive resuscitation of intravascular volume and vasoactive drugs - Patients in septic shock, admitted in ICUs, are aggressively fluid challenged and treated with vasoactive drugs. Our notions of the circulatory alterations are based on observations of this cohort, after resuscitation and hemodynamic interventions [97, 98].

3) LPS administration by continuous infusion - Endotoxins are probably released into the circulation in a continued, prolonged manner.

4) LPS administration intraperitoneal - This model mimics best the effects in cardiac output and systemic vascular resistance encountered in human sepsis.

Sepsis Models

These models, as seen in septic patients, try to recreate an episodic bacteremia associated with a systemic inflammatory response: hyperpyrexia, leukocytosis, tachycardia and MODS. Depending on the original injury site, the syndrome presents with distinctive manifestations.

The main sepsis models are the following:

1) Intravenous infusion of live bacteria - Probably constitutes a form of endotoxicosis rather than sepsis model. This condition mimics a septic focus, which is intermittently, but persistently "showering" the organism with bacteria. The patterns of pulmonary, cardiovascular and renal alterations depend on the pathogen injected. Mimics extreme clinical sepsis as seen in

meningococcemia, pneumococcal bacteremia in splenectomized individuals and gram-negative bacteremia in the setting of granulocytopenia.

2) Induction of soft tissue abscess (extremities) - Inducing any degree of tissue necrosis, followed by an infection, usually prompts this model. Inflammation of the extremity with hyperdynamic circulatory response, but no mortality is a typical characteristic of this model.

a) Intramuscular abscess

b) Intradermal abscess

3) Induction of peritonitis - Peritonitis can be elicited in several ways: bowel ischemia, bowel perforation, *inoculum* of fecal material or pure bacterial preparations.

a) *Peritoneal implantation of feces* - Sometimes results in minimal local or systemic responses, since many animal species are resistant to their own fecal material. It is an uncontrolled model concerning the dose and the strain of bacteria.

b) *i.p. administration of bacterial inoculum* - More controlled but needs an adjuvant to achieve elevated mortality. Some critics claim it is also an endotoxicosis model. This is an excellent model to study bacterial clearance or cellular response to an intra-peritoneal bacterial challenge.

c) *Intra-abdominal abscess* - This can be performed by peritoneal implantation of an adjuvant (barium sulfate, bile salts, gelatin capsules, fibrin clots) with an infective agent (feces, quantified bacterial inoculum). Easy to control and good to study specific

host-pathogen interactions or directed antibiotic strategies. It bears some similarities to acute appendicitis and diverticulitis.

d) *Organ ischemia and bowel perforation* - Claims to mimic clinical entities like cholecystitis and intestinal ischemia. Completely uncontrolled: severity of peritonitis, rate of development, intestinal bacterial contents.

e) *Cecal ligation* - Ligation of the cecum, distal to the ileo-cecal valve, creates an ischemic pouch filled with fecal content and at the same time allowing intestinal continuity. Animals present with low arterial pressure, decreased cardiac output, fever and respiratory alkalosis, but no bacteremia was reported [99, 100, 101, 102]. There is controversy if this is a SIRS or a Sepsis model, as animals cope with it fairly well. An organized and localized abscess ensues and no positive blood cultures or peritonitis signs are observed [103]. Autopsy studies 1 month after cecal ligation reveal complete cecal absorption.

f) *Cecal ligation and puncture* - Puncturing the ligated part of the cecum with a needle before replacing it to the peritoneal cavity, violated the integrity of GI barrier. This is followed by an immediate and constant bacterial leakage to the peritoneum. The procedure leads to a reproducible, simple and inexpensive model of severe sepsis and septic shock [91]. Without fluid resuscitation, this model promotes rapid onset of septic shock and mortality. After fluid challenge, mortality rate is reduced and pathophysiological responses resemble those in human sepsis [104].

4) Induction of pneumonia - Inhalation (intra-nasal) or intra-traqueal administration of the inoculum [105], [106]

5) Induction of meningitis - Intra-cisternal injection of bacterial inoculum [107].

Although constrained by many limitations, animal models will always be essential for developing new therapies for sepsis and septic shock. They offer excellent data on pharmacokinetics, toxicity and mechanism of action that would be otherwise impossible or extremely expensive.

There are many examples of improvements that can be made to these models:

- Long-term studies replicating ICU-like conditions, implying the need to create an animal ICU;
- Simulation of delayed onset organ failure;
- Altering the starting time point of therapy, mimicking the delayed intervention that we are unfortunately constrained in the clinical setting;
- Introduce models with previous debility and morbidity, like the human counterparts, that could be a pre-existing organ dysfunction (chronic renal insufficiency, diabetes, cardiac disease)
- Consider modeling in aged animals, in order to fully understand the aberrant responses that occur with age. This is one of the most relevant issues to address, as elderly patients account for 65% of sepsis cases [108]. As life expectancy increases we know that we face increasingly older population of septic patients [109] (Figure 9).

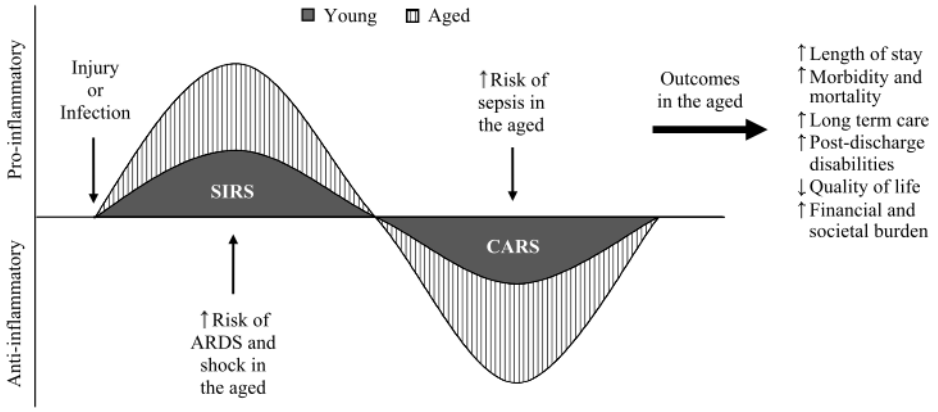

Figure 9 - Aging and SIRS after injury or infection. In older patients, an exacerbation of SIRS and CARS is observed. This triggers an increased risk for ARDS and shock during SIRS and an increased risk for secondary infections (2nd hits) during CARS. Worse outcomes are expected in aged septic populations. (adapted from Nomellini et al, 2008)

Table III - Advantages and disadvantages of the most commonly used experimental models for sepsis. Adapted from Dejager, 2011 [110]

Sepsis Model	Advantages	Disadvantages
Endotoxemia Model: systemic administration of LPS	Simple and reproducible	• LPS-mediated signaling is strictly TLR4-dependent
		• Does not reflect all complex physiological human responses
	Induced response is acute	• High, rapid and transient increase in cytokines, which differs from human sepsis
		• Rodents are endotoxin resistant, whereas humans are very sensitive
	Highly controlled and standardized model	• Different hemodynamic response compared to human sepsis
		• Variability in dose, toxin and route of administration
Sepsis Model	**Advantages**	**Disadvantages**
CLP model	Simple procedure	• Abcess formation
	Presence of an infectious focus	
	Polymicrobial sepsis model	
	Uses the complete spectrum of host enteric bacteria	
	Recreates human sepsis progression with similar hemodynamic and metabolic phases and the presence of both hyper- and hypoinflammatory phases	• Variability in severity due to differences in experimental procedures
	Prolonged and lower elevation of cytokine release, as in humans	

Sepsis Model	Advantages	Disadvantages
Bacterial inoculum model	Presence of bacteria allows insights into mechanisms of host response to pathogens	• Growth and quantification of bacteria is needed before administration
		• Single bacterium model does not reflect the diversity and combinations of infectious agents that are present in human sepsis
		• Humans are normally not challenged with a massive bacterial load, but have a septic focus that intermittently and persistently challenges the body with bacteria
		• High doses of bacteria induce an endotoxic instead of a septic shock, due to the presence of LPS after rapid bacterial lysis
		• Variability in bacterial load, route of administration and bacterial strain

Sepsis Clinical Course

It is understandable that any aggression to our organism, be it an infection, trauma injury or a severe burn, elicits a systemic inflammatory response syndrome – SIRS.

Our innate immune cells and secondary mediators are key components of this process. Almost at the same time, there is activation of a series of counter-regulatory mechanisms initiated with the purpose of limiting the excessive inflammatory process, in order to return to homeostasis. After the acute activation of the innate immune system follows an immune adaptive cell inhibition, T-cell anergy and increased apoptosis in the lymphocyte compartment of the immune system.

All these features contribute to immune-paralysis and increased susceptibility to secondary hits/infections [111].

This is the concept of compensatory anti-inflammatory response syndrome (CARS), which courses with some degree of immune-suppression or immune-paralysis. Recent clinical evidence suggests that in spleen and lungs of septic patients an increased expression of selected inhibitory receptors and ligands are accompanied by an expansion of suppressor cell populations in both organs [112].

Classical descriptions of the SIRS/CARS paradigm define CARS as a hypo-immune, hypo-inflammatory state that follows SIRS. A syndrome that is responsible for dampening SIRS deleterious collateral effects [113]. It is also a

period of characteristic second hit infections that can hinder the already frail balance of these patients [114].

In the current pathophysiologic model, a patient that is subjected to severe injury (which can be either trauma or infection) develops SIRS. This is followed by a compensatory anti-inflammatory or immune-suppressive response syndrome.

Exacerbated inflammation is responsible for the adverse outcome, which is related with second hits prompted by opportunistic infections, viral reactivation or even surgical stress [115] (Figure 10).

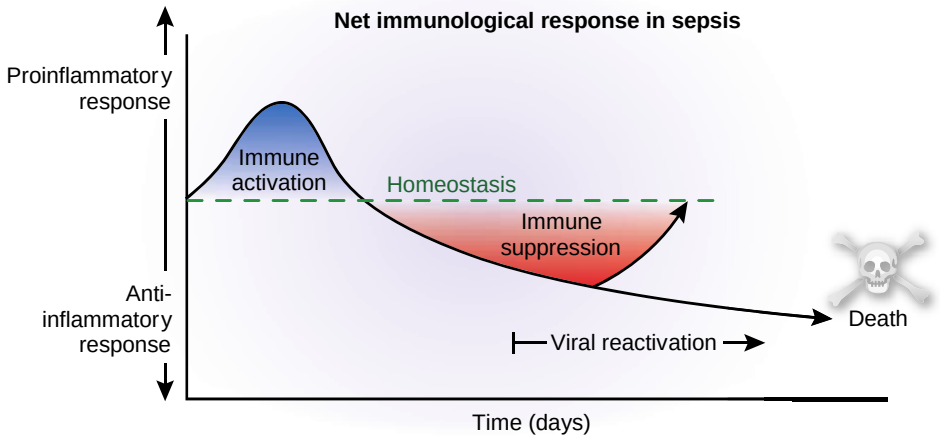

Figure 10 – Inflammatory and immunologic response in sepsis over time. In the early phases of sepsis, both pro- and anti-inflammatory responses are activated. However, in the beginning the pro-inflammatory response phenotype predominates. As sepsis progresses, the anti-inflammatory response becomes predominant, and it is during this later phase that secondary infections (nosocomial) and viral reactivation occur. Early deaths during the pro-inflammatory response phase are considered to be cytokine storm–mediated events, whereas later deaths during the anti-inflammatory phase are attributed to the failure to control pathogens. (Adapted from Hotchkiss, 2009)

The accepted paradigm of SIRS/CARS describes the adverse outcomes in patients subjected to a series of excessive pro-inflammatory responses (SIRS) followed temporally by compensatory anti-inflammatory responses (CARS) and varying degrees of adaptive immunity suppression. Any subsequent insult leads to a more severe, recurrent SIRS that can end in organ dysfunction and death.

However, recent studies of leukocyte transcriptome in critically injured patients shed new light on the subject and challenged the current paradigm [116].

The genomic response to trauma, burns and endotoxemia did not differ much and the patterns were consistent with an organized and reproducible response to severe inflammatory stress. Gene expression patterns were not qualitatively different between patients that recovered and those that did not. In patients with adverse outcome, the pattern changes were larger and did not return to baseline.

This data is consistent with the "non-resolving inflammation hypothesis" [41], in which patients with severe inflammatory responses who will subsequently die from their injuries have the same immune response as those that will recover. The distinction between them is the intensity and length of the uncontrolled acute inflammatory response. The evidence that patients subjected to severe injury have higher 1-year mortality rates than non-injured matched controls, suggesting that there is an underlying sustained chronic inflammation, also supports this hypothesis [117].

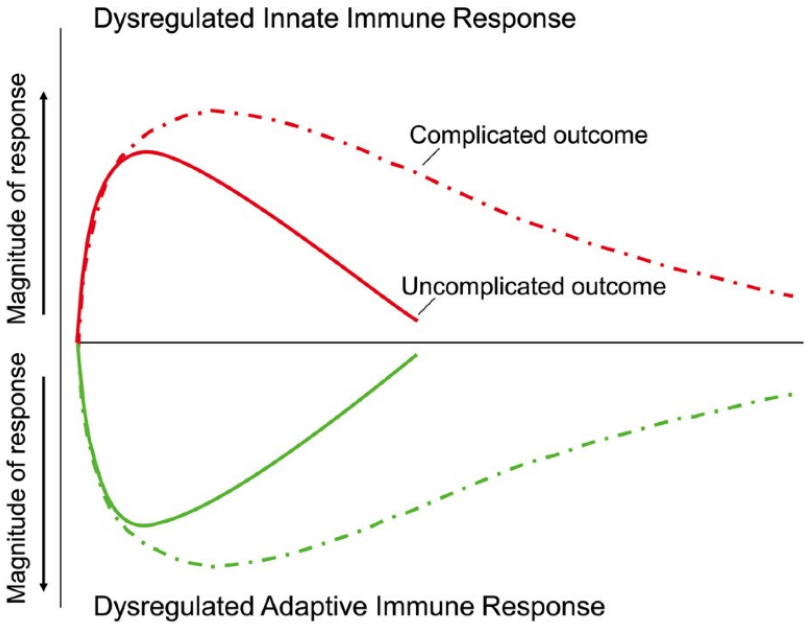

Figure 11 – Severe inflammatory syndrome progression. The hypothesis postulates that the systemic response to severe injury relies on simultaneous and swift induction of innate (both pro- and anti-inflammatory genes) and suppression of adaptive immunity genes. Adverse outcomes represented by delayed recoveries, result from a persistent, deregulated immune–inflammatory state. (Adapted from Xiao, 2011)

This recent hypothesis postulates that the onset of a pro-inflammatory response to injury occurs simultaneously with an anti-inflammatory and immune adaptive response to stress [116]. There is a concurrent, and not sequential, up-regulation of innate immune-related genes and the suppression of adaptive immune-related genes, regardless of clinical outcome. The later is dependent on the degree of deregulation of the pro-inflammatory innate immune response and also on the phenotype of immune-suppression or immune-paralysis that can take a while to manifest (Figure 11).

Sepsis is the result of a complex systemic immune response, simultaneously balancing inflammatory and anti-inflammatory stimulus. The magnitude of the end result probably depends on the underlying predisposing factors, the characteristics of the infection, the response of the "host" and the degree of organ dysfunction [6].

Despite all the progress of intensive care procedures, development of new antibiotics and aggressive organ support measures, patients that survive the initial exacerbated inflammatory phase enter a stage of delayed immune suppression. This is a phase where secondary infections - nosocomial infections – develop, producing a "2nd hit effect".

Severe sepsis can progress as two characteristically portrayed syndromes: septic shock and severe sepsis with multiple organ dysfunction. What is still not clear is if they represent the extremes of a spectrum of syndromes or just two manners of presenting the same syndrome, depending on the nature of the pathogen involved and the genetic background of the host.

Nevertheless, in the clinical setting we are faced with the classically described:

- **Septic shock** - which is a high lethality syndrome with sudden cardiovascular failure, killing in 24-48h
- **Severe sepsis with multiple organ failure** - a sub-acute disorder that has a more insidious course, developing sequential organ shutdowns and kills in 7-14 days

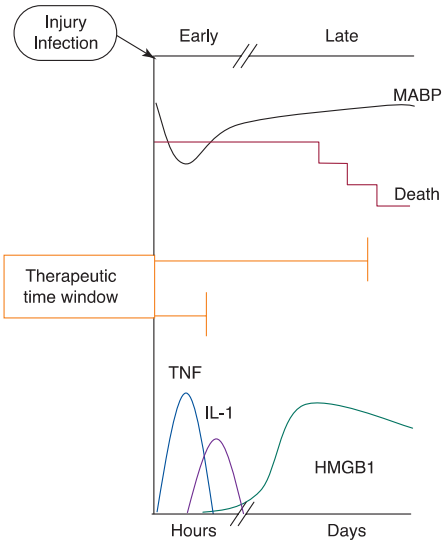

Figure 12 – Cytokine profiles in sepsis development and progression. (Adapted from Ulloa and Tracey, 2005)

The early initiating step is dependent on IL-1 and TNFα (Figure 12). These cytokines represent prototypes of acute activation of the immune system and are also associated with a low mean arterial blood pressure pattern. If patients survive the acute episode, they enter the delayed, slow progressing phase, mainly dependent on late mediators such as High Mobility Group Box-1 - HMGB1 [118, 31, 119]. This is characterized by a progressive dysfunction of the target organs: lung, kidney, liver and heart.

Cytokines play a role in sepsis as a secondary endogenous danger signal, associating with PAMPs and activating the PRRs of immune effector cells.

Cytokine patterns vary between patients and even during the course of the disease in the same patient. They are quickly secreted, in a matter of

minutes they can be detected in serum of patients, and their effects are almost immediate. The use of anti-cytokine therapies in sepsis, it had to be done pre-emptively. On the other hand, even TNFα plays an important role in regulating resolution of inflammation and tissue repair, leading to the return to homeostasis. Its abrogation could hamper the control of inflammation. These are probably the main reasons why anti-cytokine therapy aimed at stopping the development of SIRS and Sepsis has failed [120].

Multiple Organ Dysfunction Syndrome

Multiple Organ Dysfunction Syndrome (MODS) can be defined as the onset of diminished organ function in an acutely ill patient, which requires specific intervention to maintain homeostasis. It is considered present when at least two previously healthy organs fail, simultaneously or in a sequential manner [3].

In current sepsis literature, it is accepted that the severity of each organ dysfunction is correlated with prognosis [121, 122]. Several scores were developed in an attempt to categorize and stratify patients according to their illness severity and expected mortality. Sepsis mortality can be separated in ICU mortality (deaths in the ICU), hospital mortality (30-day mortality) and long-term mortality (deaths occurring 1 year after ICU discharge).

There are several clinical scores that can translate into quantifying variables the severity of MODS. Some, like the Multiple Organ Dysfunction Score, try to predict the ICU mortality rate [123], while others try to assess 30-day mortality, like the New Simplified Acute Physiology Score (SAPS II) [15] and Acute Physiology And Chronic Health Evaluation II (APACHE II) [14].

Other groups of clinical scores do not try to predict mortality, but are very useful in monitoring each of the dysfunctional organs. An example is the Sequential Organ Failure Assessment (SOFA) [122]. This scoring system records the respiratory, renal, hepatic, cardiovascular, hematologic and neurological abnormalities in a daily basis. This way, the effect of all therapeutically and interventional measures taken can be assessed.

It is not yet established why some patients develop sepsis and progress to MODS, even after all intensive care measures. Correcting mean arterial pressure (MAP) values and systemic oxygen delivery are not enough to prevent organ failure. Whether it is dependent on increased systemic inflammatory mediators, depletion of systemic anti-inflammatory molecules, microvascular thrombotic angiopathy or impaired mitochondrial oxidative phosphorylation, remains to be settled.

Common sense suggests it might be a combination of the following etiologic factors:

1. Vascular endothelium dysfunction
2. Cytopathic hypoxia
3. Inflammatory cytokines

Vascular Endothelium Dysfunction

Any dysfunction in the normal activity of endothelial cells (EC) can lead to: disruption of blood vessel lining; changes in vasomotor tone; or compromise in the normal antithrombosis, profibrinolysis, inhibition of platelet and leucocyte adhesion functions [124, 125].

ECs express anticoagulant molecules that accelerate inactivation of coagulation proteases thus controlling the extrinsic pathway of the clotting cascade [126]. The interaction of pro-inflammatory mediators and ECs can potentiate a strong pro-coagulant phenotype, especially in the microvasculature. Increased coagulation and altered fibrinolysis can promote tissue ischemia, necrosis and ultimately MODS [127, 128].

ECs, in the presence of pro-inflammatory stimuli, increase the expression and secretion of adhesion molecules: ICAMs, E-selectins and platelet EC adhesion molecules[129], [130]. These promote activated leucocyte migration and tissue infiltration by neutrophils and macrophages [131]. Inappropriate infiltration of neutrophils into target organs is also mediated by chemokines and enhanced expression of chemokine receptors in tissues remote from the initial injury [132]. ECs help to regulate vasomotor tone and peripheral control of blood pressure, by producing vasoactive molecules: nitric oxide (NO), prostacyclin, endothelin, thromboxane A2 and PAF [133].

In sepsis, compromise of the ECs breaks the balance between vasodilators and vasoconstrictors and the sum of deficient vascular relaxation, altered blood flow distribution, increased leukocyte adhesion and pro-coagulation phenotype promote microcirculation failure, tissue hypoxia and consequently MODS.

Cytopathic Hypoxia

Sepsis can induce, at the cellular level, an intrinsic deficiency in mitochondrial oxidative phosphorylation function - cytopathic hypoxia [134]. There have been several hypotheses to explain the development of sepsis related tissue hypoxia:

- Inhibition of the mitochondrial respiratory chain Cytochrome a1a3, which is aggravated by low arterial oxygen pressure
- Inhibition of Pyruvate Dehydrogenase - increases pyruvate in cells leading to an increased synthesis of lactate

- Inhibition of mitochondrial respiratory chain by accumulation of peroxynitrite

Inflammatory Cytokines

Pathogens induce a massive release of pro-inflammatory cytokines that promote inflammation, affect vascular tone, and increase leucocyte migration and vascular permeability. Simultaneously, anti-inflammatory pathways are activated to control and dampen the inflammatory response, downregulating the host response, reprogramming activated leukocytes and inducing a degree of immune-depression (discussed previously in ***Inflammation, Immunology and Pathophysiology***).

Any degree of imbalance between these two coordinated responses can lead to tissue damage and MODS.

Cardio-circulatory Dysfunction

In sepsis, cardio-circulatory dysfunction is usually the sum of insufficient peripheral circulation and depressed cardiac function. The distributive shock that typically arises is dependent on a peripheral vasodilation of the capacitance vessels and the hyper-permeability phenomenon in the microvasculature. They decrease left cardiac pre-load and impair cardiac output. Left ventricular function is, by itself, compromised by a decrease in myocardial contractility.

The cardio-circulatory pattern incorporates a high cardiac output, arterial hypotension, increased consumption and decreased transport of oxygen. In severe cases, when the syndrome is not compensated, a low cardiac output can be observed.

Septic myocardial failure can be observed in very early stages, being temporary while the syndrome attains homeostasis. If the patient recovers, these changes revert after a couple of days [135]. Changes in the cellular content of calcium, NO overproduction, increased myocyte apoptosis and circulating inflammatory mediators are responsible for this myocardial depression.

Left ventricular dysfunction and a poor prognosis in patients with sepsis, severe sepsis, or septic shock, have been associated with the observation of elevated cardiac troponin. In this population, troponin release occurs even in the absence of ischemic coronary artery disease, implying the existence of other mechanisms than thrombotic coronary occlusion, possibly a temporary loss in membrane integrity with subsequent troponin leakage or microvascular thrombotic injury [136].

Two main mechanisms have been described, to explain the loss of myocardial calcium homeostasis: a decrease in number of L-calcium channels, lowering the calcium flow [137]; decreased Ca^{2+} responsiveness in myofilaments [138]. Changes in myofilament sensitivity are linked with increased cardio-myocyte and sarcomere length, which explains the responsiveness to the "fluid-challenge" in the early phases of sepsis and the acute ventricular dilation observed after fluid infusion [139].

In sepsis, excessive synthesis of NO is associated with myocardial depression and increased expression of the inducible form of NO synthase

(iNOS) in all cardiac cavities. The degree of cardiac dysfunction is inversely correlated with the degree of iNOS expression [140].

Cytokines and secondary inflammatory mediators can produce negative inotropic effects and direct damage to the myocytes, as the case of TNFα, IL1β, myocardial depressant factor (MDF), prostaglandins and endothelins.

Moreover, increased apoptosis and necrosis of myocytes, interstitial edema and changes in the mitochondrial oxidative phosphorylation system, can directly interfere in cardiac contractility[141].

The peripheral vasodilation, characteristic of septic shock, is related to the overproduction of NO by increased synthesis from iNOS, which is induced by inflammatory mediators and cytokines. Decreased peripheral vascular resistance, is partly mediated by NO which induces an increased vascular permeability not only in the heart, but also in the liver, kidneys and all splanchnic territory [142].

Septic patients often have adrenal insufficiency, resulting in low levels of mineralocorticoids and glucocorticoids, contributing to the hemodynamic instability and the high dependency on catecholamines, despite the control of the infection source [143].

Additionally, the effect of inflammatory mediators and activated coagulation contributes to a decreased microvascular perfusion pressure, causing micro-thrombi and obstruction of the small peripheral vessels [144]. There is also a "shunt effect" at the level of skeletal muscles, intestinal villosities and diaphragm, where there is no blood flow in their capillaries [145].

The overall effect is glycolysis and increased lactic acidosis with high serum lactates.

In terms of microcirculatory dysfunction, any attempt in preserving an adequate perfusion pressure affects positively the outcome. The mean arterial pressure value that is optimal remains controversial [146].

Renal Dysfunction

Acute Renal Failure (ARF) is very common in sepsis and remains an independent risk factor for mortality, even in patients treated with hemodialysis or continuous venovenous hemodiafiltration (CVVHDF) [147]. The prevalence of ARF in sepsis is very high: 19% of all patients diagnosed with sepsis, 23% in severe sepsis and 51% in septic shock [148].

The pathogenesis of sepsis induced ARF is not well understood and the hypothesis derive mainly from animal studies and *in vitro* models.

Excessive NO synthesis induces arterial vasodilation, leading to hypotension and a consequent fall in renal blood flow, associated with a selective renal vasoconstriction. So far, the known mechanisms behind septic ARF include: glomerular afferent arteriolar vasoconstriction [149], tubular hypoperfusion [150] and redistribution of the cortical to medullary blood flow [151]. This is dependent on two sets of causes:

- Failure of homeostatic mechanisms that normally compensate hemodynamic alterations: abnormal activation of the sympathetic system, the renin-angiotensin-aldosterone system, the kinin-kallikrein system and the release of α-atrial natriuretic peptide (αANP) [147] .

- Direct effect of systemic inflammatory mediators (TNFα, IL1β, IL8) can decrease ultrafiltration coefficient, change the renal vascular tone and induce tubulo-interstitial lesions [152].

Respiratory Dysfunction

Sepsis is usually associated with respiratory failure and/or an increased risk of respiratory dysfunction.

More than 50% of patients in sepsis will develop acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) and will need mechanical ventilator support. The criteria for defining ALI/ARDS take into account: the chest radiograph, oxygenation parameters and pulmonary artery wedge pressure (PWP). Both respiratory syndromes have acute onset, bilateral infiltrates in chest X-ray and PWP of 18mmHg or no evidence of left atrial hypertension. The difference lies in the oxygenation pattern: ALI has a ratio of PaO2/FiO2$\leq$300mmHg, as for ARDS a ratio of PaO2/FiO2$\leq$200mmHg has to be observed, regardless of the PEEP value.

This respiratory failure is not only common in pneumonia (a frequent cause of sepsis), but in other systemic inflammatory response syndromes such as severe burns, pancreatitis, hemorrhagic shock and trauma [153], [154]. In SIRS, the inflammatory reaction induces diffuse endothelial cell injury and increased capillary permeability via TNFα, IL1β, IL-6 and PAF [155].

These events lead to interstitial pulmonary edema, alveolar flooding and collapse. EC lesion may progress to microvascular thrombosis and alveolar

hemorrhage, reducing the available surface to exchange gas. Pneumocytes are also involved in this mechanism, as their dysfunction leads to decreased surfactant, which is essential for the alveolar surface tension and maintaining alveolar oxygen exchange [156].

The net result is an important ventilation-perfusion mismatch, establishing a physiologic shunt with severe hypoxemia.

Neurologic Dysfunction

In sepsis syndromes, cerebral dysfunction or neurologic damage is present in 9 to 71%, depending on the definition [157] [158]. Its' related mortality is dependent on the severity of brain dysfunction. Sepsis associated delirium (SAD) or sepsis encephalopathy describe the cognitive dysfunction occurring during sepsis or SIRS. This is an exclusion diagnosis and requires the absence of direct infection of the central nervous system (CNS), head trauma, fat embolism and side-effects from drugs [159].

Inflammatory mediators profoundly affect ECs and astrocytes, as they disrupt the blood-brain barrier and compromise neuronal function. Compromised oxygen delivery to the brain, enhanced by free radical lesion, allows for aromatic amino acids to enter the brain and disturb neurotransmission. Dysfunction in mitochondrial respiratory mechanism leads to apoptosis of brain cells and neuronal injury [160].

Hematologic Dysfunction

Sepsis and systemic inflammation induce a pro-coagulant phenotype in the endothelium, translating from a mild thrombocytopenia to a disseminated intravascular coagulation (DIC).

Thrombocytopenia is an independent risk factor for developing MODS. Thrombocytopenia-associated multiple organ failure is a thrombotic micro-angiopathy syndrome which varies from thrombocytopenic purpura to DIC [161].

DIC occurs in less than 20% of septic syndromes and is characterized by *purpura fulminans*, high levels of D-dimers, low platelet counts and increased prothrombin time (PT) and activated partial thromboplastin time (aPTT). Establishment of DIC is so deleterious to the prognosis in these patients that even with shock reversal, the coagulopathy remains a predictor of mortality [161].

Hepatic Dysfunction

The causes underlying hepatic failure in SIRS remain poorly understood. Direct damage to the hepatocytes and alterations in hepatic microcirculation are thought to be main reasons of liver dysfunction in sepsis [162].

Cytotoxicity from activated Kupffer cells, NO dependent reduction in the portal blood flow, decrease in the perfusion pressure of liver sinusoids, increased platelet adherence and activated leukocyte-endothelial interactions all contribute to impaired hepatic perfusion, leading to hypoxia and hepatotoxic cellular damage [163, 164].

Organ Dysfunction in Sepsis: Index of Severity or a Defense Mechanism?

There are numerous reports from the literature acknowledging the disparity between histology findings and the degree of organ failure in patients dying with sepsis [113]. Usually, cell death and tissue necrosis is limited and does not account, by itself, as the cause of organ failure. Moreover, we know that in survivors there is a complete recovery of organ function, as in the case of acute tubular necrosis or hematologic dysfunction.

These observations have been pointed by some authors, building the hypothesis that organ failure in SIRS is a protective mechanism, like a process of "hibernation" or "cell stunning", allowing the organs to recover after a period of lethal hypoxia. Mitochondria, not only involved in the pathophysiology of severe sepsis, are the main organelles postulated to be responsible for this protective cellular hibernation [165].

Therapeutic Issues

The key principles in treating an infection are still the removal of the causative agent and prompt antibiotic therapy. The delay in antibiotic administration is one of the main variables to dictate survival in sepsis. From the onset of symptoms and signs of severe sepsis, there is an 8% increase in mortality for each hour that passes without appropriate antibiotic coverage [166].

In sepsis it is mandatory to initiate fluid resuscitation and institute vasopressors to reverse hypotension and to preserve normal tissue perfusion. Several degrees of organ replacement therapies are available in the ICU setting. However, as it was stated before, with all these advances in care a significant decrease in mortality did not occur in the past 50 years.

Anti-septicemia therapies

In the early 1980s a human trial with anti-endotoxin serum reported favorable outcomes, but it was never reproduced [167]. The use of endotoxin-neutralizing agents and endotoxin antagonists did not improve survival and had serious toxicity issues.

Trials using intravenous administration of polyclonal immunoglobulin gave uncertain results and its use was abandoned [168].

Anti-inflammation and Immune-modulation therapies

In the late 1980s, there was a large number of experts in the field believing that sepsis and sepsis-syndromes were inflammation-driven, and not so much pathogen-dependent. After the 1991 Consensus Conference, a lot of interest fell on anti-inflammation approaches [3].

High-dose corticosteroids were used in several clinical settings, but the evidence showed that short courses of high-dose steroids worsened survival in sepsis [169].

Low-dose corticosteroids gained a renewed interest and were administrated in septic patients with suspected adrenal gland failure and vasopressor refractory hypotension. This "physiologic" or "stress" dose was shown to improve survival in patients with predicted high mortality [170]. The main benefit of low-dose steroids, reported in large multicenter trials of high-risk patients, has been the shortening of time to reverse the cardio-circulatory dysfunction. This is especially true in cases of adrenal gland dysfunction, where low dose steroids can diminish the dependency of norepinephrine and other vasopressor drugs.

The interest of targeting single pro-inflammatory mediators (cytokines, platelet-activating factor - PAF, bradykinin, cyclooxigenase), has been weaning in the last few years, as the excellent results in vitro and animal studies did not match the poor outcomes of several clinical trials [171].

In retrospective subgroup analysis, several agents - namely anti-TNF antibodies - may have improved survival in severe patients but were often harmful to the less sick. The main issue is the bedside assessment of which patients should benefit from these specific agents.

From the current immunologic knowledge, it seems likely that eliminating or blocking a single mediator or even a single signaling pathway will not affect the overall septic progress. Also, when tampering with immune-suppression in the critically ill one must carefully consider the risk/benefit ratio of exposing this already frail host to possible opportunistic invaders.

Anti-coagulation therapies

One of the most publicized new sepsis therapies was the administration of recombinant anticoagulant proteins, in order to block or revert thrombosis-related organ injury.

OPTIMIST trial tested the efficacy and safety of tifacogin (recombinant tissue factor pathway inhibitor) [172]. Initially it appeared to save lives, but the efficacy lowered by mid-trial analysis and at the end, authors concluded that the all-cause mortality was not lowered and that tifacogin was associated with an increased risk for bleeding and serious adverse events.

The PROWESS trial was intended to test the safety and efficacy of drotrecogin (human recombinant activated protein C). It was stopped prematurely because of a dramatic 6.1% decrease in overall mortality [12].

The US Food and Drug Administration approved Xigris® in 2001 and the European Commission in 2002, for the treatment of severe sepsis in the highest-risk patients. Subsequent trials to assess its effectiveness in low-risk population were stopped because of futility. In successive randomized trials, the rate of serious bleeding events and the risk of death increased significantly, in comparison to the original trials. In October 2011, consequent to a rising

body of literature suggesting the risks outweighed the potential benefits, the company withdrew Xigris® from the market.

Clinicians keep formulating questions at the bedside of their patients and continue to hope for new ways to treat this devastating illness. Immunologists, biomedical scientists and evolutionary biologists keep searching for new approaches to restore immune competence of the host and new ways to enhance the recovery of the injured organism.

In brief, after more than 50 years and enormous progress in biomedical research, we are still left with good control of the site of infection, appropriate and timely antibiotic administration and standard organ support measures to treat severe sepsis.

The Problem

After the onset of an uncontrolled or unresolved systemic inflammatory response, homeostasis is disrupted. PAMPs and DAMPs released from invading pathogens and/or injured host tissue stimulate pattern-recognition receptors in immune cells, unleashing several signaling cascades of the innate immune system, including inflammatory, complement, coagulation and fibrinolysis, autonomic and endocrine responses. The complexity of interactions between these networks is one of the main problems in addressing any question in Sepsis.

Stimulated immune cells can secrete excessive amounts of pro-inflammatory mediators, leading to a "cytokine storm", free radicals and enzymes. In this situation, a normal beneficial effect of inflammation is altered into an excessive response that damages the host.

Overstimulation of the adrenergic pathway of the autonomic nervous system (ANS) and/or reduced activity of the cholinergic anti-inflammatory pathway (parasympathetic branch of the ANS) intensifies the pro-inflammatory responses of neutrophils, macrophages and dendritic cells in sepsis.

The hypothalamic-pituitary-adrenal axis coordinates the neuroendocrine integration and any disturbance in its' feedback loops can contribute to an immune-depressed state. The existence of bacteria circulating in the blood activates the complement system, inducing the production of complement anaphylatoxins, which by themselves can prompt several harmful effects.

A pro-inflammatory *milieu* and the damaged endothelium synchronously activate the coagulation cascade and inhibit fibrinolysis, creating an environment suitable for DIC. DIC acts in amplifying the systemic inflammatory response syndrome. Complement, coagulation and fibrinolysis are directly connected through a system of serine proteases. Alterations in any of the cascades are reflected and balanced by positive-feedback loops.

Changes in the leukocyte population, dysfunction of neutrophils or altered apoptosis, are responsible for degrees of immunosuppression and immunoparalysis that occur during severe sepsis. This immune dysfunction is responsible for an increased susceptibility to secondary infections.

Sepsis is a heterogeneous and dynamic syndrome caused by immune dysfunction that might assume different aspects temporally and in different components of the immune system. For instance, the early stage of sepsis is characterized by a hyper-inflammatory environment due to overwhelming activation of innate immune responses by infection or tissue damage, while later time points might be better described as an immunosuppressive state [173]. The early initiating step is dependent on TNF and IL-1β and the delayed, slow progressing phase, dependent on late mediators such as HMGB1 [174].

I have started this work by asking what would be the effect of inhibiting the exaggerated systemic inflammatory response that occurs in sepsis by blocking simultaneously the two key early initiators of sepsis. In the following chapters, I will describe the journey that began with the identification of drugs that can block the secretion of TNF and IL-1β and culminated in the description of the mechanisms that potentially mediate the therapeutic effect of anthracyclines against severe sepsis.

Chapter II - Methods

In vitro chemical screen

Our group has developed and validated an *in vitro* model that simulates the cytokine environment of SIRS, in order to study the secretion of the key cytokine mediators acting in the early phase of severe sepsis (IL-1β and TNFα). These early sepsis mediators are key components of the subsequent systemic response.

Little is known about the exact cellular mechanisms responsible for the ensuing immunological response. Working on the premise that if we are able to interfere with these pathways and/or modulate the immune response, we can probably arrest the release or stop the amplification of the implicated cytokines, thus delaying the progression of severe sepsis.

We have recapitulated the early monocyte/macrophage PRRs activation by bacterial components, in order to select drugs that interfere simultaneously in the production and secretion of TNFα and IL-1β.

We have chosen for this screen the Microsource "Spectrum Collection"®. This drug library has over 2000 biologically active and structurally diverse compounds of known drugs, including most of the compounds that are currently approved for clinical use, experimental bioactives, and pure natural products. All drugs present in the "Spectrum Collection" library were tested.

THP-1 cells (monocyte/macrophage cell line - American Tissue Culture Collection-ATCC TIB-202) were plated in 96 well plates at 10^6 cell/ml and incubated with each one of ≈2320 compounds included in the Spectrum collection (Microsource Discovery Systems, Gaylordsville, CT) at 10mM for 1 hour.

The ensuing stimulus was 4% PFA- fixed DH5a *E. coli* at a Multiplicity of Infection (MOI) of 10 bacterial cells per THP-1 cell for an additional 24 hours. After a 24h period, cell viability was assessed by Alamar Blue test (Invitrogen®), according to manufacturer's instructions, and cell supernatants were collected. IL-1β and TNF cytokines were quantified by DAS-ELISA, using Human IL-1β /IL-1F2 DuoSet® and Human TNF DuoSet® (R&D Systems) respectively, according to company's protocol.

All data values from IL-1β and TNF secretion assays were normalized by dividing the amount of IL-1β and TNF in the conditioned media 24, 12, 8, 6, 4 or 2 hours after *E. coli* stimulation by the number of cells in each well and then by the average concentration per cell of the plate. Results were logarithmic natural transformed.

Scores were sorted in ascending order and graphed.

Pharmacologic Screening Protocol

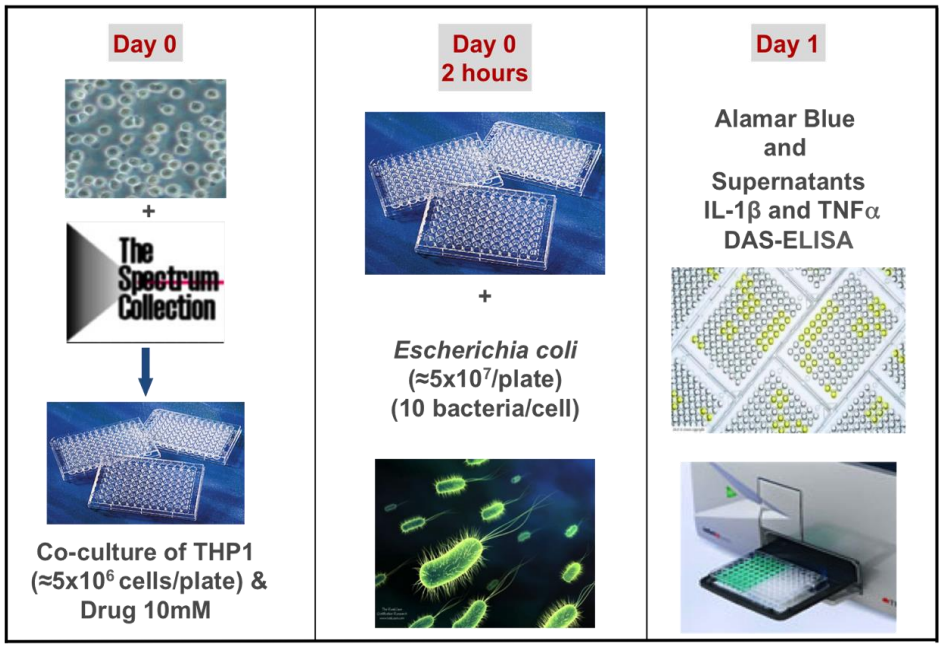

Figure 13 – Chemical screen protocol

In vitro shRNA-based screen and The RNAi Consortium Library

RNAi Screen

Building on the work of Fire *et al.* [175], we have used short-hairpin RNA (shRNA) mediated RNA interference to generate loss-of-function phenotypes and screen for genes with a role in primary sepsis mediators secretion (IL-1β and/or TNFα).

shRNA Screening Protocol

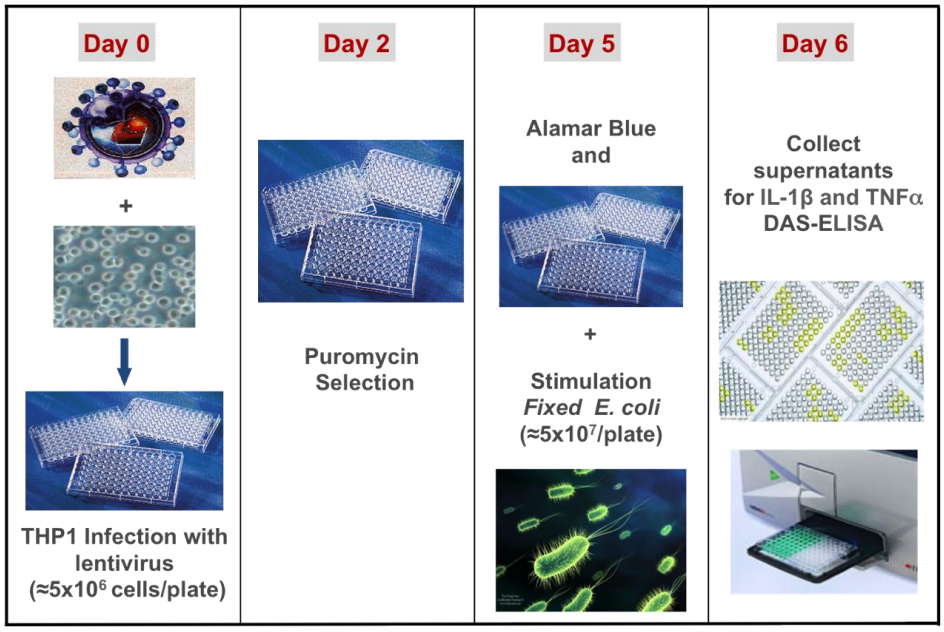

Figure 14 - RNAi screen protocol

Using the same principle as in the pharmacologic screen, we assessed the amount of cytokines in the supernatants of cells infected with a subset of an RNAi Lentiviral library of the "Broad Institute of MIT and Harvard" (Boston, USA) [176, 176, 175, 174, 177].

We generated a working subset of The RNAi Consortium (TRC) shRNA lentiviral vector [176] library that allows for the silencing of most of the genes that are either human kinases or phosphatases. This subset was composed of 1440 individually arrayed lentiviral shRNA vectors targeting ≈ 700 genes, after selecting the most efficient shRNAs (two on average) based on available silencing efficiency data from the Broad Institute of MIT and Harvard.

THP-1 cells were plated in 96 well plates at 10^6 cell/ml and infected with shRNA-expressing lentivirus. 48 hours later, the infected cells were selected with puromycin. After the 3 days of selection, plates were duplicated.

One of the plates was used to measure the cell number using Alamar Blue® cell viability assay (Invitrogen®), according to manufacturer's instructions.

In the other plate, cells were stimulated with 4% PFA-fixed DH5a *E. coli* at a Multiplicity of Infection (MOI) of 20 bacterial cells per THP-1 cell. Twenty-four hours after stimulation, cell supernatants were collected and IL-1β and TNF cytokines quantified by DAS-ELISA. All data values from IL-1β and TNF secretion assays were normalized by dividing the amount of IL-1β and TNF in the conditioned media 24, 12, 8, 6, 4 or 2 hours after *E. coli* stimulation by the number of cells in each well and then by the average concentration per cell of the plate. Results were logarithmic natural transformed. Scores were sorted in ascending order and graphed.

We calculated 1.5 SDEVs above and below the mean to identify the genes that changed IL-1β and TNF secretion when silenced. The selected genes were submitted to two additional rounds of phenotypic validation.

TRC Library

Detailed description of the RNAi Consortium (TRC) lentiviral RNAi library used in this study was originally published in [176] (see www.broad.mit.edu/rnai/trc/lib for additional details).

Virus-mediated integration of an RNAi expression cassette is considered to be the most efficient method for long-term gene suppression and use in a broad range of cell types [178]. When integrated, the cassette produces a short dsRNA molecule, normally in the form of a hairpin structure, short hairpin RNA (shRNA), which is then processed into active small interfering RNA (siRNA).

In order to amplify viral titers and diminish resistance to plasmid recombination, the pLKO.1 vector was selected by the consortium as the library vector. It was shown by Moffat *et al.,* that even after 10 rounds of sequential regrowth of several library clones, no evidence of recombination was observed [176]. The pLKO.1 lentiviral vector originates from pRRLSIN.cPPT.PGK/GFP/WPRE [179], and is a third-generation self-inactivating lentiviral vector [180, 181]. A human U6 promoter was introduced to drive the expression of shRNAs, and the vector contains a PGK promoter driving

expression of the puromycin resistance gene to allow selection of transduced cells (Figure 15).

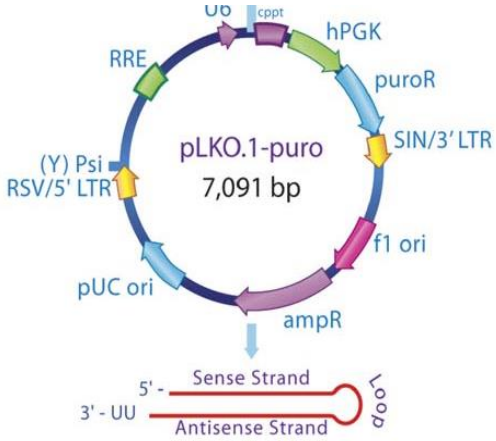

Figure 15 - Map for the pLKO.1 lentiviral vector (adapted from Moffat *et al.,* 2006).

Third generation lentiviral vectors use a three plasmid packaging system (i.e. the different necessary components of the viral genome are introduced in separate plasmid vectors, along with a viral envelope) to minimize the potential for recombination and creation of replication competent viruses. A schematic representation of a lentiviral vector production for stable shRNA expression is shown in Figure 16.

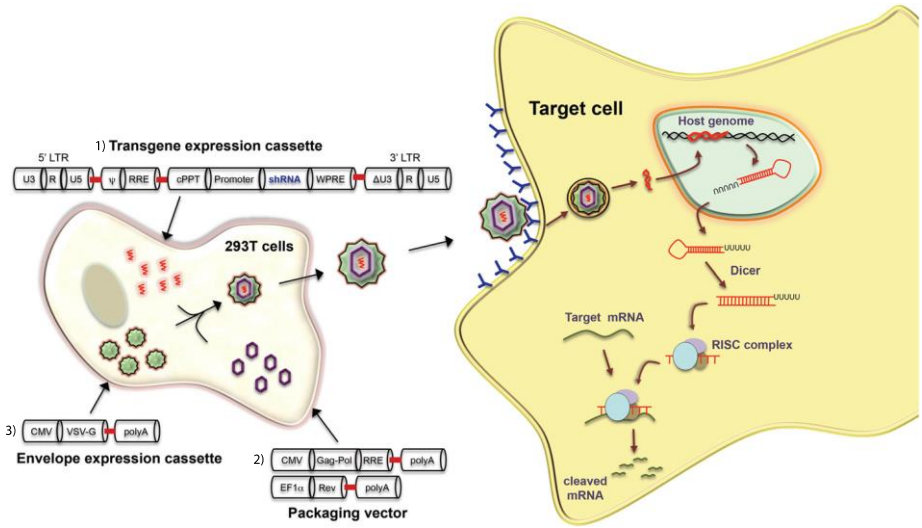

Figure 16 - Lentivirus expressing shRNAs production using three plasmid packaging system. Packaging host cells (in this case 293T cells) are transfected with a mixture of plasmids consisting of: 1) an shRNA expression cassette; 2) a packaging cassette and 3) a heterologous (commonly VSV-G) viral envelope expression cassette. The generated lentivirus is then used to transduce the target cell type of option for shRNA expression. Only the vector containing the shRNA expression cassette is integrated into the genome of the target cells, and as such, shRNA is continually expressed but infectious virus is not produced (adapted from Manjunath *et al.,* 2009).

The hairpin sequences contain stems of 21 nucleotides that exactly match the target transcript, and are selected using an algorithm designed to increase likelihood of good target gene knockdown, to avoid off-target effects and to disperse the 21-mer sequences across the target transcript [176]. For each target there is an average of five distinct shRNA constructs and at least four shRNAs are available for over 96% of targeted genes. Also, for the majority of the genes, the library contains shRNAs that target both coding sequence (CDS) and the 3' untranslated region (UTR) of their transcripts.

Animal Model

Animal care and experimental procedures were conducted in accordance with Portuguese and US guidelines and regulations after approval by the respective local committees (Instituto de Medicina Molecular and Instituto Gulbenkian de Ciência, and also by the Comissão de Ética para a Saúde do CHLN/FML).

All mice used were 8–12 weeks old. Mice were bred and maintained under specific pathogen-free (SPF) conditions. C57BL/6 and C57BL/6 *ATM*$^{-/-}$ were obtained from the Instituto Gulbenkian de Ciência (a kind gift from Dr. Vasco Barreto).

C57BL/6 *Nrf2*$^{-/-}$ mice were provided originally from the RIKEN BioResource Center (Koyadai, Tsukuba, Ibaraki, Japan) and subsequently at the Instituto Gulbenkian de Ciência.

LC3b$^{-/-}$ (B6129PF2/J background) and NMRI mice were purchased from Jackson and Charles River laboratories, respectively.

miR-146 mice were generated in the Baltimore's laboratory [182]. FancD2$^{-/-}$ were generated by the Grompe laboratory [183].

ATG7$^{loxP/loxP}$ were generated in by Masaaki Komatsu and obtained from the Green laboratory. ATM$^{loxP/loxP}$ mice were generated and obtained from the F.W. Alt's laboratory.

Cecal Ligation and Puncture Model

Anesthesia and Surgery

In pre-warmed heat pads, animals were anesthetized using xylazine/ketamine mixture (0.8 ml 2% xylazine + 1.2 ml ketamine + 8 ml saline - 10μl/g body weight) or isoflurane as it induces good relaxation of skeletal muscles. Mice were monitored by toe pinch prior to the beginning of surgery to ensure that they were adequately anesthetized. The abdomen was disinfected with three alternating applications of chlorhexidine scrub and solution, betadine, and alcohol to thoroughly disinfect the skin area (Figure 17 A).

To expose the cecum a longitudinal para-midline incision was made ensuring that the peritoneal cavity was not penetrated (Figure 17 B). Scissors are then used to extend the incision and to incise the peritoneal cavity (Figure 17 C). The cecum was located (typically found on the left of the abdomen) and pulled out using blunt ended forceps (Figure 17 D). The remaining of the small intestine and large bowel was left in place. Feces were pushed to the tip of cecum. The cecum was ligated at 50% the distance between the distal pole and the base of the cecum with black braided silk non-absorbable surgical spool suture 4-0. The extent of ligation determined the severity of the sepsis. C57BL/6 background: 50% cecal ligation results in 40% mortality at 72h, adding 20% mortality between D5-D7; ≥75% cecal ligation results in 100% mortality at D4 (Figure 17 E).

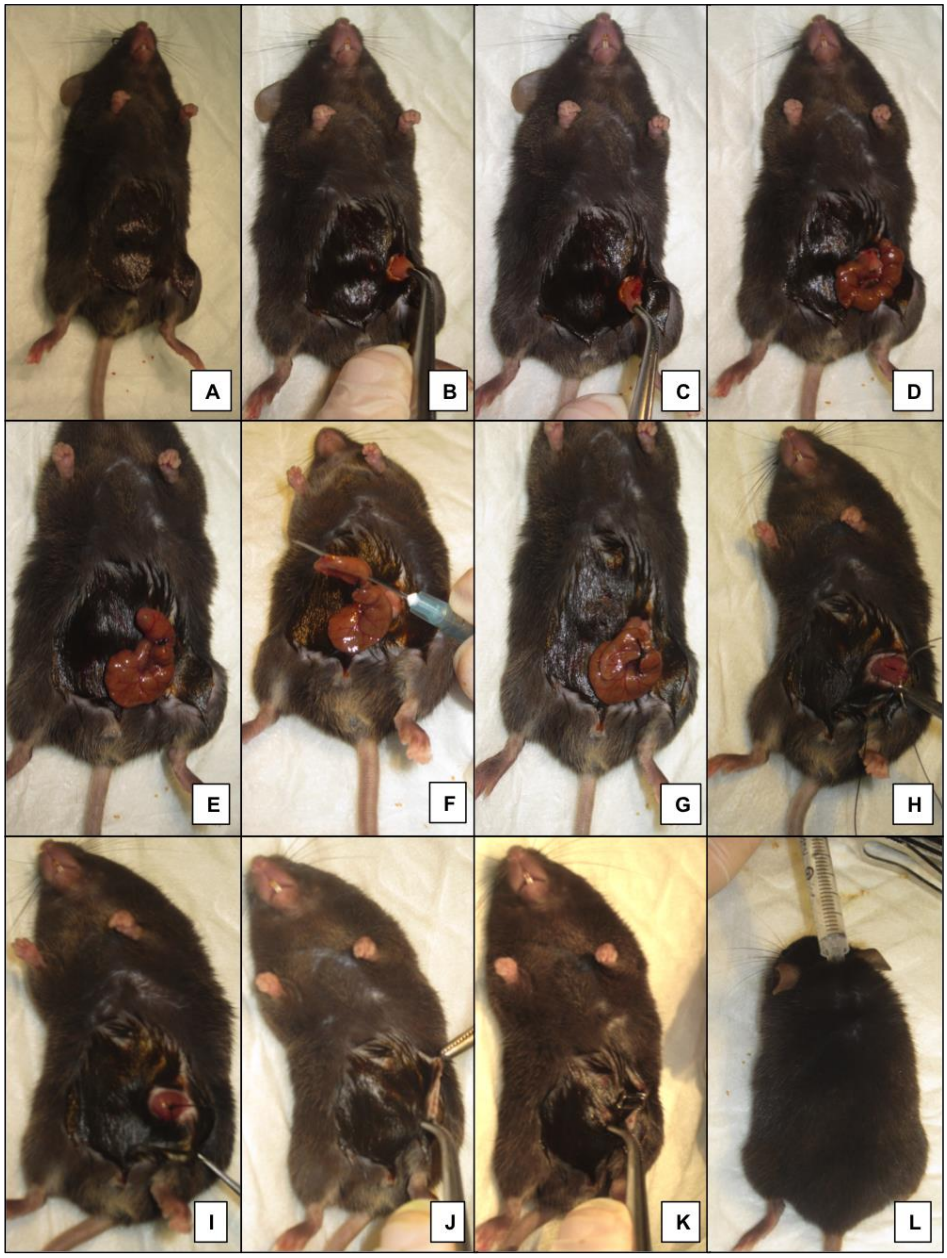

Figure 17 – Cecal Ligation and Puncture model.

The cecum was perforated by a single “through and through” puncture midway to the ligation and the tip of the cecum with a 23 G needle in a mesenteric to antimesenteric direction (Figure 17 F). Careful attention was paid to ensure that no damage occurred to mesenteric blood vessels. Upon removal of the needle tiny droplet of feces is extruded from both the mesenteric and antimesenteric holes to assess patency (Figure 17 G). The cecum was then relocated into the abdominal cavity without spreading feces on the abdominal wall or wound margins.

Closure and post-operative care

The peritoneum and abdominal muscles were closed with running sutures using wax coated braided absorbable polyglactin 910 surgical sutures 4-0 (Figure 17 H-I). The skin was closed using metallic wound clips (Figure 17 J-K)). Animals were rehydrated by injecting prewarmed saline (37C, 5mL per 100g of body weight) subcutaneously, allowing animals to demonstrate the early hyperdynamic phase of sepsis (Figure 17 L).

Surviving animals were euthanized at day 7-9 post-sepsis induction.

Endotoxemia Model

This model was performed by injecting intraperitoneally (i.p.) a single dose of 50 μg/g body weight of LPS (from *E. coli* serotype 026:B6; Sigma-Aldrich).

Pulmonary Infection Model

Pulmonary monostrain infections were carried out as described previously [184]. Intranasal injection of *Klebsiella pneumoniae* (ATCC13803) 8x10^7 CFU and *Pseudomonas aeruginosa* (ATCC27853) 5x10^6 CFU.

Pharmacologic Compounds

Epirubicin (Sigma-Aldrich), doxorubicin (Sigma-Aldrich), daunorubicin (Sigma-Aldrich) were dissolved in PBS, etoposide (Sigma-Aldrich) was dissolved in DMSO, aliquoted and stored at –80ºC. Meropenem (AstraZeneca, Lisbon) was dissolved in PBS.

Epirubicin and daunorubicin (0.6µg/g body weight), doxorubicin (0.5µg/g body weight), etoposide (2µg/g body weight) were injected intraperitoneally at 0 and 24 hours following CLP. Meropenem (20µg/g body weight b.i.d.) was injected i.p. for 5 consecutive days.

Additional Methods

Colony-Forming Units Assay

Blood samples from septic or mock CLP mice were collected by cardiac puncture at indicated times after surgery. Mice were subsequently perfused *in toto* with 10ml ice cold PBS and spleen, liver and kidneys were surgically removed and homogenized in 5ml of sterile PBS. Serial dilutions of blood and tissue homogenates were immediately plated on Trypticase Soy Agar II plates supplemented with 5% Sheep Blood. CFUs were counted after 12 hours of incubation at 37°C.

Serology and cytokine measurement

Plasma from blood samples obtained 24 hours post CLP was collected after centrifugation. LDH, CK, ALT and urea levels were measured using the BioAssay Systems kits (BioAssay Systems, California) according to company's protocol. Levels of TNF-alpha, IL-1β and IL-6 were measured using the murine ELISA kits (R&D Systems, Minneapolis) according to company's protocol. Levels of HMGB1 were assessed using the ELISA kit (Shino Test Corporation, Tokyo) according to company's protocol.

Histology

Mice were euthanized, perfused *in toto* with 10ml ice cold PBS and lungs, livers and kidneys were surgically removed.

Livers were placed in 10% phosphate buffered formalin for 24 hours after which were embedded in paraffin. Sections were subsequently incubated with a primary antibody reactive to HMGB1 (Abcam) followed by incubation with biotinylated secondary antibody and then with biotinylated horseradish peroxidase.

Staining was developed by addition of diaminobenzidine (DAB) substrate (Vector Labs, Burlingame, CA) and counterstained with hematoxylin. Lungs were embedded in Tissue-Tek OCT (Sakura), and snap-frozen in liquid nitrogen. Lung sections (7 μm) were fixed in 1% paraformaldehyde in PBS for 2 minutes, followed by methanol at -20ºC for 10 minutes and then in acetone for 2 minutes.

Detection of LC3b and histone γH2AX was performed by incubating sections overnight at 4ºC with rabbit polyclonal antibodies specific for, respectively, LC3b (L7543, Sigma Aldrich, USA) and γH2AX (phosphoS139) (ab2893; Abcam, Cambridge, UK); incubation with a secondary DyLight 488-coupled antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was for 1 hour at room temperature.

Sections were counterstained with DAPI (0.5 μg/ml) to visualize DNA and mounted in Vectashield (Vector Laboratories Inc., Burlingame, CA) before confocal microscopy.

Samples were examined with a Zeiss LSM 510 META laser scanning confocal microscope (Carl Zeiss, Jena, Germany). The acquired images were analyzed using a MATLAB (Mathworks; Natick, MA) routine developed in-house to perform automatic threshold segmentation and enumeration of individual cell nuclei stained with DAPI.

In vivo viral infection and viral titer assay

Murid herpesvirus-4 infection and viral particle quantification was performed as previously described [185]. Briefly, mice were intranasally inoculated with 1000 PFU of MuHV-4 strain 68 in 20 µl of PBS under light isofluorane anaesthesia.

At 6 and 12 days post-infection, lungs were removed and homogenised in 5ml of Glasgow's modified Eagle's medium (GMEM). Infectious viral titers in freeze-thawed lung homogenates were determined by serial diluted suspension assay using Baby hamster kidney cells (BHK-21) cells cultured in GMEM supplemented with 10% fetal bovine serum, 10% tryptose phosphate broth, 2mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (GMEM). Plates were incubated for four days, fixed with 10% formal saline and counterstained with toluidine blue. Viral plaques were counted with a plate microscope.

Cre-adenoviral vector intranasal infection was carried as described before[186]. Briefly, the *Cre*-adenovirus was prepared as a calcium-phosphate co-precipitate and incubated for 20 min at room temperature. $Atg7^{loxP/loxP}$ and

$ATM^{loxP/loxP}$ were subjected to light isofluorane anaesthesia and allowed to inhale 125 µl of virus at a concentration of 10×10^7 PFU. Additionally, wildtype C57Bl/6 mice were included as controls. Mice were allowed to recover for 5 days after inhalation, after which they were subjected to CLP.

Staining and flow cytometry

Peritoneal infiltrating leukocytes from either wildtype or LC3b-GFP transgenic animals were obtained 24 hours post CLP by lavage with 5 ml of sterile ice-cold PBS (Sigma), washed and blocked with mouse Ab anti-FcγIII/II (clone 93) receptor mouse Ab diluted in PBS containing 2% FCS (v/v) for 20 minutes at 4°C.

Surface markers were detected by incubating for 30 minutes at 4°C with mouse Ab anti-CD4 (clone GK1.5), -CD8 (clone 53-6.7), -CD19 (clone 6D5), -Ly-6G (clone 1A8) (all Biolegend) and -neutrophils monoclonal antibody (clone 7/4) (Abcam). Dead cells were excluded by co-staining with propidium iodide. Total cell number was determined by flow cytometry using a fixed number of latex beads (Beckman Coulter) co-acquired with a pre-established volume of the cellular suspension.

For phosphoATM intracellular staining, stimulated THP-1 cells were washed and fixed with ice-cold methanol. Mouse Ab anti-phosphoATM pS1981, clone 10H11.E12 (IgG1k) (Rockland) was incubated for 60 minutes at room temperature followed by an incubation of secondary Ab conjugated with Alexa 488 (Molecular Probes). Fluorescence was measured by flow cytometry, and data analysed using FlowJo software.

Bone marrow-derived macrophages (BMDM)

BMDM were derived as previously described. Briefly, total bone marrow cells were flushed from the femurs and tibiae of wildtype and $ATM^{-/-}$ mice and cultured in RPMI supplemented with 10% fetal bovine serum, 2 mM glutamine, 1mM sodium pyruvate, 10mM HEPES, 50 µM 2-mercaptoethanol and 20 U/ml penicillin and 20 µg/ml streptomycin (all from Invitrogen) supplemented with 30% M-CSF-producing L929 cells conditional medium for 7 days. Adherent cells (i.e. macrophages) were collected by gentle scrapping and replated in 96-well, flat-bottomed plates (BD Biosciences), pre-incubated with epirubicin for 1 hour and challenged with 4% PFA-fixed DH5a *E.coli* at MOI of 20 bacterial cells per BMM cell for an additional 4 hours.

Immunoblotting

Mouse phosphoATM (4526, Cell Signaling, Danvers, MA, 1:1000 dilution), rabbit total ATM (2873, Cell Signaling, Danvers, MA, 1:1000 dilution), rabbit LC3b (Sigma, 1:1000 dilution) and the rabbit FancD2 (Novus Biologicals, 1:1000 dilution) antibodies were used overnight at 4ºC. Primary antibodies were detected using peroxidase conjugated secondary antibodies (1 hour; RT) and developed with SuperSignal chemiluminescent detection kit (Pierce, Carcavelos, Portugal).

RT-qPCR

Total RNA was extracted with TRIzol (Invitrogen) and reverse transcribed with Superscript II Reverse Transcriptase (Invitrogen). Quantitative PCR reaction was performed with Power SYBRgreen (Applied Biosystems) on Rotor-Gene 6000 real-time thermal cycler (Corbett Life Science /QIAGEN).

Primer sequences were retrieved from Primer Bank (http://pga.mgh.harvard.edu/primerbank/) or designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Values were normalized to GAPDH.

Assessment of autophagy and ROS content

THP-1 cells were stimulated as described earlier. Autophagy was assessed by incubating cells with Cyto-IDTM Green detection Reagent (Enzo Life sciences) according to the manufacturer's instructions. Generation of cellular free radicals was assessed by incubating cells for 20 minutes with 0.5mM of the broad free radical probe 5-(and 6)-chloromethyl-2'7'-dichlorodihydrofluoscein diacetate acetyl ester (CM-H2DCFDA; Molecular Probes). Fluorescence was measured by flow cytometry, and data analyzed using FlowJo software.

Chapter III - Results and Discussion

Sepsis syndromes and their immune response are triggered by a combination of pro-inflammatory mediators leading to cellular injury and organ dysfunction, and also an exaggerated synthesis of anti-inflammatory mediators that induce an immune-suppressive status. Cytokines play a key role in the onset and maintenance of all inflammatory responses to pathogens and endogenous alarm signals. On one hand, they facilitate activation of the immune cells that have to be in place to fight infection; on the other, these same mediators contribute to the pathogenesis and lethal consequences of endotoxemia and sepsis.

My initial question was: ***"Is it possible to dampen the overwhelming inflammatory response without compromising the ability to fight infection?"***

During a clinical documented SIRS, we know that most cytokine levels correlate with other inflammatory mediators (lactate, C-reactive protein, activated complement fragments), clinical status (fever, organ dysfunction, shock), clinical severity scores (SOFA, SAPS II) and outcome.

The most predictive pattern of a good outcome is the absence of detectable levels over time, instead of an absolute elevated/diminished

cytokine level. For at least 20 years we know that sustained elevated levels of some cytokines (TNFα) are predictive of MODS [187].

TNFα and IL-1β act synergistically to mount an effective inflammatory response, by activating neutrophils, promoting the pro-coagulant flank of the coagulation cascade, and up-regulating gene expression of adhesion molecules to promote leukocyte diapedesis. All these features potentiate tissue injury and ultimately organ dysfunction.

Several studies have demonstrated, in animal models, that TNFα neutralization was beneficial before LPS or bacterial challenge and that TNFα-KO mice were protected from LPS induced shock [188]. In contrast, LPS challenge in IL-1β deficient mice produced the same phenotype as in wild type mice. This effect was attributed to the compensatory actions of other IL-1-family cytokines (IL-1α, IL-18, IL-33). This hypothesis was further supported by the observation that mice deficient in Caspase-1 are resistant to endotoxic shock. However, previous caspase-1 KO mice are actually double KO for caspase-1 and caspase-4. It was only recently shown, by having single KO mice for each of these caspases that Caspase-4 (also known as Caspase-11) is critical for Caspase-1 activation and IL-1β production in response to certain bacterial strains [189]. These authors demonstrated that Caspase-4, not Caspase-1, was required for non-canonical inflammasome-triggered macrophage cell-death, and that Caspase-4 orchestrates Caspase-1-dependent and -independent production of IL-1β. Therefore, it might be the loss of Caspase-4, instead of Caspase-1, that protects mice from lethal endotoxemia.

IFNγ has pro-inflammatory and antibacterial properties, contributing to enhanced phagocytosis, ROS production and increased activity of macrophages

and neutrophils. However, IFNγ-KO mice are resistant to lethal endotoxemia and have reduced lethality in bacterial sepsis models, showing decreased bacterial load and diminished systemic inflammation [190].

Macrophage migration inhibitory factor (MIF) is synthesized in response to IFNγ and TNFα, promoting the secondary expression of multiple pro-inflammatory cytokines. MIF-deficient mice are protected from LPS and bacterial challenge [191].

High-mobility group box 1 (HMGB1) is a nuclear transcription factor-like protein that has been identified as a late inflammatory mediator, after its extracellular release. When it is found outside the cell, either from passive release or by active secretion, HMGB1 induces the expression of TNFα and IL-1β.

HMGB1 seems to be a necessary and sufficient mediator in severe sepsis because: systemic HMGB1 is detected in serum of patients and *in vivo* experimental models of severe sepsis [31]; administration of recombinant HMGB1 in mice can recapitulate the syndrome of MOF representative of severe sepsis [192]; and the inhibition of HMGB1 secretion/activity is protective in LPS- or bacteria-induced models of MOF [192]. In clinical studies, HMGB1 levels correlate with the severity of certain sepsis syndromes [193].

HMGB1 production is postulated to be the connection between apoptosis on the final common pathway to organ damage and death, in severe sepsis [194].

The compensatory anti-inflammatory cytokines (IL-10, IL-4, IL-13, TGFβ and IFNγ) down-regulate the production of pro-inflammatory cytokines, inducing a compensatory release of soluble TNFα receptors and synthesis of IL-1

receptor antagonist (IL-1Ra) [195, 196, 197]. All these compensatory anti-inflammatory cytokines have shown a protective effect against lethality in LPS- or bacteria-induced sepsis models. However, they also contribute to the immune-dysfunction observed in septic patients.

The "Cytokine Storm" is an historical expression that emerged from observing this devastating inflammatory reaction associated with increased pro-inflammatory cytokine synthesis, severe illness and organ dysfunction.

This heterogeneous syndrome is one of the problems we brought from the clinical side. Having this in mind, we devised an "in vitro" model as a simple starting point to approach the extreme complexity and heterogeneity.

Using the Spectrum® library and a monocyte-macrophage human cell-line, we screened for immune-modulatory compounds that could simultaneously down-regulate the secretion of TNF and IL-1β. Our objective was to decrease but not to completely block cytokine secretion, because of the well-known dual role of pro-inflammatory mediators [198 199 200].

Anthracyclines inhibit the secretion of TNF and IL-1β in vitro

In order to identify small molecules that simultaneously inhibit the secretion of TNF and IL-1β, we have performed a chemical screen using a library of over 2320 compounds.

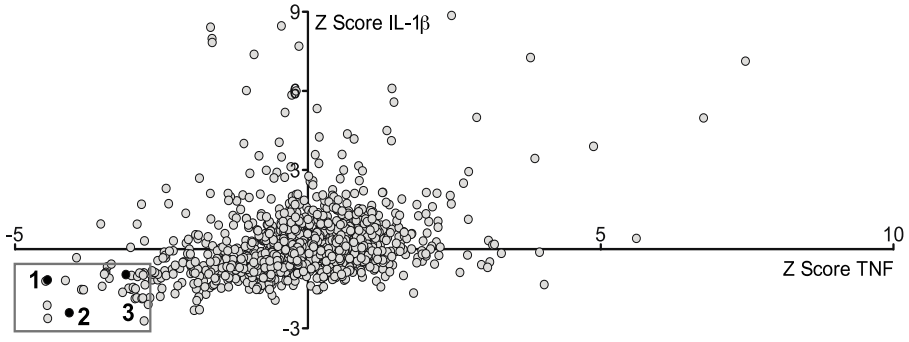

Figure 18 - Two-dimension plot of TNF and IL-1β production Z scores calculated upon THP-1 cells challenge with PFA-fixed E. coli for 24 hours in the presence of 10μM of each compound. The grey square defines the area in which compounds are considered primary hits, i.e., inhibiting both TNF and IL-1β. Black dots identify epirubicin (1), daunorubicin (2) and doxorubicin (3)

We identified 45 leading candidates (Figure 18 and Table IV) that inhibited the secretion of both cytokines. Among these, we found 3 representative members of the anthracycline family of chemotherapeutic agents (epirubicin, doxorubicin and daunorubicin).

Table IV - List of drug candidates with a simultaneous effect on TNF and IL-1b secretion sorted according to the TNF score.

ID	MOLECULE NAME	Z score TNF	Z score IL-1β	BIOACTIVITY
1505708	***EPIRUBICIN HYDROCHLORIDE***	***-4,4749***	***-1,2129***	***antineoplastic***
300037	CRASSIN ACETATE	-4,4487	-2,1263	antiviral
1501193	ERYSOLIN	-4,4424	-1,1627	antiproliferative
1504079	TOMATINE	-4,4402	-2,6261	antifungal, antibacterial, anti-inflammatory
330001	DACTINOMYCIN	-4,1376	-1,1878	antineoplastic, intercalating agent
1505483	***DOXORUBICIN***	***-4,0711***	***-2,4121***	***antineoplastic***
200007	GAMBOGIC ACID	-3,8624	-1,5297	anti-inflammatory, cytotoxic
200090	OBTUSAQUINONE	-3,8294	-1,5322	
200022	AKLAVINE HYDROCHLORIDE	-3,5117	-1,4089	antibacterial, antineoplastic
1504181	PRISTIMERIN	-3,4749	-0,9526	antineoplastic, anti-inflammatory
1505955	COLISTIN SULFATE	-3,4573	-0,9186	antibacterial
1504082	DIHYDROCELASTROL	-3,3875	-1,1020	
1505908	MANGOSTIN TRIMETHYL ETHER	-3,1628	-1,6342	
1504218	ACRISORCIN	-3,1061	-0,9596	antifungal
300549	ACETYL ISOGAMBOGIC ACID	-3,0568	-1,4812	
201522	GAMBOGIC ACID AMIDE	-3,0139	-1,5172	caspase inhibitor
1500223	***DAUNORUBICIN***	***-3,0071***	***-0,9955***	***antineoplastic***

ID	MOLECULE NAME	Z score TNF	Z score IL-1β	BIOACTIVITY
201604	PYRROMYCIN	-2,9806	-1,4564	antibacterial
1500260	PYRITHIONE ZINC	-2,9518	-1,8723	antibacterial, antifungal, anti-seborrheic
201664	CELASTROL	-2,9413	-1,4996	antineoplastic, anti-inflamatory, NO synthesis inhibitor, chaperone stimulant
1500319	GRAMICIDIN	-2,9186	-1,8456	antibacterial
1503006	BENZYL ISOTHIOCYANATE	-2,9086	-1,0343	antineoplastic, antibacterial, antifungal
1503904	PATULIN	-2,8746	-0,9027	antibacterial
1503640	PARTHENOLIDE	-2,8423	-0,9220	5HT antagonist, antineoplastic, smooth muscle relaxant
100005	ANTHOTHECOL	-2,8344	-1,8687	
100009	CEDRELONE	-2,8172	-1,8526	
1504098	PHENOTHRIN	-2,8108	-0,9829	ectoparasiticide
1505438	HYDROCORTISONE VALERATE	-2,7943	-2,7229	antiinflammatory, glucocorticoid
1504240	1,4-NAPHTHOQUINONE	-2,7780	-1,3490	
1505450	PREDNISOLONE HEMISUCCINATE	-2,7243	-2,0959	anti-inflammatory, glucocorticoid
1500315	GENTIAN VIOLET	-2,6964	-1,8357	antibacterial, anthelmintic
310010	HELENINE	-2,6839	-0,9437	anthelmintic, antibacterial, antineoplastic
310035	SANGUINARINE SULFATE	-2,6516	-1,1600	antineoplastic, antiplaque agent
1503074	ALEXIDINE HYDROCHLORIDE	-2,6482	-0,9412	antibacterial

ID	MOLECULE NAME	Z score TNF	Z score IL-1β	BIOACTIVITY
1503278	MITOXANTHRONE HYDROCHLORIDE	-2,5627	-1,1589	antineoplastic
1505723	BETAMETHASONE ACETATE	-2,5608	-0,9505	antiinflammatory
100146	7-DESACETOXY-6,7-DEHYDROGEDUNIN	-2,5177	-1,7896	
1503432	MEPARTRICIN	-2,5050	-1,0819	antifungal, antiprotozoal
201524	DIHYDROGAMBOGIC ACID	-2,4888	-1,4900	
1505722	DESOXYMETASONE	-2,4178	-0,9951	antiinflammatory
1505726	DESONIDE	-2,2977	-0,9460	antiinflammatory, glucocorticoid
1500521	PYRVINIUM PAMOATE	-2,2681	-1,6838	antihelminthic
1505168	ETHACRIDINE LACTATE	-2,2188	-1,3892	antiseptic, abortifaceant
1501149	RITODRINE HYDROCHLORIDE	-2,1498	-1,2683	muscle relaxant (smooth)
1505125	ALCLOMETAZONE DIPROPIONATE	-1,9992	-0,9230	anti-inflammatory, glucocorticoid

To validate their effect in the inhibition of IL-1β and TNF secretion we treated THP-1 cells with increasing concentrations of the top scoring anthracyclines. For epirubicin, we found the half-inhibitory concentration (IC50) to be 0.40μM for IL-1β and 0.50μM for TNF, in the case of doxorubicin 1.02μM for IL-1β and 0.84μM for TNF, and for daunorubicin 0.22μM for IL-1β and 0.17μM for TNF (Figure 19).

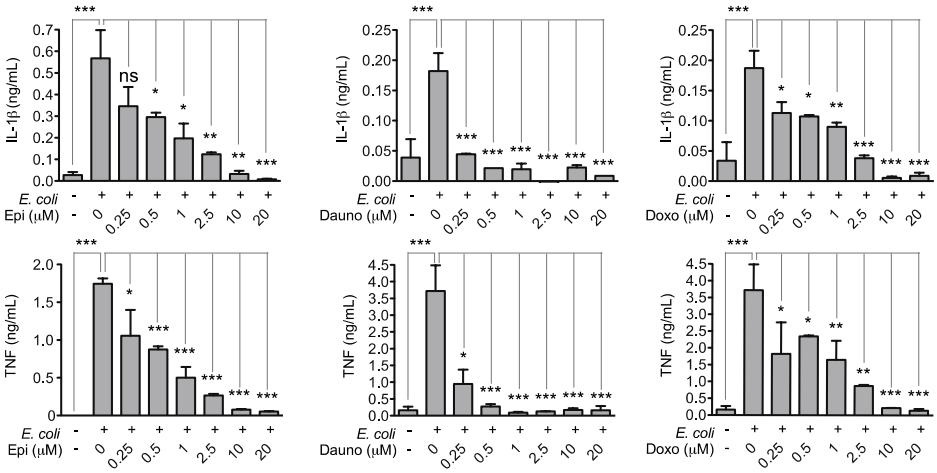

Figure 19 - IL-1β and TNF production by E. coli challenged THP-1 cells (4 hours) after a pre-incubation (1 hour) with increasing concentrations of epirubicin (left panel), daunorubicin (middle panel) and doxorubicin (right panel). Results shown represent arithmetic means ± SD from duplicate samples in one of 3 independent assays. ns, not significant; *P<0.05; **P<0.01 ***P<0.001; Mann-Whitney test.

This *in vitro* inhibitory effect was dissociated from cytotoxicity of the compounds tested on THP-1 cells (Figure 20) and therefore, the lower cytokine secretion levels cannot be explained by having less viable cells.

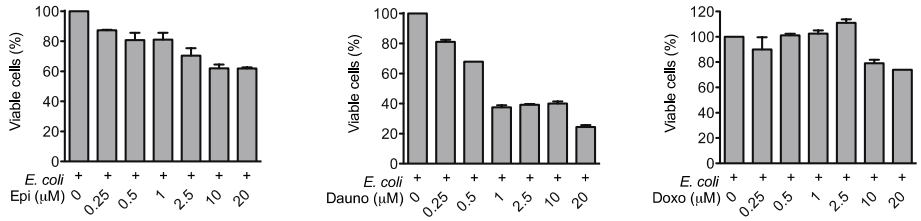

Figure 20 - THP-1 cell viability upon *E. coli* challenge (4 hours) after a pre-incubation (1 hour) with increasing concentrations of epirubicin (left panel), daunorubicin (middle panel) and doxorubicin (right panel).

Epirubicin confers protection against severe sepsis

To investigate the *in vivo* effects of epirubicin, we chose the cecal ligation and puncture (CLP) mouse model [201].

In the CLP model, sepsis results from a polymicrobial infection of abdominal origin, leading to bacteremia and a systemic inflammatory response [201]. We have adjusted CLP severity to a high-grade sepsis, where at least 80% of C57BL/6 mice die within 48hrs after the initial procedure. Under these conditions, epirubicin administered i.p. at the time of CLP and again 24hrs later in a total of 1.2μg/g of body weight reproducibly increased the survival of C57BL/6 mice subjected to CLP by nearly 70% (Figure 21-A).

The protection induced by this anthracycline is not dependent on mouse strain, as the "outbred" NMRI mice are similarly protected by epirubicin (Figure 21-B).

A similar protective effect is observed in epirubicin-treated animals with the same dose and schedule but administered intravenously (Figure 21-D).

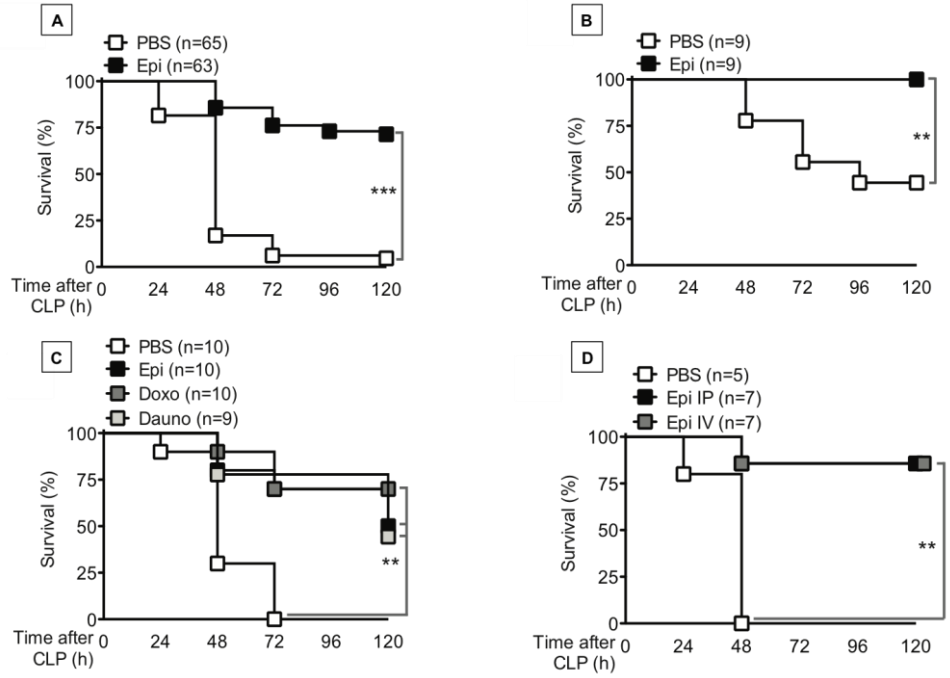

Figure 21 - Epirubicin is protective in animal models of severe sepsis. **(A)** Survival of C57BL/6 wildtype animals subjected to CLP treated with carrier (PBS) or epirubicin (Epi) (0.6µg/g body weight) at the time of procedure and 24 hours later. **(B)** Survival of NMRI mice subjected to CLP and treated with carrier (PBS) or epirubicin (Epi) as in (A). **(C)** Survival of C57BL/6 wildtype animals subjected to CLP treated with carrier (PBS), epirubicin (Epi), doxorubicin (Doxo) or daunorubicin (Dauno). Treatment schedule and doses as in (A). **(D)** Survival of C57BL/6 wildtype animals subjected to CLP treated with carrier (PBS) or epirubicin (0.6µg/g body weight) intraperitoneally (Epi IP) or intraveneouslly (Epi IV) as in (A). *P<0.05; **P<0.01; ***P<0.001; log-rank (Mantel-Cox) test.

Epirubicin is equally effective in other clinically relevant experimental models of sepsis and septic shock. We have confirmed these findings by inducing an endotoxic shock through intraperitoneal LPS injection (Figure 22-A) and pulmonary infection by intranasal inhalation of monostrain bacteria, such as *P. aeruginosa* and *K. pneumoniae* (Figure 22-B).

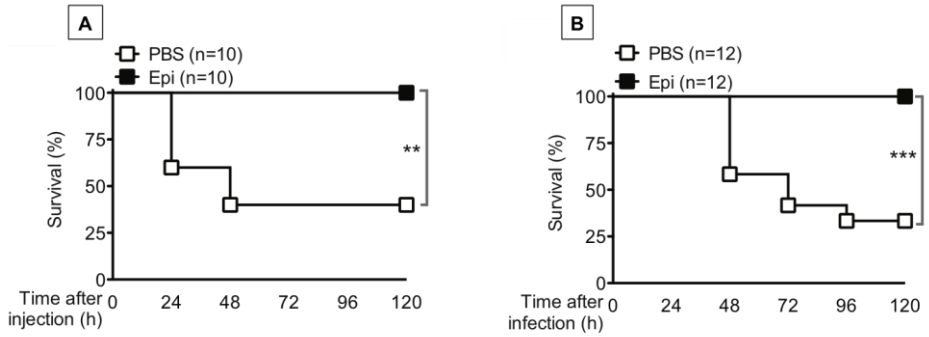

Figure 22 - (A) Survival of C57BL/6 wildtype animals following lethal LPS injection and treatment with carrier (PBS) or epirubicin (Epi) (0.6µg/g body weight) at the time of procedure and 24 hours later. **(B)** Survival of C57BL/6 wildtype animals following intranasal inoculation of Klebsiella pneumoniae and treated with carrier (PBS) or epirubicin (Epi) at the time of procedure and 24 hours later. ns, not significant; *P<0.05; **P<0.01; ***P<0.001; log-rank (Mantel-Cox) test.

This appears to be a general property of the anthracycline family, a notion supported by the observation that other representative members of this family of drugs, identified in the initial chemical screen, confer similar levels of protection against CLP (Figure 21-C). This argues that epirubicin is effective in the treatment of sepsis of different origins in addition to peritoneal polymicrobial sepsis.

To document the protective mechanisms induced by anthracyclines we measured serological markers of organ damage in the serum of non-treated CLP mice and compared them with those of epirubicin-treated mice 24 hours after the initial CLP procedure. Serum concentration of LDH (an unspecific lesion marker that increases in plasma whenever there is tissue damage; usually elevated in lung and GI lesions), CK (unspecific muscle cell damage), ALT (liver lesion) and Urea (kidney dysfunction) were reduced to almost basal levels in

mice treated with epirubicin *vs* untreated mice (Figure 23-A). Our results suggest that anthracyclines provide tissue damage control and sustain organ function.

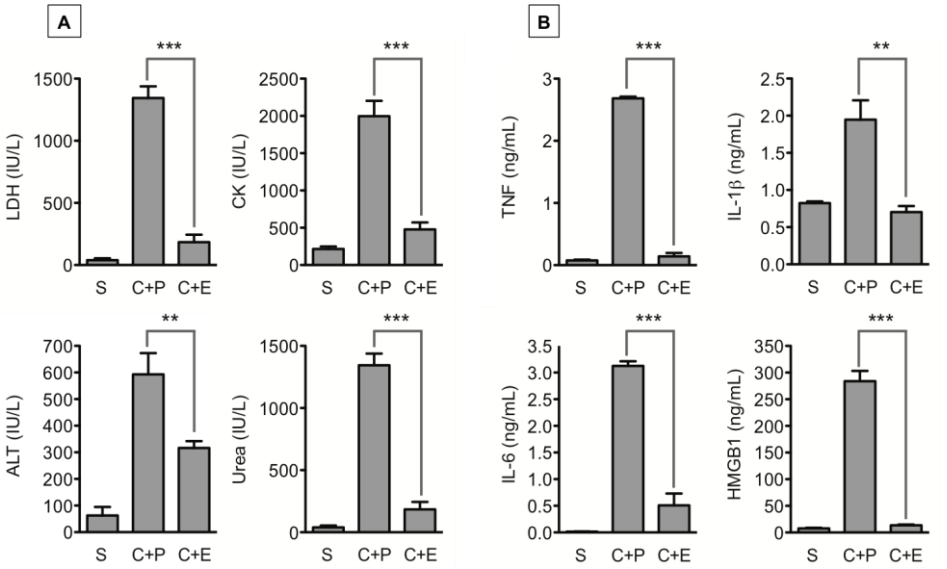

Figure 23 - Epirubicin counteracts tissue damage and inflammation associated with CLP as assessed by **(A)** LDH, CK, ALT, urea and **(B)** TNF, IL-1β, IL-6 and HMGB1 plasma concentrations in C57BL/6 wildtype animals 24 hours after mock CLP (S) (n=2) or CLP followed by treatment with PBS (C+P) (n=5) or epirubicin (C+E) (n=7) (0.6µg/g body weight) at the time of procedure and 24 hours later. Results shown represent arithmetic means ± SEM from duplicate (A) or triplicate (B) readings per animal. ns, not significant; *P<0.05; **P<0.01; ***P<0.001; Unpaired t test.

On the inflammatory side, we have also observed a significant reduction in the circulating levels of inflammatory mediators such as TNF, IL-1β and IL-6 when compared to non-treated CLP mice (Figure 23-B). The levels of the late inflammatory mediator HMGB1 were also reduced (Figure 23-B) in the plasma of epirubicin-treated mice 24hrs after the initial CLP procedure.

The main target organs in sepsis-originated MODS are: the kidneys, the liver and the lungs. When looking into the histology of these organs (H&E) at 24h after CLP, there was no significant difference in architecture or morphology

between the groups treated with the carrier PBS or epirubicin (Figures 24, 25 and 26).

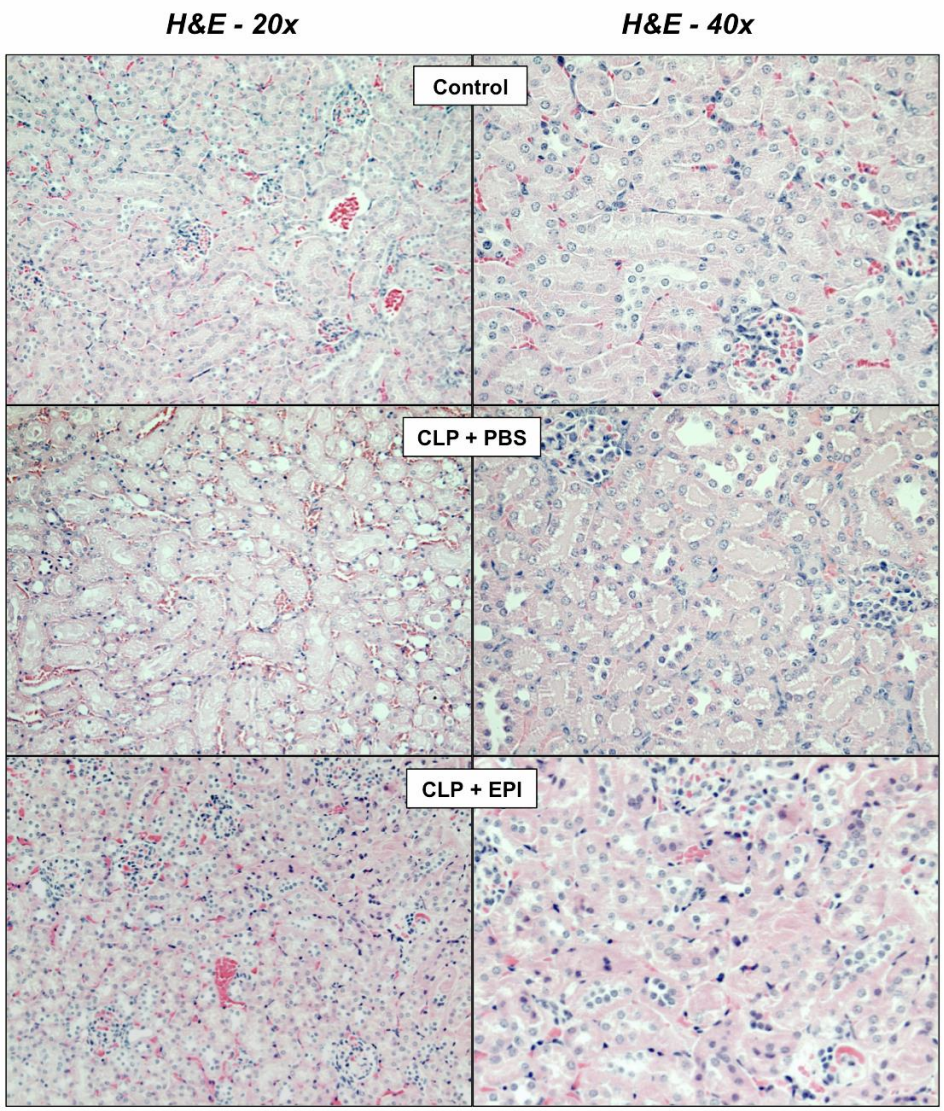

Figure 24 - Kidney histology (hematoxylin and eosin stain - H&E) 24h after CLP. C57BL/6 wildtype animals subjected to CLP treated with carrier (PBS) or epirubicin i.p. (0.6µg/g body weight), at the time of procedure. Magnification of 20x & 40x.

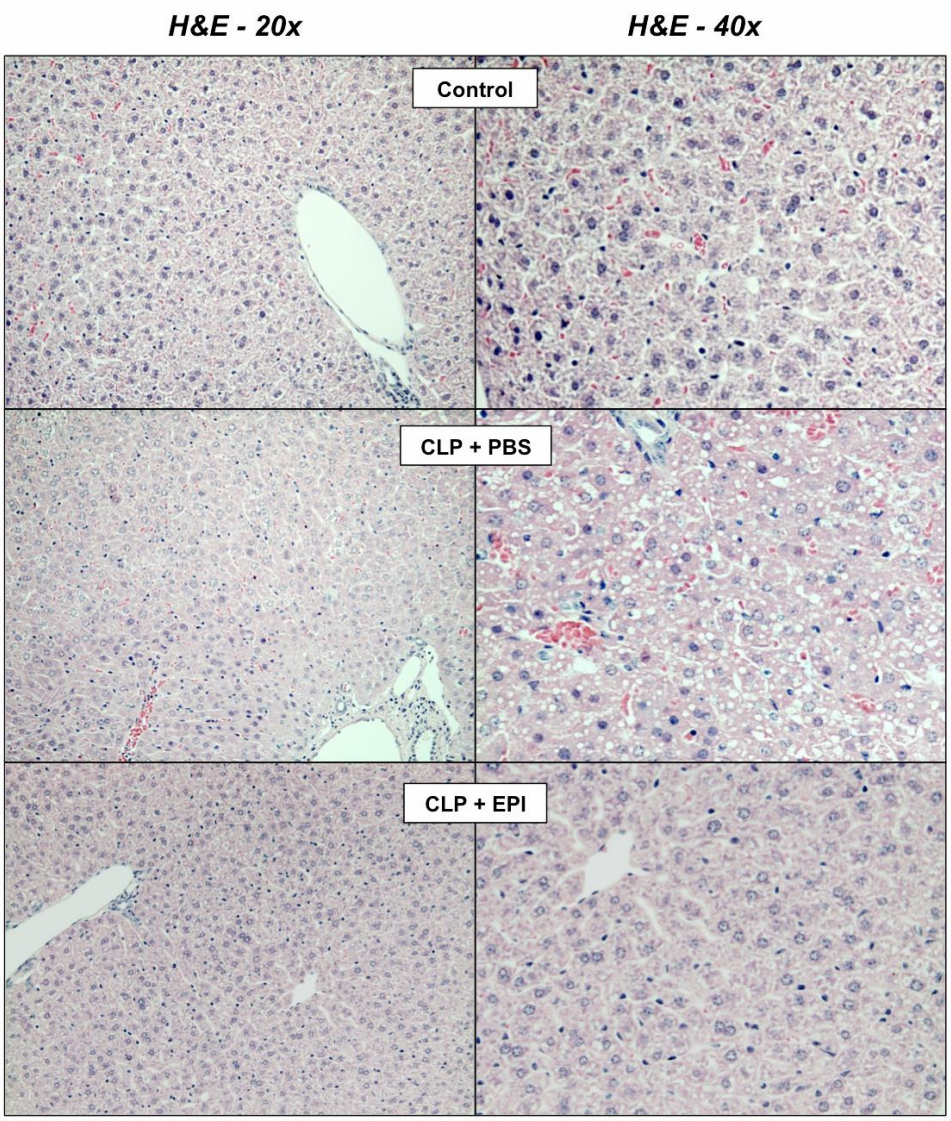

Figure 25 - Liver histology (H&E) 24h after CLP. C57BL/6 wildtype animals subjected to CLP treated with carrier (PBS) or epirubicin i.p. (0.6µg/g body weight), at the time of procedure. Magnification of 20x & 40x.

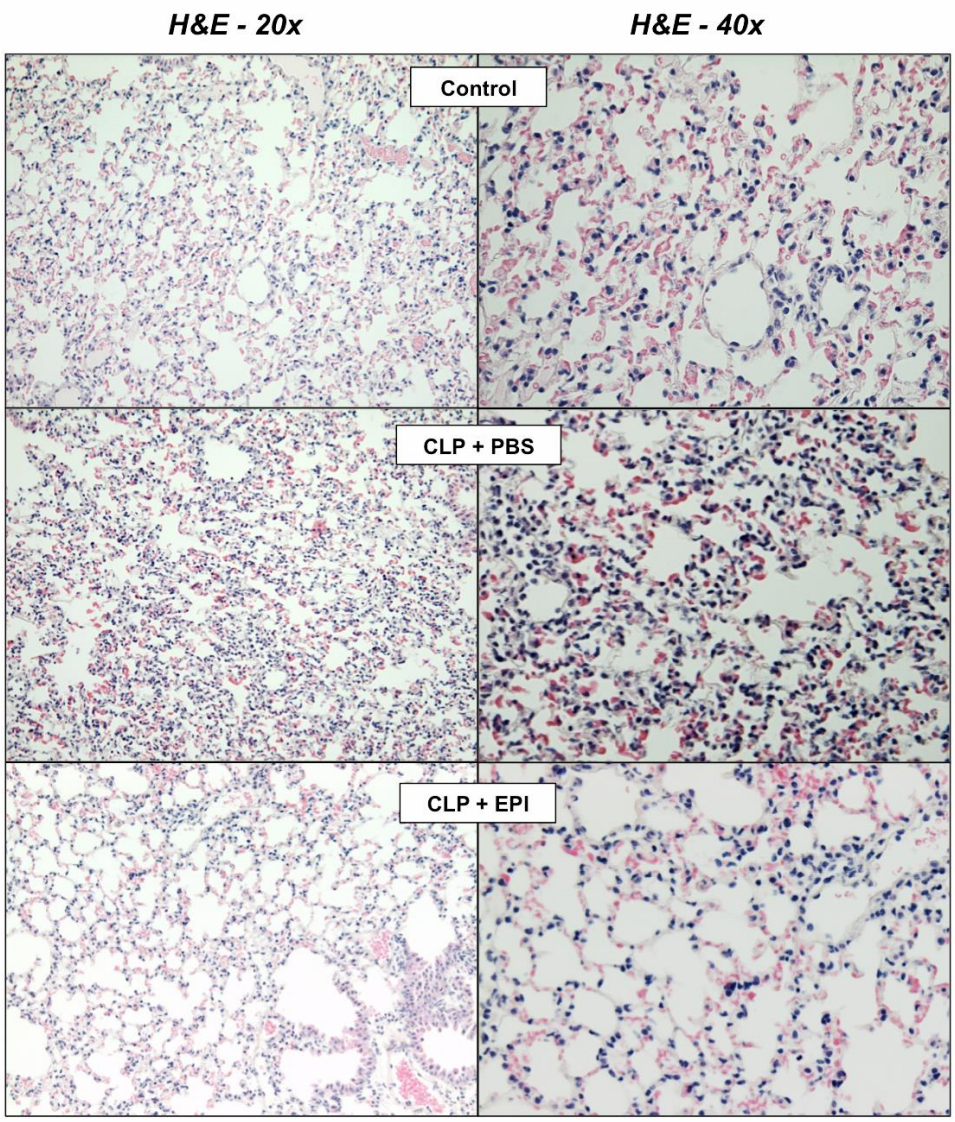

Figure 26 - Lung histology (H&E) 24h after CLP. C57BL/6 wildtype animals subjected to CLP treated with carrier (PBS) or epirubicin i.p. (0.6µg/g body weight), at the time of procedure. Magnification of 20x & 40x.

This result was somewhat unexpected as at least 50-60% of the animals were dying between 24 hours and 36 hours after severe sepsis onset. At this time, some degree of organ damage should already be apparent.

However, in immunohistochemistry assays, namely with Anti-HMGB1 stain, we could perceive marked differences between mice treated with carrier or epirubicin (Figure 27).

Our observations suggest that, despite no difference in H&E histology, a vast majority of tissues in the PBS group had already translocated HMGB1 from the nucleus to the cytoplasm. This phenomenon occurs when the cells of these organs are entering cell death.

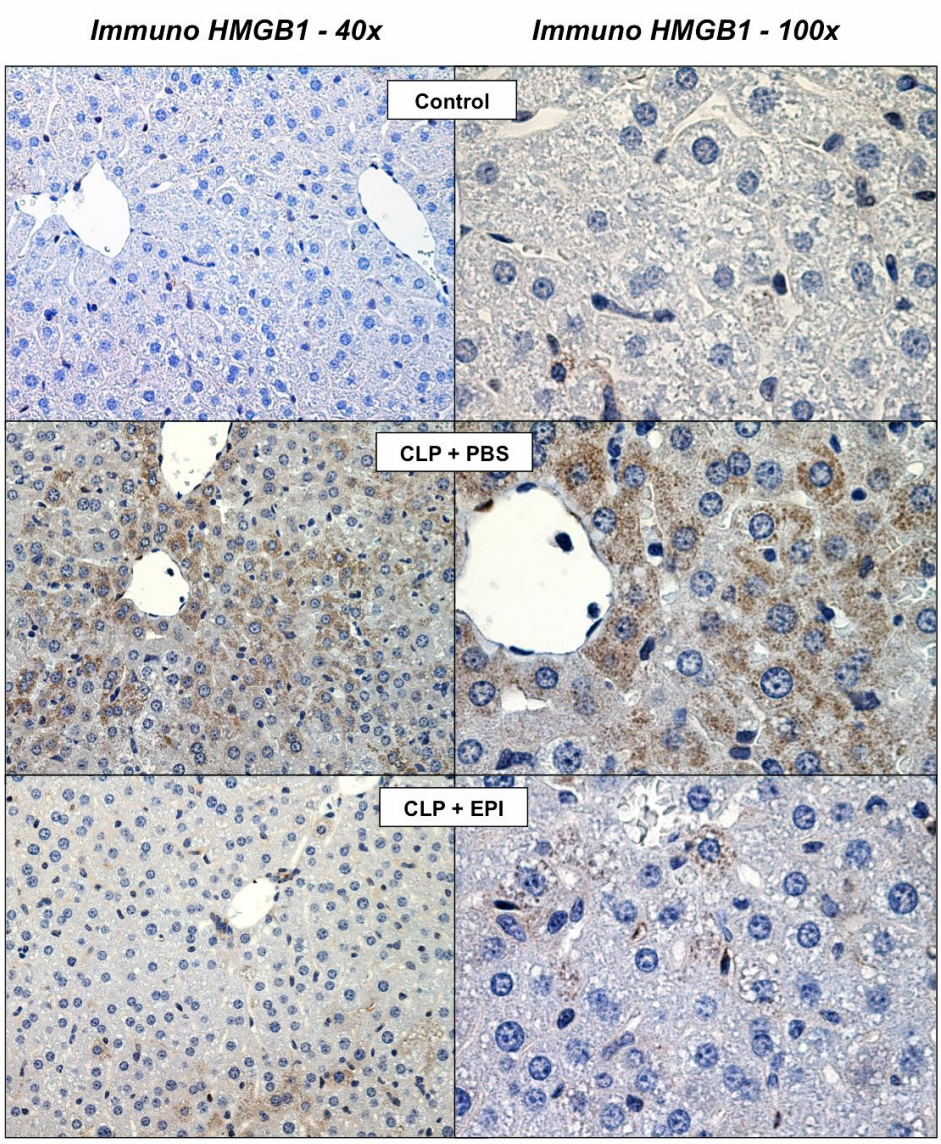

Figure 27 - Liver immunohistochemistry (IHC) for HMGB1 24h after CLP. C57BL/6 wildtype animals subjected to CLP treated with carrier (PBS) or epirubicin i.p. (0.6µg/g body weight), at the time of procedure. Magnification of 40x & 100x.

An accepted model of SIRS/CARS depicts the natural history and adverse outcomes of sepsis syndromes as a series of excessive pro-inflammatory responses (SIRS) followed temporally by compensatory anti-inflammatory responses (CARS) and varying degrees of adaptive immunity suppression. Any subsequent insult leads to a more severe, recurrent SIRS that can end in organ dysfunction and death [116].

In our model, we have administered a drug - epirubicin - with traditionally described immunosuppressive features. Although used in a dosage that is in the order of 1/10 of the usual (clinically approved in oncologic patients in combination therapy), a question of paramount importance was if this dose of epirubicin could be inducing immunosuppression.

Another question was whether our dose of epirubicin could be enhancing the late stage "immune-paralysis".

Our results demonstrate that mice previously subjected to CLP and treated with epirubicin, as in the previous experiments, are not immune-compromised. These mice can clear a secondary intranasal infection with the MuHV-4 virus similarly to control mice (Figure 28).

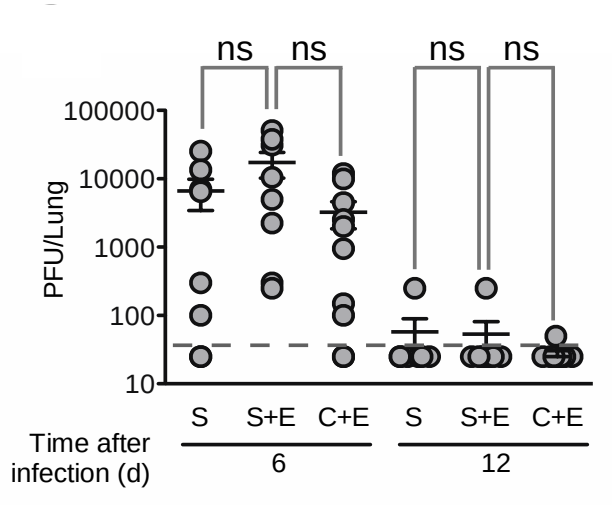

Figure 28 - Quantification of infectious viral MuHV-4 particles in lung of C57BL/6 wildtype animals previously subjected to mock CLP (S), mock CLP treated with epirubicin (S+E) or CLP treated with epirubicin (C+E). Epirubicin treatment dose and schedule was 0.6µg/g body weight at the time of procedure and 24 hours later. Mice were intra-nasally inoculated with 1000 PFU of MuHV-4 on day 3 post CLP and viral particles quantified by plaque assay at days 6 and 12 post viral infection. Each circle represents individual animals and horizontal lines indicate arithmetic means ± SEM from two independent assays. The dashed horizontal line represents the limit of detection of the assay. ns, not significant; *P<0.05; **P<0.01; ***P<0.001; Mann-Whitney test.

Recent reports make our findings counter-intuitive as doxorubicin and daunorubicin have been shown to induce acute inflammation when injected in the abdomen and to induce cytokine secretion [202, 203]. However, the concentrations of anthracyclines utilized in these studies were at least 10-fold higher than those used in our model. By using lower concentrations we may reduce the cytotoxicity of these drugs (and the resulting releasing of pro-inflammatory DAMPs by dying cells), and possibly reveal additional pharmacological effects mediated by the (surviving) target cells.

Interestingly, the antibiotic class of fluoroquinolones (bacterial type II topoisomerase inhibitors), as opposed to anthracyclines (eukaryotic type II topoisomerase inhibitors), are widely reported to have immunomodulatory effects [204] when used in supra-therapeutic concentrations. Fluoroquinolones have been shown to protect against LPS model of septic shock [205]. While the molecular mechanisms that explain these effects have not been elucidated, it has been proposed that higher doses of fluoroquinolones can inhibit mammalian topoisomerase type II enzymes in addition to their natural targets, the bacterial DNA gyrase and topoisomerase IV [204], an effect that can be achieved with very low doses of anthracyclines.

Epirubicin promotes tolerance in severe sepsis

Anthracyclines were first reported as anthracycline antibiotics. The first anthracycline to be discovered was Daunorubicin and it was initially isolated from *Streptomyces peucetius*. These compounds were never clinically used as antibacterial drugs, because of their weak bactericidal effect. Nevertheless, we had to assess whether this protective phenotype in sepsis was dependent on a bactericidal effect.

We determined that the protection conferred by epirubicin against severe sepsis was not due to an antibiotic effect of epirubicin, given that the drug also protected C57BL/6 mice from lethal septic shock subsequent to LPS administration (Figure 22-A). Moreover, epirubicin-treated mice subjected to CLP showed similar numbers of blood circulating bacteria at 24 hours post-CLP, as compared to untreated controls (Figure 29).

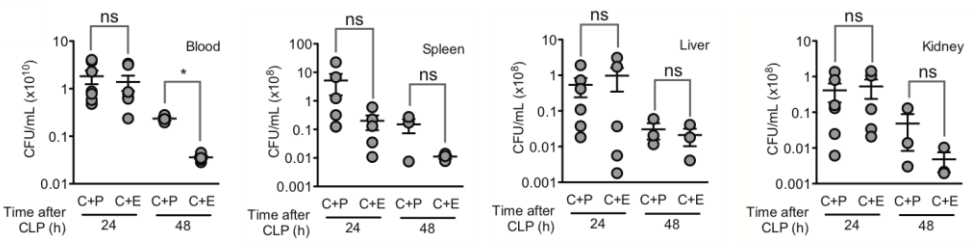

Figure 29 - Polymicrobial load (CFUs) in blood, spleen, liver and kidney, at indicated time points, of C57BL/6 animals undergoing CLP and treated with PBS (C+P) or epirubicin (C+E) as in Figure 30. Each circle represents individual animals. Horizontal lines indicate arithmetic means ± SEM. ns, not significant; *P<0.05; **P<0.01; ***P<0.001; Mann-Whitney test.

Furthermore, we assessed the bacterial load in target organs, e.g. spleen, liver and kidney. At 24h there was no significant difference between the treated and untreated groups (Figure 29). Although at 48h there was a tendency for some bacterial clearance in the treated group, especially in the blood.

A critical step in the clinical management of septic shock and severe sepsis is the prompt administration of an effective antibiotic therapy. Mortality increases on average a 7.6%, for each hour delay to antibiotic administration [166]. The early administration of appropriate antibiotics reduces mortality in patients with Gram-positive and Gram-negative bacteremia [206].

Large spectrum antibiotics such as meropenem are very effective at lowering bacteremia and are standard drugs used in sepsis syndromes [207].

We tested the efficacy of meropenem in CLP in comparison to epirubicin and found that while meropenem delays the death rate of CLP-subjected mice, it does not prevent mortality (Figure 30-A), in spite of a strong impact on bacterial burden (Figure 30-B). This observation is in sharp contrast to the action of epirubicin, which does not interfere with bacteremia (Figure 30-B) but prevents CLP-induced mortality (Figure 30-A), again arguing for a role of epirubicin in conferring disease tolerance against severe sepsis [208].

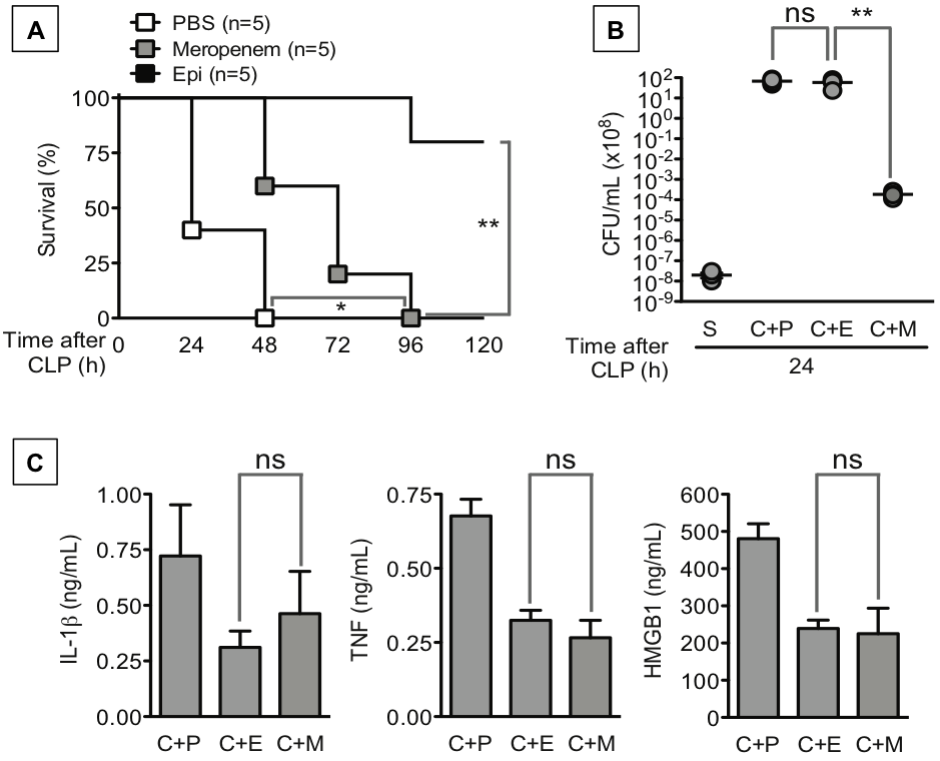

Figure 30 - **(A)** Survival of C57BL/6 wildtype animals subjected to CLP treated with carrier (PBS), meropenem (40µg/g body weight/day) or epirubicin (Epi) (dosage and schedule as before). **(B)** CFUs in blood, at indicated time, of C57BL/6 animals undergoing mock CLP (S) or CLP followed by treatement with PBS (C+P), epirubicin (C+E) or meropenem (C+M) as in (A). Each circle represents individual animals. Horizontal lines indicate arithmetic means ± SEM. **(C)** IL-1β, TNF and HMGB1 plasma concentrations in C57BL/6 wildtype animals 24 hours after CLP followed by treatment with PBS (C+P) (n=4), epirubicin (C+E) (n=5) or meropenem (C+M) (n=5) as in (A). Results shown represent arithmetic means ± SEM from triplicate readings per animal. ns, not significant; *P<0.05; **P<0.01 ***P<0.001; log-rank (Mantel-Cox) test for (A), Mann-Whitney test for (B) and unpaired t test for (C).

Both epirubicin and meropenem decrease the levels of the circulating cytokines TNF, IL-1β and HMGB1 in the serum of mice subjected to CLP (Figure 30-C). This indicates that while decreased circulating levels of inflammatory

mediators may contribute to confer protection against severe sepsis, inhibition of TNF, IL-1β and HMGB1 is not sufficient *per se* to explain the protective effect of epirubicin, which is in accordance with what is observed for other therapeutic approaches in the clinical setting [113].

Our results suggest that epirubicin, most probably, activates an anti-inflammatory program that dampens the pro-inflammatory cytokine profile while at the same time confers disease tolerance to polymicrobial infection. This is an evolutionary conserved strategy against infection that acts irrespectively of pathogen burden [208] [209]. Epirubicin appears to protect mice from lethality, inducing less tissue injury and promoting less organ dysfunction.

Our findings highlight the capacity of some compounds to provide tissue damage control. This way, pharmacologic intervention can limit disease severity irrespectively of pathogen load and represent a promising therapeutic strategy against sepsis.

According to Schneider and Ayres' theory [87], epirubicin might act through mechanisms that enable mice to cope with the burden of infection, allowing additional time for the immune system to eliminate the pathogens without inducing further harm to the target organs.

Identification of DNA Damage Response components as negative regulators of inflammation

In order to identify mechanisms underlying the protective effects of epirubicin in the CLP model, we performed an *in vitro* short hairpin RNA (shRNA)-based screen, enriched for kinases and phosphatases and using IL-1β and TNF secretion as assay readouts.

We found several negative regulators of IL-1β in response to *E. coli* challenge, including the Ataxia Telangiectasia Mutated (ATM), the Checkpoint Kinase 1 (CHEK1) and the Ataxia Telangiectasia and Rad3 Related (ATR) genes (Figure 31 and Table V).

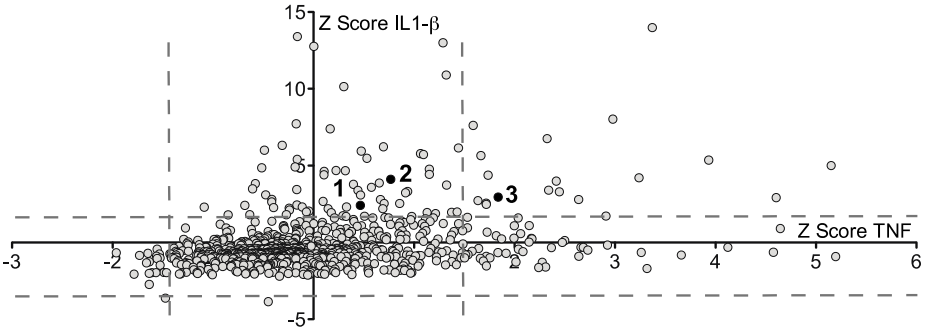

Figure 31 - Two-dimension Z score plot of TNF and IL-1β production by THP-1 cells upon target gene knockdown using a selected group of constructs of the TRC shRNA lentiviral vector library followed by PFA-fixed *E. coli* stimulation for 24 hours. Each dot represents an individual construct. Dotted horizontal and vertical lines define the area in which genes are considered primary hits. Black dots identify ATM **(1)**, ATR **(2)** and CHEK1 **(3)**.

Table V – shRNA-based identification of negative regulators of IL-1β and TNF secretion in response to *E. coli*, in THP-1 cells.

GENE SYMBOL	Z score IL-1β	Z score TNF
RXRG	13,9855	5,1961
CERK	13,3953	5,1511
CDC2L2	12,9984	4,6452
CIB3	12,7559	4,6032
PINK1	10,8947	4,5763
MAP2K1IP1	10,1435	4,1241
SSH2	8,0269	3,9327
PANK4	7,7187	3,8836
EP300	7,6234	3,6604
AK7	7,3942	3,3712
NEK8	6,7519	3,3582
OBSCN	6,3214	3,3184
PKMYT1	6,2266	3,2620
HRAS	6,1495	3,2392
TRAF3IP3	6,0024	3,1707
NR1I3	5,9519	2,9780
MKNK1	5,7793	2,9255
LRRK1	5,7194	2,9090
RIPK2	5,6610	2,7289
GMIP	5,4791	2,6844
EGFR	5,4123	2,6614
Gabra3	5,3594	2,6400
TAF1	5,0110	2,6379
PGK2	4,9178	2,4989
PPP2R5A	4,8571	2,4762
UNK	4,7714	2,4462
GKAP1	4,6835	2,4154
TPD52L3	4,6736	2,3676
CNKSR3	4,6189	2,3407
INPP5D	4,4381	2,3255
ANP32A	4,3958	2,3085
ATPBD3	4,3823	2,2774
OTOF	4,2081	2,2406

GENE SYMBOL	Z score IL-1β	Z score TNF
ATR	***4,1126***	***2,1733***
CMPK	3,9938	2,1688
Gabra5	3,8749	2,1463
NR1H4	3,8238	2,1448
FASTK	3,7789	2,0836
UNK	3,7320	2,0402
ACVR1B	3,5940	2,0357
PFKP	3,4913	2,0136
NEK1	3,4474	1,9952
MYB	3,4053	1,8719
GLI2	3,3850	1,8367
LOC392265	3,3217	1,8355
PHKG2	3,3024	1,8179
CSNK1E	3,2457	1,7555
MGC16169	3,2288	1,7382
NRGN	3,0950	1,7273
PPP4R2	3,0879	1,7210
CHEK1	***2,9480***	***1,7196***
IKBKE	2,9221	1,7183
NRK	2,8027	1,7168
RET	2,7906	1,7005
NR1I2	2,7898	1,6891
PCK2	2,7260	1,6659
PIK3AP1	2,6694	1,6599
NF1	2,6400	1,6432
NME2	2,5561	1,6357
MAP3K11	2,5347	1,5880
RIMS4	2,5316	1,5777
UNK	2,4788	1,5374
KHK	2,4387	1,5320
ATM	***2,4164***	***1,5319***
IHPK3	2,4154	1,5140
RBL1	2,3158	1,5017
PRKCDBP	2,3133	1,4868
UNK	2,3016	1,4832
KRAS	2,2727	1,4823

These findings suggest that DNA Damage Response (DDR) components are negative regulators of IL-1β and TNF secretion.

While epirubicin decreased both IL-1β and TNF secretion in THP-1 cells (Figure 19), only IL-1β, but not TNF, was up-regulated after ATM or ATR silencing. Similar results were obtained using the ATM specific pharmacologic inhibitor KU-55933 (Figure 32-A).

These observations are extensible to human or mouse primary cells. Treatment of monocytes, purified from peripheral human blood, or bone marrow derived macrophages (Figure 32-B) with epirubicin inhibited IL-1β and TNF secretion and ATM inhibition by KU-55933 increased the secretion of IL-1β but not that of TNF. It should be noted however that inhibition of IL-1β secretion by epirubicin still occurs in ATM-deficient mice, suggesting that epirubicin inhibits IL-1β secretion via a mechanism that is not strictly ATM dependent (Figure 32-C).

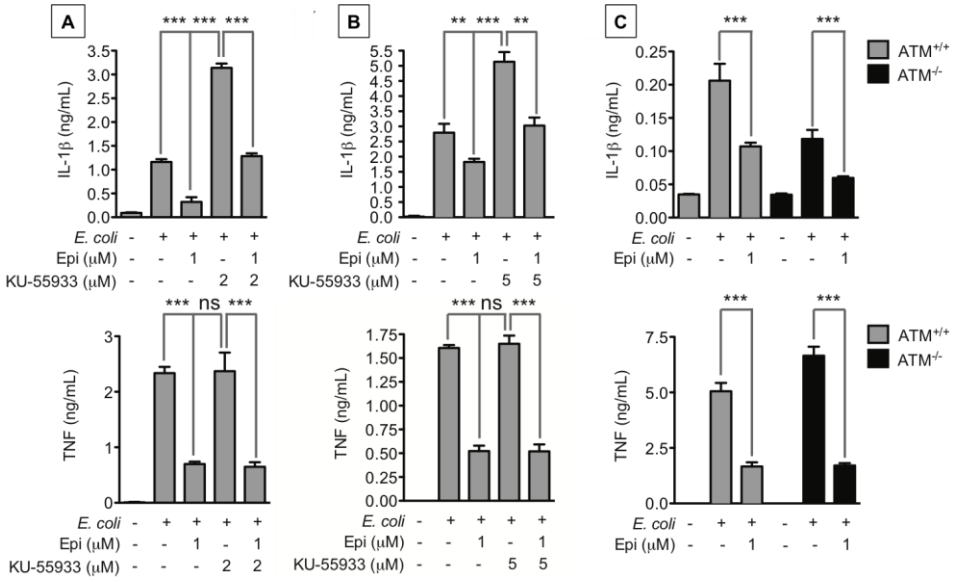

Figure 32 - **(A)** and **(B)** IL-1β and TNF production by (A) THP-1 cells and (B) BMDM following *E. coli* challenge (4 hours) after a pre-incubation (1 hour) with carrier, epirubicin or KU-55933 as indicated. Results shown represent arithmetic means ± SD from triplicate samples for one of at least 3 independent assays. **(C)** IL-1β and TNF production by $Atm^{+/+}$ and $Atm^{-/-}$ BMDM following *E. coli* challenge and pre-incubation with carrier or epirubicin as in (B). Results shown represent arithmetic means ± SD from triplicate samples in one of 3 independent assays. ns, not significant; *P<0.05; **P<0.01 ***P<0.001; unpaired t test.

Using a phospho-specific antibody against the activated form of ATM, we found that while *E. coli* alone was a poor, but reproducible ATM activator (Figure 33-A), epirubicin alone or in combination with *E. coli* triggered a robust ATM activation (Figure 33-A). This was confirmed using immunoblotting (Figure 33-B).

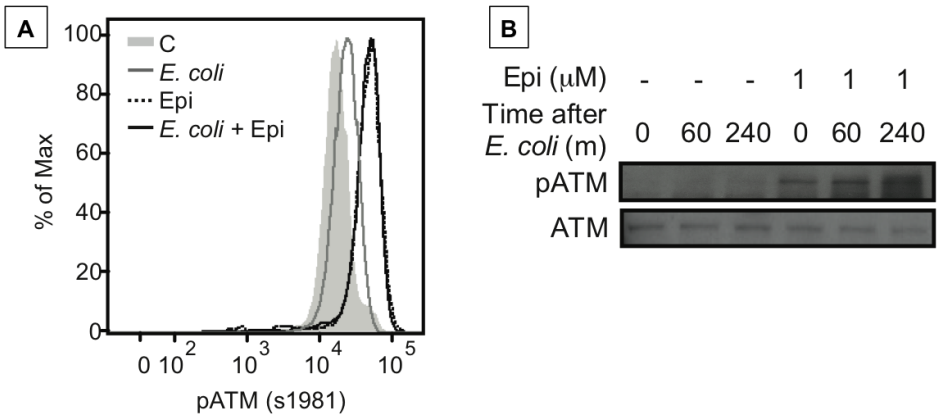

Figure 33 - **(A)** Flow cytometry analysis of the activated form of ATM, phosphorilated at serine 1981, in THP-1 cells left untreated (C) or treated with epirubicin alone (1μM) (5 hours) (Epi), challenged with PFA-fixed *E. coli* (4 hours) (*E. coli*) or *E. coli* (4 hours) plus epirubicin pre-treatment (1 hour) (*E. coli* + Epi). **(B)** Immunoblotting of total protein extracts of THP-1 cells untreated or pre-treated with epirubicin (1mM) and challenged with PFA-fixed *E. coli* at indicated timepoints probed for the total and phosphorilated (serine 1981) forms of ATM.

Epirubicin protection against sepsis is mediated by ATM

Our shRNA-based screen results point to the DDR pathway, represented by ATM, ATR and CHEK1, as being involved in the regulation of inflammation.

ATM is a master regulator of the DDR [210] and is known to be activated by anthracyclines and other DNA damaging agents [211]. Therefore we used ATM-deficient mice to test the contribution of DDR for the protective effect of anthracyclines against severe sepsis. We compared the survival of wildtype (*Atm*$^{+/+}$) *vs.* ATM-deficient (*Atm*$^{-/-}$) mice subjected to CLP and treated with epirubicin. *Atm*$^{-/-}$ mice are not protected by epirubicin against CLP and die with similar kinetics to those wild-type animals that were treated with PBS alone (Figure 34).

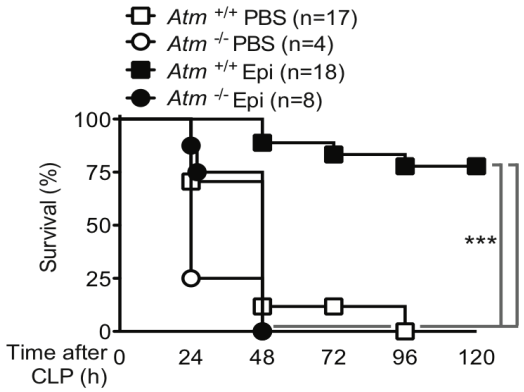

Figure 34 - Survival of *Atm*$^{+/+}$ and *Atm*$^{-/-}$ C57BL/6 animals subjected to CLP and treated with PBS or epirubicin (Epi) (0.6µg/g body weight) intraperitoneally at the time of procedure and 24 hours later. ns, not significant; *P<0.05; **P<0.01; ***P<0.001; log-rank (Mantel-Cox) test.

We conclude that ATM expression is absolutely necessary to mediate the protective effect of epirubicin. In striking contrast to Figures 23-A and B, in the absence of ATM, epirubicin no longer normalizes the serologic markers of organ lesion (Figure 35-A) or decreases the levels of inflammatory mediators (Figure 35-B).

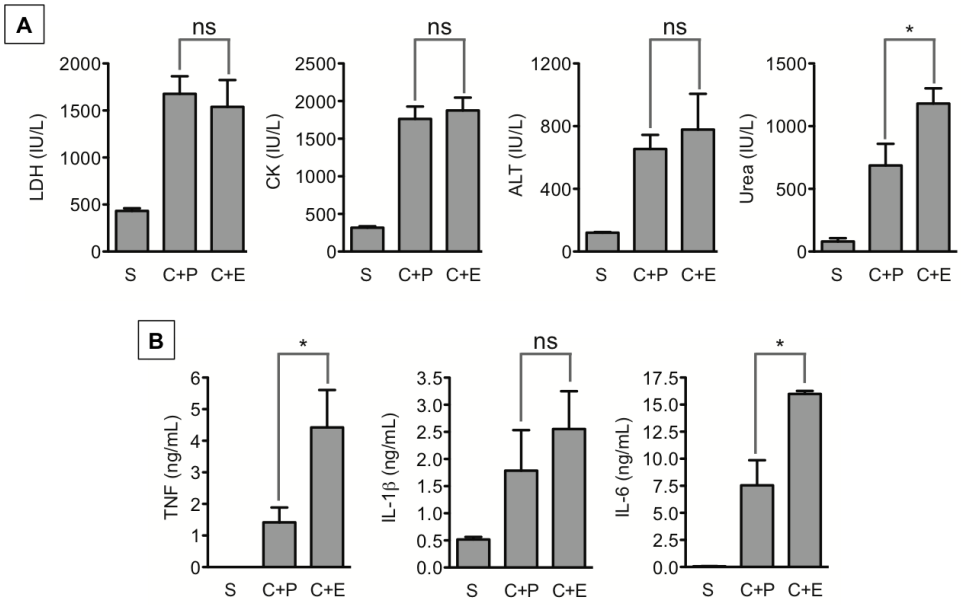

Figure 35 - **(A)** LDH, CK, ALT, urea and **(B)** TNF, IL-1β and IL-6 plasma concentrations in *Atm* $^{-/-}$ C57BL/6 animals 24 hours after mock CLP (S) (n=2) or CLP followed by treatment with PBS (C+P) (n=8) or epirubicin (C+E) (n=8) as in the previous Figure. Results shown represent arithmetic means ± SEM from triplicate readings per animal. ns, not significant; *P<0.05; **P<0.01; ***P<0.001 unpaired t test.

As ATM is necessary to elicit epirubicin-induced protection, we wanted to assess if it was also sufficient for the protection conferred by anthracyclines against sepsis. Etoposide is another cytotoxic agent that can cause DNA double strand breaks and to activate ATM-dependent pathways [211].

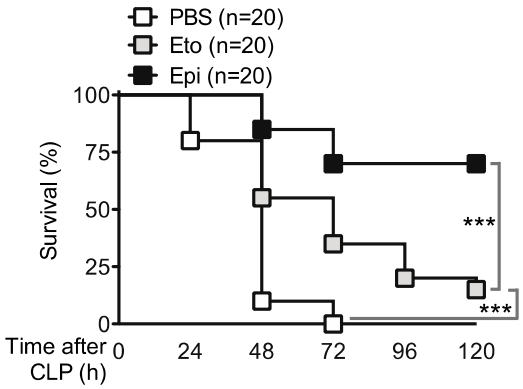

Figure 36 - Survival of PBS-, etoposide (Eto)-, and epirubicin (Epi)-treated wildtype C57BL/6 animals undergoing CLP. Etoposide dose was 2µg/g body weight and epirubicin dose was 0.6µg/g body weight i.p. administered at the time of procedure and 24 hours later. ns, not significant; *P<0.05; **P<0.01; ***P<0.001; log-rank (Mantel-Cox) test

However, in mice subjected to CLP and treated with etoposide the mortality induced by CLP is only partially rescued (Figure 36), suggesting that ATM is necessary but not sufficient for this protective effect.

Anthracyclines are potent poisons of cellular topoisomerase II, which in turn stabilizes topoisomerase-mediated DNA double strand breaks (DSBs) [212]. Exposure of cultured cells to the prototypical anthracycline doxorubicin activates both ATM and ATR kinases in a cell cycle-dependent fashion and repair of DNA damage induced by anthracyclines was shown to require both homologous recombination (HR) and nucleotide excision repair (NER) [213, 214].

Anthracyclines also cause DNA interstrand cross-links (ICL), a lesion known to be repaired by the Fanconi Anemia (FA) pathway [210]. Interestingly, FA patients have been reported to have increased levels of TNF [215, 216]. Moreover, FA protein FancD2, directly inhibits TNF promoter activity [217]. Furthermore, as

anthracyclines also form drug-DNA adducts that closely mimic interstrand crosslinks (ICLs), NER and HR components may be co-opted in the context of ICL repair systems [213] independently of topoisomerase II-mediated damage [218].

The Fanconi anemia (FA) pathway plays a key role in ICL removal, and evidence for the involvement of this pathway in the repair of anthracycline-mediated DNA damage was recently provided [219].

In the FA pathway, FancD2 that becomes mono-ubiquitylated through the ubiquitin ligase activity of the FANC complex exerts a pivotal role in coordination of the successive steps for ICL removal including nucleolytic incision, HR and translesion DNA synthesis [220].

In THP-1 cells, we observed that FancD2 is activated, in an ATM-independent manner, upon epirubicin treatment as shown by its mono-ubiquitination supporting the independence of signaling events initiated by the generation of DNA double strand breaks and DNA interstrand cross-links (Figure 37-A).

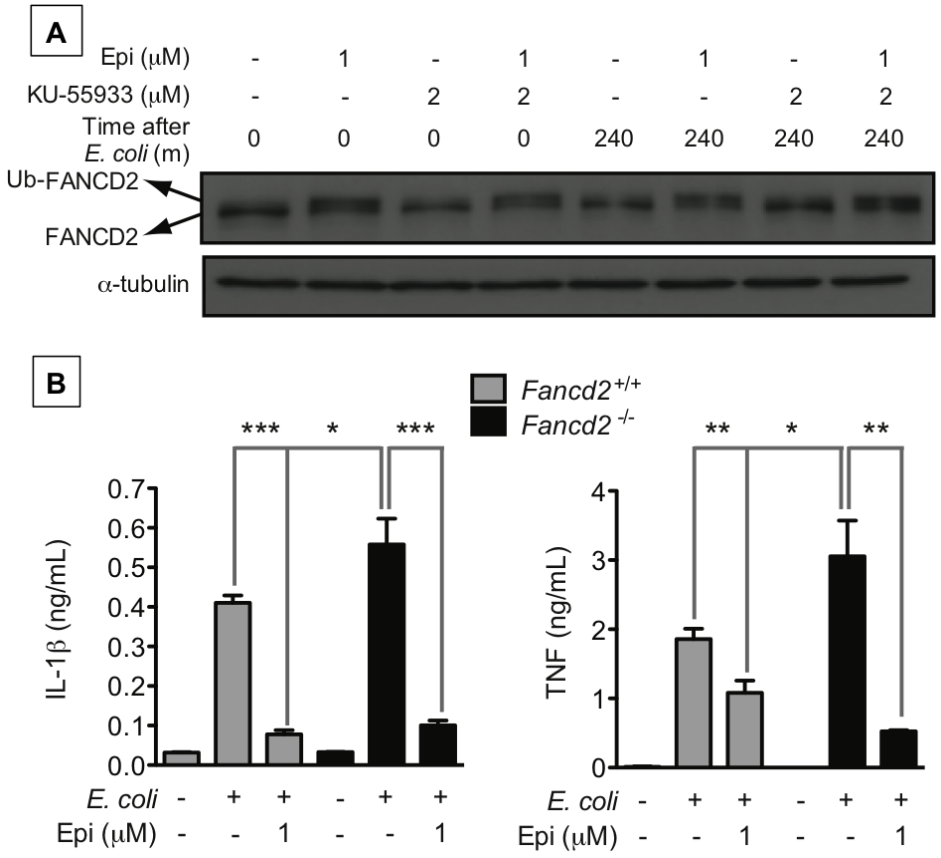

Figure 37 - **(A)** FANCD2 and Ub-FANCD2 protein levels by immunoblotting in THP-1 cells following *E. coli* challenge after a pre-incubation (1 hour) with carrier, epirubicin or KU-55933 as indicated. **(B)** IL-1β and TNF production by *Fancd2*$^{+/+}$ and *Fancd2*$^{-/-}$ BMDM following *E. coli* challenge (4 hours) after a pre-incubation (1 hour) with carrier or epirubicin. ns, not significant; *P<0.05; **P<0.01; ***P<0.001; unpaired t test for (B)

BMDM from FancD2$^{-/-}$ mice show increased secretion of TNF but epirubicin still effectively inhibits TNF secretion (Figure 37-B), leaving open the mechanisms of TNF regulation by anthracyclines.

We then used FancD2$^{-/-}$ mice to test the contribution of this pathway for epirubicin protection and found a statistically significant contribution for the protective effect of epirubicin (Figure 38).

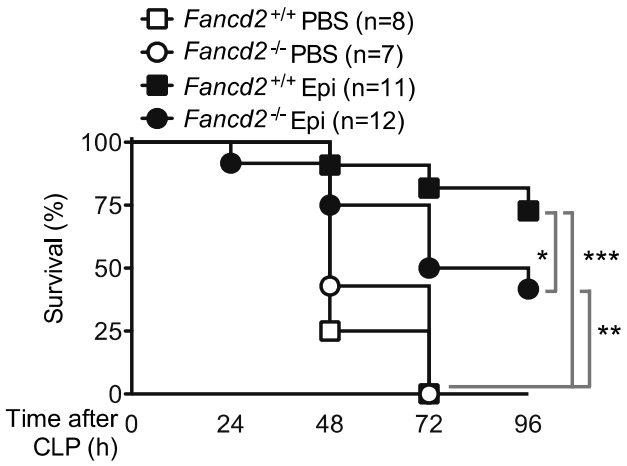

Figure 38 - Survival of *Fancd2*$^{+/+}$ and *Fancd2*$^{-/-}$ animals subjected to CLP and treated with PBS or epirubicin (Epi) 0.6µg/g body weight i.p. administered at the time of procedure and 24 hours later. ns, not significant; *P<0.05; **P<0.01; ***P<0.001; log-rank (Mantel-Cox) test

We explored several additional possible ATM-dependent mechanisms to explain the protective role of epirubicin in this sepsis model. We tested the possibilities that the ATM-mediated protection could rely on ROS scavenging, on the induction of apoptosis of inflammatory cells or even on the biogenesis of anti-inflammatory microRNAs.

Two of the most cited factors responsible for tissue lesion and organ damage are ROS and RNS. These oxidative agents are membrane permeable and known to induce cell death. Neutrophils kill bacteria by releasing granule-

associated proteases and extensively secreting ROS. Although known for many years, oxidative agents and their role in sepsis pathogenesis is still being revealed. Important as they are in microbial killing, their action in regulating inflammation is being actively studied.

Accepted paradigm states that leukocytes producing ROS are determinant to bacterial clearance, but excessive recruitment and/or impaired apoptosis may adversely affect outcome. There is recent evidence that in mice with decreased ROS production, although they have higher numbers of neutrophils and lower rates of neutrophil apoptosis, the global pathogen clearance is not hampered and there is better survival in the context of severe microbial infections [221]. The association of increased numbers of activated neutrophils with tissue lesion is not necessary true. Some of the ROS might be regulating the recruitment, activation and survival of neutrophils.

One of our hypothesis was that epirubicin, acting as a ROS scavenger, could protect from the deleterious effects of sepsis. We found that*, in vitro*, epirubicin is able to counteract the increase in ROS generated by *E. coli* challenge of THP-1 cells in an ATM-dependent manner (Figure 39-A).

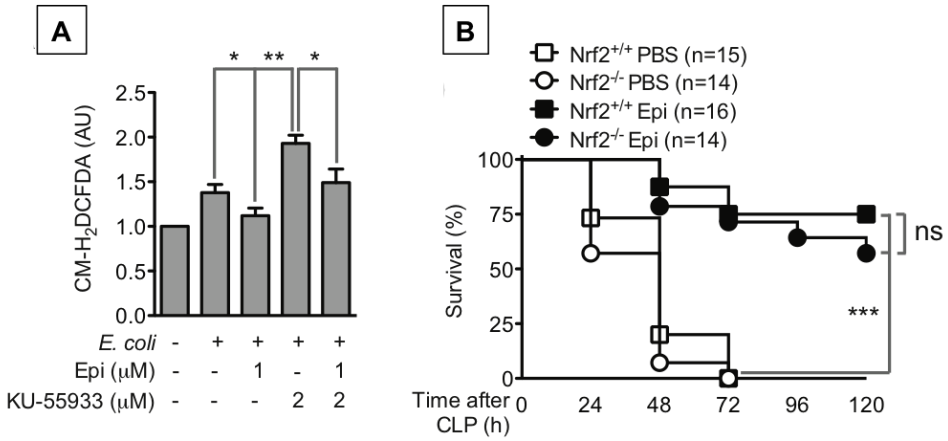

Figure 39 - **(A)** ROS content in THP-1 cells as assessed by the pan ROS probe CM-H_2DCFDA following *E. coli* challenge (4 hours) after a pre-incubation (1 hour) with carrier, epirubicin or KU-55933 as indicated. Results shown represent arithmetic means ± SEM from 3 independent assays. **(B)** Survival of *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ animals subjected to CLP and treated with PBS or epirubicin (0.6μg/g body weight) (Epi) at the time of procedure and 24 hours later. ns, not significant; *P<0.05; **P<0.01; ***P<0.001; unpaired t test for (A) and log-rank (Mantel-Cox) test for (B)

Nuclear factor (erythroid-derived 2)-like 2 (NRF2) is a master regulator of the anti-oxidative stress response [222]. In normal or basal conditions, Nrf2 is kept in the cytoplasm by another protein called Kelch like-ECH-associated protein 1 (Keap1). Oxidative stress disrupts the inhibitory association of Nrf2-Keap1.

Unbound Nrf2 is then able to translocate into the nucleus and initiate the transcription of several cytoprotective proteins: NADPH quinone oxidoreductase 1; Glutamate-cystein ligase; Heme-oxygenase 1; Glutathione-S-transferase; UDP-glucuronosyltransferase; Multi-drug resistance-associated proteins.

However, mice that are deficient for the nuclear factor (erythroid-derived 2)-like 2 (NRF2), a master regulator of ROS scavenging [223], are still protected by epirubicin against mortality due to CLP (Figure 39-B).

Therefore, epirubicin induces an ATM-dependent ROS scavenging response that may contribute to target organ protection, but it is dispensable for the overall protective effect in sepsis.

Another interesting hypothesis, to explain epirubicin protective effects, derives from the fact that increased apoptosis of neutrophils can attenuate sepsis pathogenesis [224]. This would be a simple and attractive hypothesis considering that anthracyclines initiate a DDR leading to increased apoptosis if the DNA lesion is too severe for repair [224].

Leukocytes, especially neutrophils, have to mount a fast and efficient response to protect the host against a potential lethal bacterial infection. This response has to be controlled or dampened by a mechanism that prevents organ damage from activated neutrophils. One of these protective mechanisms is programmed cell death of activated leukocytes - Apoptosis.

While induction of apoptosis in the innate immune cell compartment might be a beneficial step, controlling overwhelming inflammation, the activation of apoptosis in the target organs cells might not have the same net result. There is some evidence that inhibition of apoptosis in survivors from bacterial meningitis improves the target organ function [225].

However, our data shows that epirubicin treated mice have higher, not lower, numbers of viable neutrophils in the abdomen, excluding an important role for this mechanism (Figure 40).

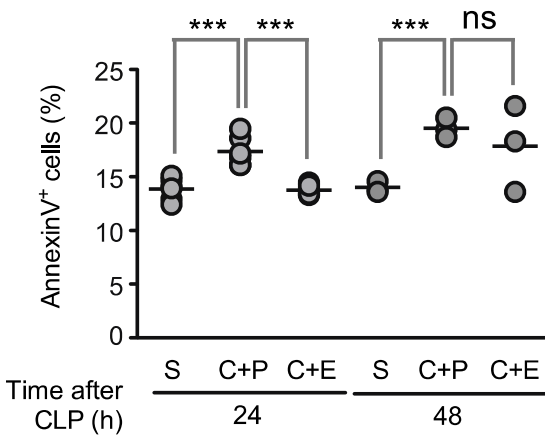

Figure 40 - Evaluation of apoptosis (AnnexinV$^+$ cells) in total splenocytes of C57BL/6 wildtype animals subjected to CLP, and treated with PBS or epirubicin (0.6μg/g body weight) at the time of procedure and 24 hours later, at the indicated times. Each circle represents individual animals and horizontal lines indicate arithmetic means ± SEM from two independent assays. ns, not significant; *P<0.05; **P<0.01; ***P<0.001; unpaired t test

MicroRNAs (miRs) comprise the largest family of small non-coding RNAs that regulate mRNA translation. The miR-mediated RNA interference mechanism acts at the translational level, regulating gene expression through these short double-stranded RNA sequences of 20-23 nucleotides, containing regions of non-complementarity. Their major function, described so far, is involved in development, differentiation and homeostasis.

Recently, changes in the expression of some miRs (namely miR-146a) have associated the development of multiple cancers and a negative regulation of the inflammatory pathway through the innate immune system [182].

miR-146a has been proposed to be an important negative regulator of inflammation, dependent on the following observations: miR-146a is expressed predominantly in immune cells; mice who have miR-146a ablation

present with several immune-related phenotypes; miR-146a $^{-/-}$ macrophages present hyper-responsiveness to LPS challenge; miR-146a$^{-/-}$ mice have an exaggerated systemic inflammatory response to endotoxin; overexpression of miR-146a$^{-/-}$ in LPS stimulated monocytes has the opposite effect, showing a dampened inflammatory phenotype; old miR-146a-null mice develop a spontaneous auto-immune disorder, characterized by lymphadenopathy, splenomegaly and multiple organ inflammation [182].

Furthermore, miR-146a expression is dependent on the transcription factor NF-κB, which in turn is an important link between carcinogenesis and inflammatory conditions.

Interestingly, the biogenesis of some miRNAs including miR-146a (a negative regulator of inflammation [226] and a proposed biomarker in sepsis [227]), is ATM-dependent [228].

We compared the survival of wild-type mice with that of miR-146a–deficient mice in the presence or absence of epirubicin. We conclude that the protection given by this drug is dependent on the presence of miR-146a (Figure 41-A).

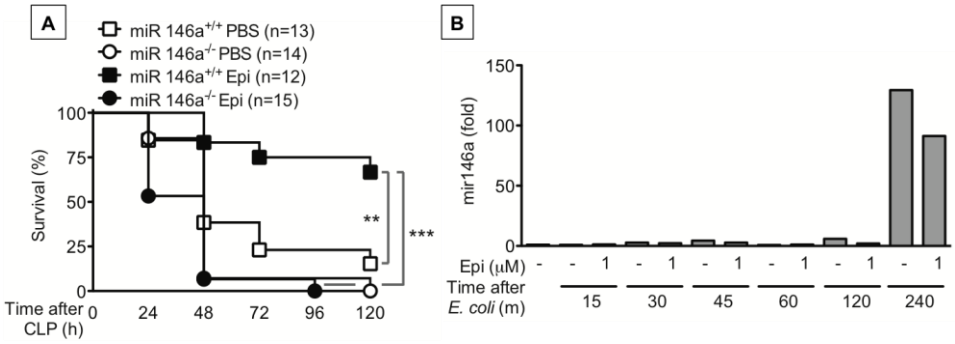

Figure 41 - **(A)** Survival of *miR 146a*$^{+/+}$ and *miR 146a*$^{-/-}$ animals subjected to CLP and treated with PBS or epirubicin (0.6μg/g body weight) at the time of procedure and 24 hours later. **(B)** *miR 146a* expression, as assessed by qRT-PCR, in THP1 cells left untreated or pre-treated with epirubicin and challenged with PFA-fixed *E. coli* for the indicated times. ns, not significant; *P<0.05; **P<0.01; *** P<0.001;log-rank (Mantel-Cox) test

However, our RT-qPCR analysis of miR-146a expression in either RAW cells or THP-1 cells (Figure 41-B) does not support a role for epirubicin in the induction of this microRNA. Therefore, direct induction of miR-146a is not the mechanism by which epirubicin protects against the LPS model of septic shock or CLP.

The protective effect of epirubicin is dependent on the autophagy pathway

ATM-dependent events are responsible for the major contribution for severe sepsis protection by anthracyclines. Autophagy induction has been previously shown to have protective effects in sepsis and septic shock models [229, 230].

We explored the requirement for autophagy in the protection afforded by epirubicin against severe sepsis, based on our prediction that ATM activation could increase autophagy. This hypothesis was derived not only from the knowledge that DNA damage induces autophagy [231] (possibly in an ATM-dependent manner [232]) but also because ATM can act as a negative regulator of mTOR, which is itself, an inhibitor of autophagy [233, 234].

To probe the role of autophagy in the protective effect of epirubicin in sepsis, we compared the survival of wild-type mice with autophagy-defective ($Lc3b^{-/-}$) mice in the presence or absence of epirubicin. Our results show that the autophagy pathway is required for the in vivo effect of epirubicin in severe sepsis protection (Figure 42).

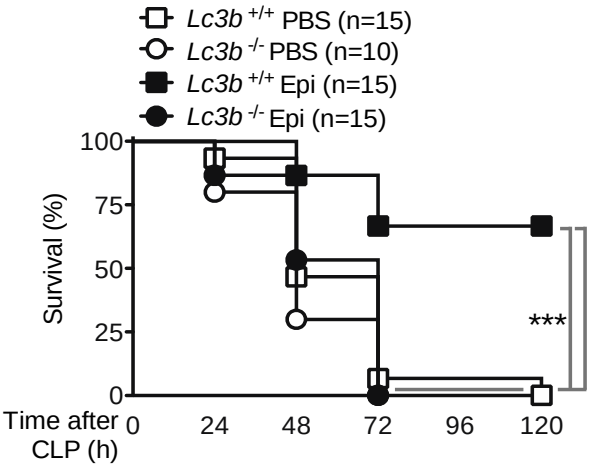

Figure 42 - Survival of *Lc3b*$^{+/+}$ and *Lc3b*$^{-/-}$ animals subjected to CLP and treated with PBS or epirubicin (0.6µg/g body weight) at the time of procedure and 24 hours later. ns, not significant; *P<0.05; **P<0.01; *** P<0.001; log-rank (Mantel-Cox) test.

Similarly to Atm$^{-/-}$ mice (Figure 35-A and B), epirubicin is not able to decrease the serologic markers associated with organ lesion (Figure 43-A) or normalize cytokine levels (Figure 43-B), except for IL-1β that is not present in wild-type animals subjected to CLP.

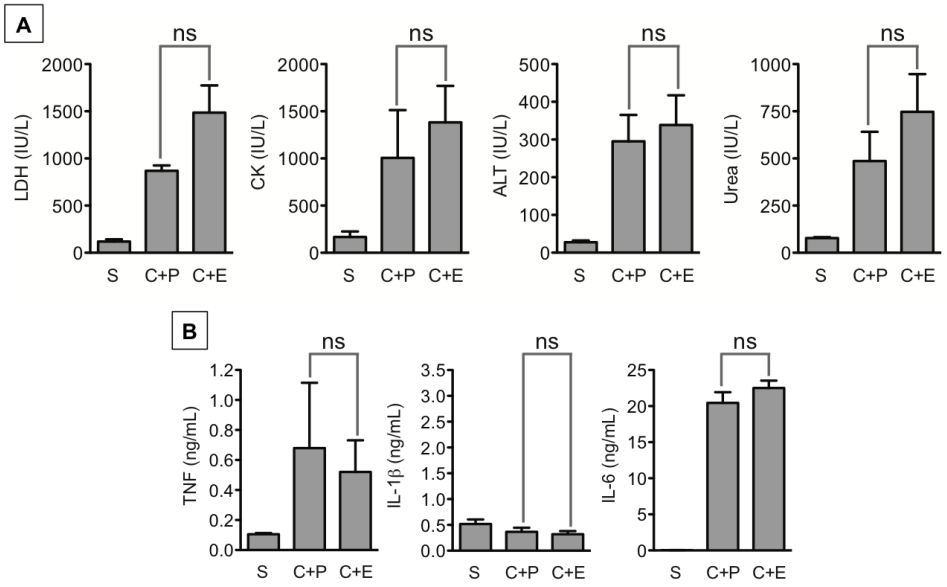

Figure 43 - **(A)** LDH, CK, ALT, urea and **(B)** TNF, IL-1β and IL-6 plasma concentrations in *Lc3b*$^{-/-}$ animals 24 hours after mock CLP (S) (n=2) or CLP followed by treatment with PBS (C+P) (n=4) or epirubicin (C+E) (n=7) as in Figure 42. Results shown represent arithmetic means ± SEM from triplicate readings per animal. ns, not significant; *P<0.05; **P<0.01; *** P<0.001; unpaired t test.

We used LC3b-GFP mice to study the contribution of the autophagy pathway in the protection conferred by epirubicin. While FACS analysis shows that CLP alone induces LC3b aggregation in different splenocyte populations, namely monocytes and neutrophils, epirubicin treatment does not increase the autophagy pathway in these critical players in sepsis (Figure 44-A).

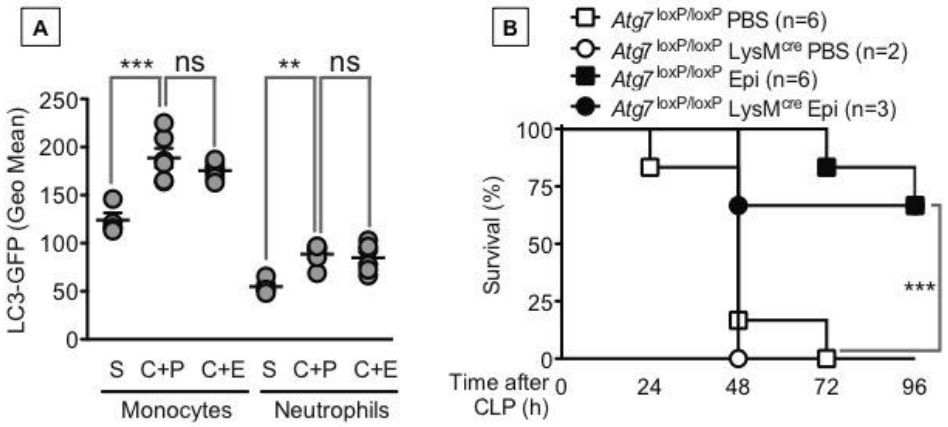

Figure 44 - **(A)** GFP expression in blood monocytes and neutrophils, isolated from transgenic LC3B-GFP animals, 24 hours after mice were subjected to mock CLP (S) or CLP followed by treatment with PBS (C+P) or epirubicin (C+E) as in (a). Each circle represents individual animals. Horizontal lines indicate arithmetic means ± SEM. **(B)** Survival of *Atg7*$^{loxP/loxP}$ and *Atg7*$^{loxP/loxP}$ *LysM*$^{cre/cre}$ mice subjected to CLP and treated with PBS or epirubicin (Epi) with same schedule and dose as in Figure 42. ns, not significant; *P<0.05; **P<0.01; ***P<0.001; Mann-Whitney test for (A) and log rank (Mantel-Cox) test for (B).

These findings were validated by assessing LC3b lipidation by immunoblotting and by testing the impact of epirubicin on the survival of a conditional depletion of *Atg7* specifically in the myeloid compartment upon CLP. In fact, *Atg7*$^{loxP/loxP}$ *LysM*^{Cre} GFP-LC3b animals are equally protected by epirubicin as compared to control mice (Figure 44-B).

Following up on this observation, that the autophagy pathway is not required in the hematopoietic compartment, we looked at target organs of sepsis (lung, liver and kidney) using immunobloting to identify lipidation of LC3b as an indication of the autophagy pathway activation. We found that

epirubicin specifically induces lipidation of LC3b in the lung at 6hrs, but not in the liver or kidney (Figure 45). LC3 is transiently lipidated after CLP in the liver at 6 and 24hrs as previously reported [229] but epirubicin does not change the levels in treated animals (Figure 45).

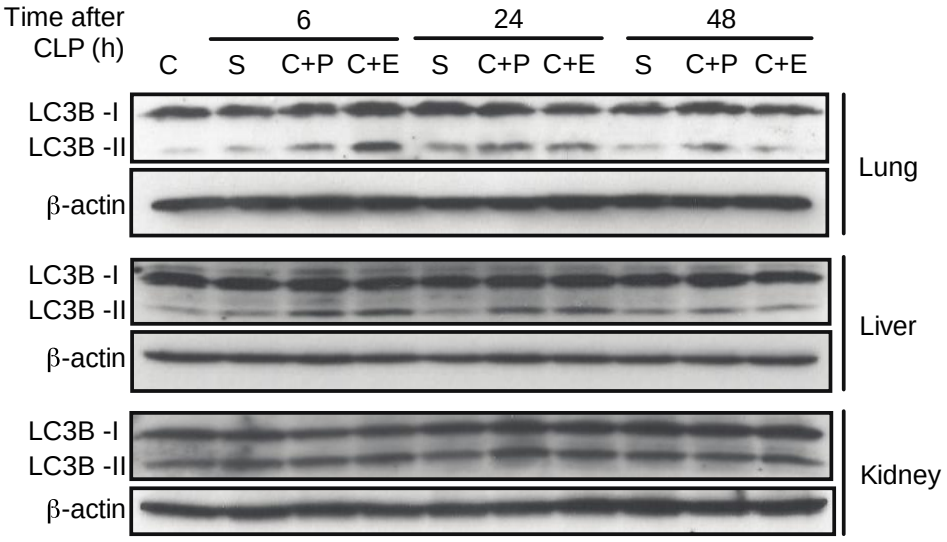

Figure 45 - LC3B-I and -II protein levels by immunoblotting using a specific antibody against LC3B in lung, liver and kidney, isolated at the indicated times, of naïve C57BL/6 animals (C) or mice subjected to mock CLP (S) or CLP followed by treatment with PBS (C+P) or epirubicin (C+E) as in Figure 42.

Additionally, we have confirmed that autophagy is induced in the lung as shown by the increase of LC3b positive vesicles in lung sections at 6hrs and 24hrs comparing epirubicin treated and non-treated mice (Figure 46).

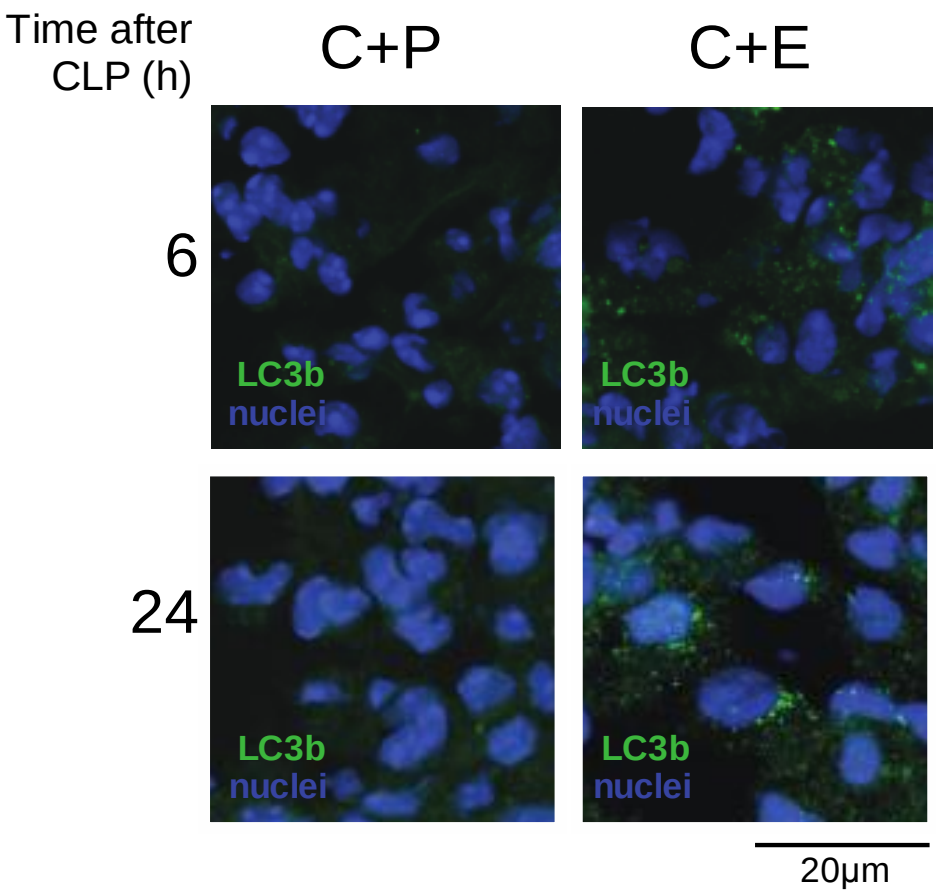

Figure 46 - Representative sections of LC3B staining in lungs, isolated at the indicated times, of mice subjected to CLP followed by treatment with PBS (C+P) or epirubicin (C+E) as in Figure 42.

Furthermore, by making use of a adenovirus-expressing CRE (Ad^{cre}) to intranasaly infect *Atg7*$^{loxP/loxP}$ mice, thus deleting *Atg7* specifically in the lung, we show that when subjected to CLP these mice are no longer protected by epirubicin treatment (Figure 47).

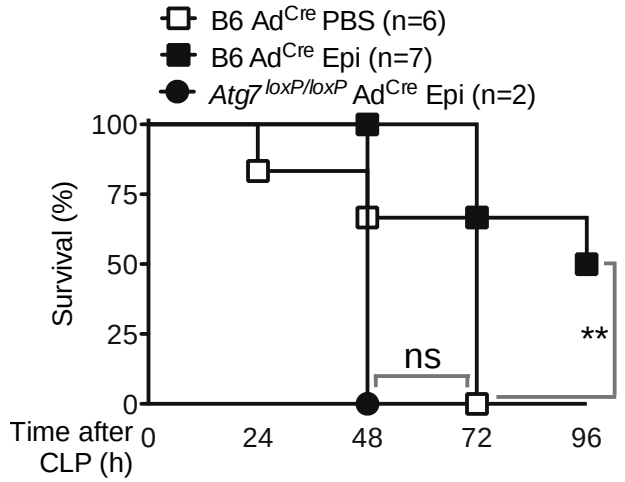

Figure 47 - Survival of wildtype and *Atg7*$^{loxP/loxP}$ animals subjected to CLP and treated with PBS or epirubicin (Epi) with same schedule and dose as in Figure 42, 5 days after inhalation of adenoviral vector encoding Cre (Ad^{Cre}). ns, not significant; *P<0.05; **P<0.01; *** P<0.001; log-rank (Mantel-Cox) test.

By assessing the levels of γH2AX, a surrogate marker of ATM activation [210], in the lung of control or epirubicin treated CLP-subjected mice, we found a significant increase in the number of cells with γH2AX foci in lungs of epirubicin treated mice (Figure 48-A and 48-B).

To test whether ATM activation is also required specifically in the lung, we used *Atm*$^{loxP/loxP}$ mice and adenovirus expressing CRE to delete ATM specifically in the lung. Upon Ad^{cre}-mediated ATM deletion in the lung, mice are no longer protected against sepsis by treatment with epirubicin (Figure 48-C).

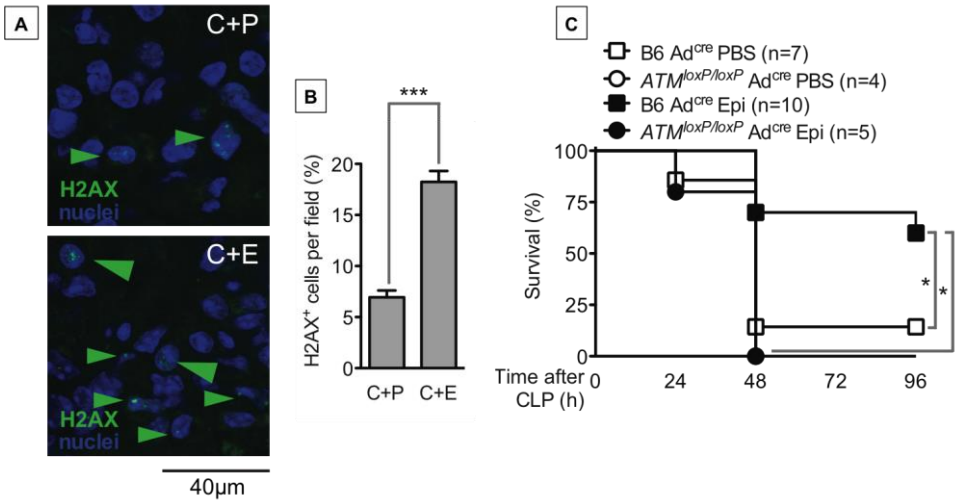

Figure 48 - **(A)** Representative sections of γH2AX staining and **(B)** percentage of $H2AX^{+}$ cells per field, in lungs isolated 6 hours after mice were subjected to CLP followed by treatment with PBS (C+P) or epirubicin (C+E) as in Figure 42. Results shown represent arithmetic means ± SD from 10 fields. **(C)** Survival of wildtype and $Atm^{loxP/loxP}$ animals subjected to CLP and treated with PBS or epirubicin (Epi) with same schedule and dose as in Figure 42, 5 days after inhalation of Ad^{Cre}. ns, not significant; *P<0.05; **P<0.01; ***P<0.001; Mann-Whitney test for (B) and log rank (Mantel-Cox) test for (C).

We conclude that the protective effect of epirubicin in sepsis is, at least in part, due to the ATM-dependent induction of autophagy in target organs, namely the lung.

Epirubicin specifically induces LC3b lipidation in the lung, a response that is low or absent in this organ without anthracycline treatment, but occurs spontaneously and transiently in the liver, and according to some reports [235] also in the kidney by sepsis induction alone.

This finding is also clinically relevant because we know that in sepsis syndromes, all target organs have a sequential and often dependent

dysfunction. The protection of epirubicin in the lung is especially important, as this organ is often the first to fail in septic patients and can potentially drive the failure of other target organs particularly the kidney and later the liver [113].

The hypothesis that arises from our observations is that in some organs (liver) there is an innate response to cope with the aggression, while in others (lung) this response does not exist in a constitutional manner. A common belief in the medical community is that in septic patients the sequential organ failure is dependent on a physiologic reserve. This has arisen from the observation that the lung is one of the first, and the liver is often one of the last, organs to fail. Functional reserve may not be the only reason for this failing sequence.

Based on our results we can speculate that by inducing this protective autophagic response in organs that usually do not have this pathway activated, anthracyclines can confer additional time for septic hosts to deal with the pathogen burden.

While it is not fully understood by what mechanisms autophagy protects in sepsis, the protective phenotype of epirubicin is strikingly similar to that of RIPK3-deficient mice [236], suggesting that epirubicin-mediated, ATM-dependent, autophagy induction can possibly prevent TNF-driven necroptosis in such key organs in sepsis pathology as the lung.

In fact, there have been recent works that support the role of autophagy in the inhibition of necroptosis [237, 238, 239, 240].

Epirubicin has a 24 hour therapeutic window

During the development of our research project, we always had present the idea of devising a clinical application for the benefit of our patients.

At this point, we wanted to assess if the protective effects elicited by epirubicin could be translated to the clinical setting. If this anthracycline demonstrated a therapeutic window of action, it might become a useful drug in our clinical armamentarium.

We therefore studied the therapeutic window of epirubicin in mice. When given alone, epirubicin confers protection at the time of the procedure or until 3hrs after the initiation of CLP (Figure 49-A). When administered only 6 hours after CLP, epirubicin quickly loses its protective effect (Figure 49-A).

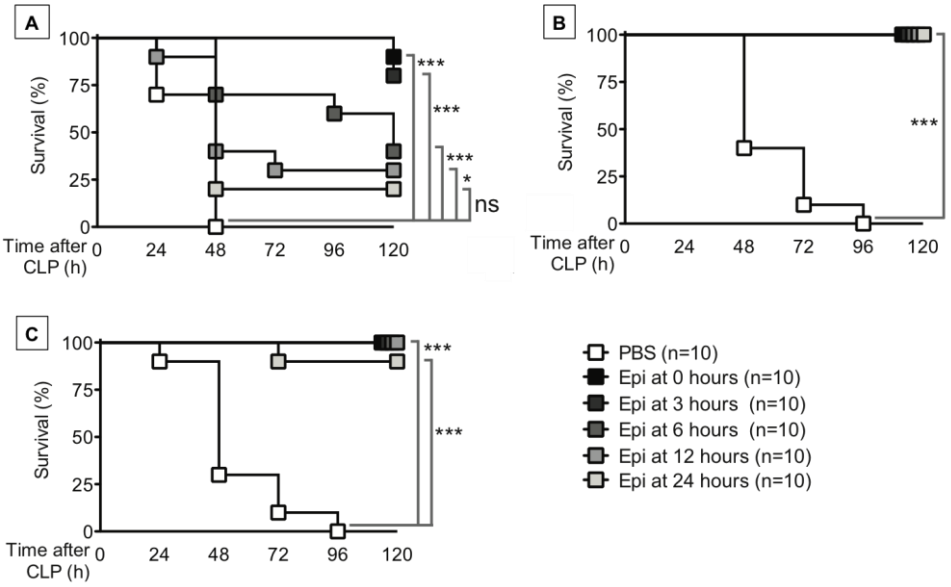

Figure 49 - Survival of C57BL/6 wildtype animals subjected to CLP treated with PBS or epirubicin (0.6µg/g body weight) at indicated times in: **(A)** the absence of meropenem; **(B)** with administration of meropenem (40µg/g body weight/day) starting at the time of the procedure; or **(B)** with meropenem treatment starting 12 hours after CLP. ns, not significant; *P<0.05; **P<0.01; ***P<0.001; log-rank (Mantel-Cox) test.

However, when given in combination with meropenem, even if this antibiotic is only administered 12 hours after CLP, low dose epirubicin confers protection until at least 24 hours after the initial onset of the septic syndrome (Figure 49-B and 49-C).

Apparently, epirubicin has a 24-hour therapeutic window that may be sufficient to make this drug useful in the clinic to reduce the mortality from severe sepsis and septic shock.

This is especially relevant for the group of patients that are either in the hospital or seek medical attention within the first few hours after the onset of symptoms.

Chapter IV - Concluding Remarks

This has been a long and exciting journey!

My interest in sepsis has begun during my training as a surgeon. Sepsis is one of the most frequent and challenging conditions for which we (surgeons and physicians) have little to offer. In spite of decades of tremendous progress in medical care and research in the field of inflammation and innate immunity, sepsis remains a poorly understood systemic inflammatory condition with high mortality rates and limited therapeutic options. In addition to organ support measures and large spectrum antibiotics, we still have a poor clinical *armamentarium*.

In fact, in the last five decades, there have not been any significant contributions to the therapeutic possibilities for this condition that were able to alter the natural course of this syndrome.

Most often, sepsis is triggered by a bacterial infection that causes an excessive production of pro-inflammatory mediators, including the initial critical tumor necrosis factor and interleukin 1β, leading to the activation of spiraling signaling cascades causing multiple organ dysfunction and ultimately death.

My project was initiated by asking whether simultaneous inhibition of the secretion of these cytokines could be beneficial in a mouse model of

sepsis. To approach this question, I have started with a drug screen to find molecules that were able to block these two sepsis mediators. I have been fortunate to identify, among other potential candidates, the clinically approved group of anthracyclines as potent in vitro inhibitors of two key initiators of sepsis, TNF and IL-1β.

In a murine model of peritonitis, anthracyclines confer strong protection against severe sepsis induced by cecal ligation and puncture (CLP). This protective effect relies on the induction of autophagy and on an anti-inflammatory program that increase the tolerance to infection without reducing bacterial burden.

Unraveling this protective autophagic pathway, and other cellular defense mechanisms, are becoming increasingly important clues to a better understanding of the pathophysiology involved in sepsis.

The field of Sepsis has been gradually moving its focus from the pathogen to the "dual combat" of the driving mechanisms of pro-inflammatory and of the compensatory anti-inflammatory pathways.

For the past centuries we have been focused on the fight against the pathogens that initiated these syndromes but not giving enough attention to the cellular mechanisms that have been evolutionary selected to allow the human body to withstand infection.

In a more holistic point of view, we have unraveled some of the "off-target" properties of a drug that can give an infected organism an additional time to fight the attacking microorganisms. Meanwhile, instead of blocking inflammatory pathways (a strategy that has repeatedly failed in the past) these drugs dampen the inflammatory milieu (soften the "cytokine storm") and

confer protection to target organs, against the deleterious pro-inflammatory effects that usually ensue this septic aggression.

Using an shRNA-based screen we identified the Ataxia Telangiectasia Mutated (ATM) as a mediator of the protective effect of anthracyclines. ATM deficient ($Atm^{-/-}$) mice are refractory to this protective effect succumbing to severe sepsis with similar kinetics to the non-treated wild-type mice.

If anthracyclines are administered in combination with a broad-spectrum antibiotic such as meropenem, they protect from CLP even if they are given only 24 hours after the procedure, provided meropenem is administered within the initial 12 hours after CLP. This means that epirubicin, and more generally the group of anthracyclines, are very effective at conferring protection against severe sepsis in mice, even when used up to 24 hours after the onset of infection.

This therapeutic window is likely to be sufficient to make these drugs useful in the clinical setting to reduce the mortality of sepsis in most patients that are either in the hospital or seek medical attention within the first few hours of symptoms initiation. Therefore, our results identify the group of anthracyclines as an effective therapeutic option in sepsis, and ATM as a potential molecular target in inflammation-driven conditions.

What started with a rather naïve assumption (inhibition of the secretion of two of the initiators of sepsis could change the course of the disease), has led to the identification of a family of clinically approved and well known drugs that are very effective in a mouse model of sepsis. The molecular

basis of protection is complex and goes substantially beyond cytokine and inflammatory mediator inhibition.

We now know that the activation of a DNA damage response and the induction of autophagy are critical components in this protection, which is likely to happen at the level of target organs, the lung in particular.

In addition to finding drugs that are clinically approved and therefore can be tested for their therapeutic potential in sepsis to treat real patients in the very near future, my work raises interesting biological questions that are relevant themselves and should be followed in future research.

One important example is to understand how DNA damage leads to the induction of autophagy. A related question and less explored issue is to understand how the induction of autophagy is protective. If the reason relates to the inhibition of necroptosis (a controlled form of necrosis), for which there is supportive literature, it will be of great interest to study the fine molecular mechanisms that explain this process.

This will be of relevance, because understanding the molecular mechanisms of necroptosis inhibition is likely to be clinically important. Understanding this mechanism can open new opportunities to develop drugs that can be beneficial in conditions that are driven by cell death and can include, other than sepsis, ischemia-reperfusion injury, and neuro-degeneration.

An additional question that my work raises and requests further attention is the reason for evolutionary association between inflammation and DNA damage responses. This work suggests that at least several key initiators

of DNA damage response are negative regulators of inflammation. It makes sense that a microbial infection would cause collateral DNA damage and therefore a response to repair the DNA would be initiated at the risk of lethal and irreversible DNA damage and inflammation with significant costs for the individual and its offspring.

However, these mechanisms are likely to be complex, and it is difficult at this point to anticipate which pathways might be involved. Whatever they may be, their elucidation is likely to be a significant contribution to fundamental science. These same mechanisms are also of potential interest to be explored in the development of therapies targeting inflammation and related conditions.

Finally, I consider that the more exciting possibility to continue this work is the obvious potential to convert these findings in the mouse model of sepsis into a clinically useful and novel approach to treat patients with sepsis. To this aim, I have begun to take the first steps into the design and development of a clinical trial to test if epirubicin at low doses is safe and can constitute a therapeutic option in well-selected sepsis patients.

At this point I feel that I have come a full cycle: from bedside to the bench and back.

As a physician-scientist, I truly look forward to the next step…

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Publications

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Abstract:	Severe sepsis remains a poorly understood systemic inflammatory condition with high

	mortality rates and limited therapeutic options in addition to organ support measures. Here we show that the clinically approved group of anthracyclines acts therapeutically at a low dose regimen to confer robust protection against severe sepsis in mice. This salutary effect is strictly dependent on the activation of DNA damage response and autophagy pathways in the lung, as demonstrated by deletion of the ataxia telangiectasia mutated (Atm) or the autophagy-related protein 7 (Atg7) specifically in this organ. The protective effect of anthracyclines occurs irrespectively of pathogen burden, conferring disease tolerance to severe sepsis. We propose that lung protection is an early priority in sepsis management and that anthracyclines are potential effective therapeutic options in sepsis.
Suggested Reviewers:	Peter Vandenabeele peter.vandenabeele@dmbr.vib-ugent.be RIP kinase-dependent necrosis drives lethal systemic inflammatory response syndrome. Duprez L, Takahashi N, Van Hauwermeiren F, Vandendriessche B, Goossens V, Vanden Berghe T, Declercq W, Libert C, Cauwels A, Vandenabeele P. Immunity. 2011 Dec 23;35(6):908-18. doi: 10.1016/j.immuni.2011.09.020.
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Cover Letter

INSTITUTO DE MEDICINA MOLECULAR
Faculdade de Medicina de Lisboa

Lisbon, February 22nd, 2013

Dear Dr. Stacey,

We submit for your consideration the manuscript "***Anthracyclines induce autophagy-mediated protection against severe sepsis***" for publication in *Immunity*.

Sepsis is the leading cause of death in intensive care units and the third cause of overall hospital mortality. In spite of tremendous progress in the fields of innate immunity, microbiology and related biomedical areas, the physiopathology of sepsis remains poorly understood. As a result, the basic elements of treatment – early antibiotics, prompt source control of infection and organ support - have not changed significantly in the last fifty years, and attempts to translate basic research results into effective new interventions have been met with limited or no success.

Here we show in an experimental mouse model that anthracyclines confer strong protection against sepsis by increasing disease tolerance to infection, that is, acting irrespectively of pathogen burden. We further show that ATM (ataxia telangiectasia mutated) kinase and the induction of autophagy are strictly required for the in vivo protection against sepsis. These molecular pathways provide strong damage control in target tissues, specifically in the lung, possibly by blocking necroptosis, raising interesting connections to recent publications in *Immunity* (Immunity. 2011 Dec 23;35(6):908-18).

After reading the manuscript, I hope you agree that our results are novel, exciting and relevant, not only because they point to a possible effective treatment for sepsis, but

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also because we identify the DNA damage response and autophagy pathways in the lung as critical processes in the physiopathology of sepsis.

You will find that we have previously submitted our original (perhaps preliminary) observations to *Immunity* back in May 2011 as a pre-submission inquiry. Based on the information available in the abstract, you have expressed concerns about the mechanistic depth provided. We have since improved our manuscript dramatically, mainly by considerably expanding on the mechanistic basis for the protective phenotype of anthracyclines.

We hope that you find our work suitable for publication in *Immunity*.

I look forward to hearing from you, best regards,

Luís F. Ferreira Moita, M.D., Ph.D.
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*Manuscript

Anthracyclines induce autophagy-mediated protection against severe sepsis.

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Key Words: Sepsis; ATM; Autophagy; Anthracyclines

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Running Title

Anthacyclines induce tolerance in sepsis

Summary

Severe sepsis remains a poorly understood systemic inflammatory condition with high mortality rates and limited therapeutic options in addition to organ support measures. Here we show that the clinically approved group of anthracyclines acts therapeutically at a low dose regimen to confer robust protection against severe sepsis in mice. This salutary effect is strictly dependent on the activation of DNA damage response and autophagy pathways in the lung, as demonstrated by deletion of the ataxia telangiectasia mutated (*Atm*) or the autophagy-related protein 7 (*Atg7*) specifically in this organ. The protective effect of anthracyclines occurs irrespectively of pathogen burden, conferring disease tolerance to severe sepsis. We propose that lung protection is an early priority in sepsis management and that anthracyclines are potential effective therapeutic options in sepsis.

INTRODUCTION

Sepsis is a life-threatening condition that arises as a systemic inflammatory response to an infection (Bone et al., 1992; Levy et al., 2003). It includes a continuum of clinical severity ranging from systemic inflammatory response syndrome (SIRS), sepsis, severe sepsis and to septic shock (Suffredini and Munford, 2011). It is the leading cause of death in intensive care units and the third cause of overall hospital mortality (Angus and Wax, 2001; Ulloa and Tracey, 2005). In spite of significant improvement in diagnosis and support measures, the global annual mortality rate is ~28% (Hotchkiss and Karl, 2003), ranging from less than 10% in SIRS to up to 70% in septic shock (Angus and Wax, 2001; Annane et al., 2003). The pathophysiology of sepsis remains poorly understood. As a result, the basic elements of treatment – early antibiotics, prompt source control of infection and organ support - have not changed significantly in the last fifty years, and attempts to translate basic research results into effective new interventions have been met with limited or no success (Suffredini and Munford, 2011). In the same period, the incidence of sepsis and its economic burden has increased by 1% each year (Martin et al., 2003; Ulloa and Tracey, 2005), indicating the urgent need for novel therapeutic options.

Inflammation is a response to harmful stimuli that limits tissue damage and aims at restoring homeostasis (Medzhitov, 2008). Pathogen-associated molecular patterns (PAMPs) on microorganisms and damage-associated molecular patterns (DAMPs) originating from dying cells are sensed by the host through germline-encoded pattern recognition receptors (PRRs) that recognize conserved signature structures in non-self and self (Janeway and Medzhitov, 2002). These sensors are present in both

professional (including neutrophils, macrophages and dendritic cells) and non-professional immune cells and their activation initiates intracellular signaling cascades leading to the transcriptional expression of inflammatory mediators, such as cytokines and chemokines. Inflammation needs to be effectively terminated after removal of the original trigger and repair of damaged tissue. In the susceptible host, overproduction of inflammatory mediators or an exaggerated response to their presence can lead to septic shock, tissue destruction or permanent loss of function (Takeuchi and Akira, 2010).

There are two evolutionarily conserved defense strategies against infection that can limit host disease severity. One relies on reducing host pathogen load, i.e. resistance to infection, while the other provides tissue damage control, limiting disease severity irrespectively of pathogen load, i.e. tolerance to infection (Raberg et al., 2009; Schneider and Ayres, 2008). As demonstrated originally for plants and thereafter in *Drosophila*, tolerance to infection also operates in mammals, as revealed for *Plasmodium* (Raberg et al., 2007; Seixas et al., 2009) and polymicrobial infections in severe sepsis (Larsen et al., 2010).

Here we show in an experimental mouse model that anthracyclines confer strong protection against sepsis by increasing disease tolerance to infection, that is, acting irrespectively of pathogen burden. We further show that ATM (ataxia telangiectasia mutated) kinase and the induction of autophagy are strictly required for the in vivo protection against sepsis. These molecular pathways provide strong damage control in tissues, specifically in the lung.

RESULTS

Antracyclines confer strong protection against severe sepsis

In an in vitro chemical screen using ~2320 compounds, we identified several lead candidates capable of inhibiting inflammatory cytokine production by the THP-1 macrophage line (Figure S1a and Supplementary Table I). This inhibitory effect was dissociated from cytotoxicity of the compounds tested on THP-1 cells (Figure S1b). Among these, we found 3 representatives of the anthracycline family of chemotherapeutic agents namely epirubicin, doxorubicin and daunorubicin, and validated their inhibitory activity on cytokine production (Figure S1c).

We then used the cecal ligation and puncture (CLP) mouse model of experimental sepsis to investigate the *in vivo* effects of epirubicin (Rittirsch et al., 2009). In CLP, sepsis results from a polymicrobial infection of abdominal origin, leading to bacteremia and a systemic inflammatory response (Rittirsch et al., 2009). We adjusted CLP severity to a high-grade sepsis, where at least 80% of C57BL/6 mice succumbed within 48 h after the initial procedure. Under these conditions, epirubicin administered i.p. at the time of CLP and again 24 h later in a total of 1.2μg/g of body weight reproducibly and significantly increased the survival of C57BL/6 mice subjected to CLP by nearly 80%, without the use of antibiotics (Figure 1a). A similar protective effect was observed in epirubicin-treated animals with the same dose and schedule but administered i.v. (Figure S2). This appeared to be a general property of the anthracycline family because other representative members of this family of drugs identified in the initial chemical screen conferred similar levels of protection against CLP (Figure 1b). The protective effect of anthracyclines was not dependent on the mouse strain as outbread NMRI mice were similarly protected by epirubicin (Figure

1c). Epirubicin was equally effective against another clinically relevant pathogen causing sepsis, *K. pneumoniae* administered intranasally (Figure 1d), arguing that epirubicin can be effective in the treatment of sepsis of different origins in addition to peritoneal sepsis. Mice previously subjected to CLP and treated with epirubicin were not immunocompromised as they could clear a secondary intranasal viral infection similarly to control mice (Figure 1e).

Epirubicin acts therapeutically to promote disease tolerance to severe sepsis

We found that in epirubicin-treated mice subjected to CLP the bacterial load in blood and target organs of sepsis, e.g., spleen, liver and kidney, 24 h post-CLP did not differ from that of untreated controls (Figure 2a). While at 48 h post-CLP we noticed a trend towards a lower bacterial load in the target organs of epirubicin-treated animals, the differences were not statistically significant, even if most untreated control animals die between 24 and 48 h after the CLP procedure. These results raised the possibility that the protective effect of epirubicin *in vivo* is related to disease tolerance without directly affecting the pathogen burden (Medzhitov et al., 2012). This idea was supported by the observation that the serum concentrations of several markers of tissue damage such as LDH (lung and general cellular damage), CK (muscle), ALT (liver) and urea (kidney) were significantly reduced to almost basal levels in epirubicin-treated mice, 24 h after CLP, compared to untreated mice (Figure 2b). In addition, we observed a significant reduction in the levels of inflammatory mediators including TNF, IL-1β, IL-6 and HMGB1 compared to non-treated CLP mice (Figure 2c and 2d). To explore this further in the absence of bacteria, we found that the drug protected C57BL/6 mice from lethal septic shock caused by lipopolysaccharide (LPS, endotoxin) (Figure 2e).

Large spectrum antibiotics such as meropenem are very effective at lowering bacteremia and are standard drugs used in sepsis (Russell, 2006). We tested the efficacy of meropenem in CLP in comparison to epirubicin and found that while meropenem delayed the death rate of CLP-subjected mice, it did not prevent mortality (Figure 2f), in spite of a strong impact on bacterial burden (Figure 2g). This was in sharp contrast to the action of epirubicin, that did not interfere with bacteremia (Figure 2g) but prevented CLP-induced mortality (Figure 2f), again arguing for a role of epirubicin in conferring disease tolerance against severe sepsis (Larsen et al., 2010; Medzhitov et al., 2012).

Both epirubicin and meropenem decreased the levels of TNF, IL-1β and HMGB1 in the serum of mice subjected to CLP (Figure 2h). This indicates that while decreased circulating levels of inflammatory mediators may contribute to confer protection against severe sepsis, inhibition of TNF, IL-1β and HMGB1 is not sufficient *per se* to explain the protective effect of epirubicin, which is in accordance with what is observed for other therapeutic approaches in the clinical setting (Hotchkiss and Karl, 2003). Taken together these data suggest that epirubicin acts through an additional alternative mechanism to cytokine inhibition to confer disease tolerance to sepsis.

Epirubicin protection against sepsis is mediated by ATM

Next, in order to explore the molecular mechanism behind the protective effects of anthracyclines, we used our *in vitro* assay system to perform a short hairpin RNA (shRNA)-based screen in THP-1 cells, focusing on kinases and phosphatases and using IL-1β and TNF secretion as assay readouts. While our *in vivo* results suggested

the possibility that anthracyclines ameliorate the lethal effects of sepsis by a mechanism affecting tissue tolerance, we reasoned that our *in vitro* assay would be useful for the identification of candidate pathways mediating the anthracycline effects. We found several negative regulators of IL-1β in response to *E. coli* challenge, including the Ataxia Telangiectasia Mutated (ATM), the Checkpoint Kinase 1 (CHEK1) and the Ataxia Telangiectasia and Rad3 Related (ATR) genes (Figure S3 and Supplementary table II). These findings suggest that DNA damage response (DDR) components are negative regulators of IL-1β secretion. Using a phospho-specific antibody against the activated form of ATM, we found that while *E. coli* alone was a poor, but reproducible ATM activator (Figure S3), epirubicin alone or in combination with *E. coli* triggered a robust ATM activation (Figure S3). This was confirmed using immunoblotting (Figure S3).

ATM is a master regulator of the DDR (Ciccia and Elledge, 2010) and is known to be activated by anthracyclines and other DNA damaging agents (Siu et al., 2004). Therefore we used ATM-deficient mice to test the contribution of the DDR to the protective effect of anthracyclines against severe sepsis. ATM-deficient ($Atm^{-/-}$) mice were not protected by epirubicin against CLP and died with similar kinetics to those of wild-type ($Atm^{+/+}$) animals that were treated with PBS alone (Figure 3a). We conclude that ATM expression is necessary to mediate the protective effect of epirubicin in sepsis. In striking contrast to wild-type mice (Figures 2b and c), in the absence of ATM, epirubicin no longer normalized the serologic markers of organ lesion (Figure 3b) or decreased the levels of inflammatory mediators (Figure 3c). However, in mice subjected to CLP and treated with etoposide, an agent known to cause DNA double strand breaks and to activate ATM-dependent pathways

(Montecucco and Biamonti, 2007), mortality induced by CLP was only partially rescued (Figure 3d), suggesting that ATM is necessary but not sufficient for the protection conferred by anthracyclines against sepsis.

In addition to double strand breaks (repaired in an ATM-dependent manner), anthracyclines also cause DNA interstrand cross-links, a DNA lesion known to be repaired by the Fanconi Anemia (FA) pathway (Ciccia and Elledge, 2010). Interestingly, FA patients were reported to have increased levels of TNF (Briot et al., 2008; Vanderwerf et al., 2009), and FA protein FancD2, directly inhibits TNF promoter activity (Matsushita et al., 2011). In THP-1 cells, we observed that FancD2 is activated in an ATM-independent manner upon epirubicin treatment, as shown by its mono-ubiquitination (Figure 3e). These findings support the independence of signaling events initiated by the generation of DNA double strand breaks and DNA interstrand cross-links. We examined the contribution of this pathway for epirubicin protection of CLP and found that *FancD2*$^{-/-}$ mice were slightly but significantly impaired for the protective effects (Figure 3f).

The protective effect of epirubicin is dependent on the autophagy pathway

While it is possible that the dominant ATM-mediated protection against sepsis might rely on ROS scavenging (Cosentino et al., 2010), on the induction of apoptosis of inflammatory cells (Garrison et al., 2011), on the preservation of genomic stability (Westbrook and Schiestl, 2010), or on the biogenesis of anti-inflammatory microRNAs such as miR-146a (Zhang et al., 2011), we found no significant contribution for any of these processes (Figure S4). We, therefore, explored a possible role for autophagy in this process, given that ATM is a negative regulator of mTOR,

which is itself, an inhibitor of autophagy (Alexander et al., 2010a; Alexander et al., 2010b). Using autophagy-defective ($Lc3b^{-/-}$) mice, we found that the autophagy pathway is required for the *in vivo* effect of epirubicin (Figure 4a). Similarly to $Atm^{-/-}$ mice (Figure 3b and c), epirubicin was not able to decrease the serologic markers associated with organ lesion (Figure 4b) or to normalize cytokine levels in autophagy-defective mice (Figure 4c).

We then used LC3b-GFP mice to study the contribution of the autophagy pathway in the protection conferred by epirubicin. While FACS analysis shows that CLP alone induces LC3b aggregation in different splenocyte populations, namely monocytes and neutrophils, epirubicin treatment did not increase the autophagy pathway in these critical players in sepsis (Figure 5a). We then tested the impact of epirubicin on the survival of a conditional depletion of *Atg7* specifically in the myeloid compartment upon CLP, using $Atg7^{loxP/loxP}$ $LysM^{Cre}$ GFP-LC3b animals. Strikingly, these animals were equally protected by epirubicin as compared to control mice (Figure 5b), suggesting that the autophagy pathway is not required in the myeloid compartment for the protective effects of epirubicin against sepsis.

Autophagy can be effectively monitored by the conversion and immobilization of LC3 (Kabeya et al., 2000). Because the autophagy pathway was not required in the hematopoietic compartment for protection against sepsis by epirubicin, we then looked at target organs of sepsis (lung, liver and kidney) using immunobloting to identify lipidation of LC3b as indicative of activation of the autophagy pathway. We found that epirubicin specifically induced lipidation of LC3b in the lung at 6 h, but not in the liver or kidney (Figure 5c). Although LC3 was transiently lipidated after

CLP in the liver at 6 and 24 h as previously reported (Chien et al., 2011), levels of LC3 were not altered by epirubicin treatment (Figure 5c). We have further confirmed that autophagy was induced in the lung as shown by the increase of LC3b positive vesicles in lung sections at 6 h and 24 h comparing epirubicin treated and non-treated mice (Figure 5d).

We then deleted *Atg7* specifically in the lung, using an adenovirus-expressing CRE (Ad^{cre}) to intranasaly infect $Atg7^{loxP/loxP}$ mice (Komatsu et al., 2005). When subjected to CLP, these mice were no longer protected from CLP by epirubicin treatment (Figure 5e). By assessing the levels of H2AX, a surrogate marker of ATM activation (Ciccia and Elledge, 2010), in the lungs of control or epirubicin-treated CLP-subjected mice, we found a significant increase in the number of cells with H2AX-positive foci in lungs of epirubicin-treated mice (Figure 5f). To test whether ATM activation was also required specifically in the lung, we used $Atm^{loxP/loxP}$ mice and Ad^{cre} to delete ATM specifically in the lung. Upon Ad^{cre}-mediated ATM deletion in the lung, mice were no longer protected against sepsis by treatment with epirubicin (Figure 5g). We therefore conclude that the protective effect of epirubicin in sepsis is, at least in part, due to the ATM-dependent induction of autophagy in target organs, namely the lung.

Epirubicin has a 24 h therapeutic window to protect against sepsis

Finally, we studied the therapeutic window of epirubicin in mice. When given alone, epirubicin conferred strong protection at the time of the procedure or until 3 h after the initiation of CLP (Figure 6a). When administered only 6 h after CLP, epirubicin quickly lost its protective effect (Figure 6a). However, if given in combination with

meropenem, even when this antibiotic is only administered 12 h after CLP, low dose epirubicin conferred complete protection until at least 24 h after the initial procedure (Figure 6b and 6c). These results suggest that anthracyclines can be used not only to prevent sepsis, but also that they can act therapeutically when their administration is combined with a large spectrum antibiotic.

Discussion

Here we report that epirubicin, and more generally the group of anthracyclines, are very effective at conferring protection against severe sepsis in mice, even when used up to 24 h after the onset of infection. This therapeutic window is likely to be sufficient to make these drugs good candidates for useful therapeutic options in the clinic to reduce the mortality of sepsis in most patients that are either in the hospital or seek medical attention within the first few hours of symptoms initiation.

Although we began our investigation of the use of anthracyclines in sepsis by virtue of their effects in inhibiting inflammatory cytokine expression in myeloid cells *in vitro*, our studies have identified a novel mode of protection that seems to be much stronger and perhaps completely independent of such effects, and rather manifests at the level of DNA damage-induced, ATM-dependent autophagy in the lung. Thus, our findings uncover an unexpected role for this pathway in tissue (lung) tolerance to the pathological consequences of infection. These novel findings are especially relevant given that agents discovered in studies over the last few years targeting various pro-inflammatory cytokines have had limited success in humans. Our studies suggest a critical role for protecting host tissues thereby conferring protection against sepsis.

Recent studies have highlighted the role of tissue tolerance to infection as an important aspect of host pathology (Medzhitov et al., 2012).

Interestingly, the protective effect of epirubicin seems to act irrespectively of the host pathogen burden, revealing that it confers disease tolerance to polymicrobial infection (Larsen et al., 2010; Raberg et al., 2009; Schneider and Ayres, 2008). This finding reveals that pharmacologic agents that provide tissue damage control can limit disease severity irrespectively of pathogen load and represent a promising therapeutic strategy against sepsis. Moreover, based on our identification of ATM as a major mediator of epirubicin effects, we propose that this protein and other components of the DNA damage response machinery constitute novel regulators of tolerance, without affecting pathogen resistance mechanisms.

Recent reports make our findings counter-intuitive as doxorubicin and daunorubicin have been shown to induce acute inflammation when injected in the abdomen where they induce cytokine secretion (Krysko et al., 2011; Sauter et al., 2011). However, the concentrations of anthracyclines utilized in these studies were more than 10-fold higher than those used here. By using lower concentrations we may reduce the cytotoxicity of these drugs and the resulting release of pro-inflammatory DAMPs by dying cells and reveal the additional pharmacological effects mediated by the surviving target cells. Interestingly, fluoroquinolones that are bacterial type II topoisomerase inhibitors, as opposed to anthracyclines, which are eukaryotic type II topoisomerase inhibitors, were reported to have immunomodulatory effects (Dalhoff and Shalit, 2003) when used in supra-therapeutic concentrations. Fluoroquinolones have been shown to protect against LPS model of septic shock (Khan et al., 2000).

While the molecular mechanisms that explain these effects have not been elucidated, it has been proposed that higher doses of fluoroquinolones can inhibit mammalian topoisomerase type II enzymes in addition to their bacterial targets (Dalhoff and Shalit, 2003), an effect that can be achieved with very low doses of anthracyclines.

Autophagy induction has been previously shown to have protective effects in sepsis and septic shock models (Chien et al., 2011; Nakahira et al., 2010). ATM likely mediates the induction of autophagy by epirubicin, which is critical for the protection conferred by this drug in the CLP mouse model of sepsis. Importantly, epirubicin specifically induces LC3b lipidation in the lung, a response that is low or absent in this organ in the absence of anthracycline treatment, but occurs spontaneously and transiently in the liver, and according to some reports (Hsiao et al., 2011) also in the kidney by sepsis induction alone. This finding is likely relevant, because it focuses the protective effect of epirubicin to the lung, an organ that often fails in septic patients and drives the failure of other target organs particularly the kidney and later the liver (Hotchkiss and Karl, 2003). Interestingly, the protective phenotype of epirubicin is strikingly similar to that of RIPK3-deficient mice (Duprez et al., 2011), suggesting that epirubicin-mediated, ATM-dependent, autophagy induction can possibly prevent TNF-driven necroptosis in such key organs in sepsis pathology as the lung. In fact, there have been recent works that support the role of autophagy in the inhibition of necroptosis (Bray et al., 2012; Degenhardt et al., 2006; Lu and Walsh, 2012; Shen and Codogno, 2012). The molecular mechanisms at the basis of epirubicin-induced protection in sepsis by autophagy are certainly an interesting topic for future studies.

Experimental Procedures

Animal Model and Anthracycline Treatment

Animal care and experimental procedures were conducted in accordance with Portuguese and US guidelines and regulations after approval by the respective local committees (Instituto de Medicina Molecular and Instituto Gulbenkian de Ciência). All mice used were 8–12 weeks old. Mice were bred and maintained under specific pathogen-free (SPF) conditions. C57BL/6 and C57BL/6 *ATM*$^{-/-}$ were obtained from the Instituto Gulbenkian de Ciência (a kind gift from Dr. Vasco Barreto). C57BL/6 *Nrf2*$^{-/-}$ mice were provided originally from the RIKEN BioResource Center (Koyadai, Tsukuba, Ibaraki, Japan)[23] and subsequently at the Instituto Gulbenkian de Ciência. LC3b-/- (B6129PF2/J background) and NMRI mice were purchased from Jackson and Charles River laboratories, respectively. miR-146 mice were generated in the Baltimore's laboratory (Boldin et al., 2011). FancD2-/- mice were generated by the Grompe laboratory (Houghtaling et al., 2003). ATG7$^{loxP/loxP}$ were generated in by Masaaki Komatsu and obtained from the Green laboratory. ATM$^{loxP/loxP}$ mice were generated and obtained from the F.W. Alt's laboratory. CLP was performed as described previously (Rittirsch et al., 2009). The endotoxemia model was performed by injecting intraperitoneally (i.p.) a single dose of 50 µg/g body weight of LPS (from *E. coli* serotype 026:B6; Sigma-Aldrich). Pulmonary monostrain infections were carried out as described previously (Weber et al., 2011), using intranasal injection of *Klebsiella pneumoniae* (ATCC13803) at 8x10^{7} cfu. Epirubicin (Sigma-Aldrich), doxorubicin (Sigma-Aldrich), daunorubicin (Sigma-Aldrich) were dissolved in PBS, etoposide (Sigma-Aldrich) was dissolved in DMSO, aliquoted and stored at –80ºC. Meropenem (AstraZeneca, Lisbon, Portugal). Epirubicin and daunorubicin (0.6µg/g body weight), doxorubicin (0.5µg/g body weight), etoposide (2µg/g body weight) were injected intraperitoneally at 0 and 24 h following CLP. Meropenem (20µg/g body weight b.i.d.) was injected i.p. for 5 consecutive days.

Colony-Forming Units Assay Blood samples from septic or mock CLP mice were collected by cardiac puncture at indicated times after surgery. Mice were subsequentially perfused *in toto* with 10mL ice cold PBS and spleen, liver and kidneys were surgically removed and homogenised in 5ml of sterile PBS. Serial dilutions of blood and tissue homogenates were immediately plated on Trypticase Soy Agar II plates supplemented with 5% Sheep Blood. CFUs were counted after 12 h of incubation at 37 C.

Serology and Cytokine Measurement

Plasma from blood samples obtained 24 h post-CLP were collected after centrifugation. LDH, CK, ALT and urea levels were measured using the BioAssay Systems kits (BioAssay Systems, California) according to company's protocol. Levels of TNF-alpha, IL-1b and IL-6 were measured using the murine ELISA kits (R&D Systems, Minneapolis) according to company's protocol. Levels of HMGB1 were assessed using the ELISA kit (Shino Test Corporation, Tokyo) according to company's protocol.

Histology

Mice were euthanased, perfused *in toto* with 10mL ice cold PBS and lungs and livers were surgically removed. Livers were placed in 10% phosphate buffered formalin for 24 h after which were embedded in paraffin. Sections were subsequently incubated with a primary antibody reactive to HMGB1 (Abcam) followed by incubation with biotinylated secondary antibody and then with biotinylated horseradish peroxidase. Staining was developed by addition of diaminobenzidine (DAB) substrate (Vector Labs, Burlingame, CA) and counterstained with hematoxylin. Lungs were embedded in Tissue-Tek OCT (Sakura), and snap-frozen in liquid nitrogen. Lung sections (7 µm) were fixed in 1% paraformaldehyde in PBS for 2 min, followed by methanol at -20°C for 10 min and then in acetone for 2 min. Detection of LC3b and histone gH2AX was performed by incubating sections overnight at 4°C with rabbit polyclonal antibodies specific for, respectively, LC3b (L7543, Sigma Aldrich, USA) and gH2AX (phosphoS139) (ab2893; Abcam, Cambridge, UK); incubation with a secondary DyLight 488-coupled antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was for 1 h at room temperature. Sections were counterstained with DAPI (0.5 µg/ml) to visualize DNA and mounted in Vectashield (Vector Laboratories Inc., Burlingame, CA) before confocal microscopy. Samples were examined with a Zeiss LSM 510 META laser scanning confocal microscope (Carl Zeiss, Jena, Germany). The acquired images were analyzed using a MATLAB (Mathworks; Natick, MA) routine developed in-house to perform automatic threshold segmentation and enumeration of individual cell nuclei stained with DAPI.

In vivo Viral Infection and Viral Titer Assay

Murid herpesvirus-4 infection and viral particle quantification was performed as previously described (Marques et al., 2008). Briefly, mice were intranasally inoculated with 1000 PFU of MuHV-4 strain 68

in 20 µl of PBS under light isofluorane anaesthesia. At 6 and 12 days post-infection, lungs were removed and homogenised in 5ml of Glasgow's modified Eagle's medium (GMEM). Infectious virus titers in freeze-thawed lung homogenates were determined by serial diluted suspension assay using Baby hamster kidney cells (BHK-21) cells cultured in GMEM supplemented with 10% fetal bovine serum, 10% tryptose phosphate broth, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (GMEM). Plates were incubated for four days, fixed with 10% formal saline and counterstained with toluidine blue. Viral plaques were counted with a plate microscope. *Cre*-adenovirus were obtained from the University of Iowa, prepared as a calcium-phosphate coprecipitate and incubated for 20 min at room temperature. $Atg7^{loxP/loxP}$ and $ATM^{loxP/loxP}$ were subjected to light isoflurane anesthesia and allowed to inhale 125 µl of virus at a concentration of 2.5×10^7 PFU. Additionally, wild-type C57Bl/6 mice were included as controls. Mice ere allowed to rest for 5 days after inhalation after which were subjected to CLP.

Stainings and Flow Cytometry

Peritoneal infiltrating leukocytes from either wild-type or LC3b-GFP transgenic animals were obtained 24 h post CLP by lavage with 5 ml of sterile ice-cold PBS (Sigma), washed and blocked with mouse Ab anti-FcγIII/II (clone 93) receptor mouse Ab diluted in PBS containing 2% FCS (v/v) for 20 min at 4°C. Surface markers were detected by incubating for 30 min at 4°C with mouse Ab anti-CD4 (clone GK1.5), -CD8 (clone 53-6.7), -CD19 (clone 6D5), -Ly-6G (clone 1A8) (all Biolegend) and -neutrophils monoclonal antibody (clone 7/4) (Abcam). Dead cells were excluded by co-staining with propidium iodide. Total cell number was determined by flow cytometry using a fixed number of latex beads (Beckman Coulter) co-acquired with a pre-established volume of the cellular suspension. For phospho-ATM intracellular staining, stimulated THP-1 cells were washed and fixed with ice-cold methanol. Mouse Ab anti-phosphoATM pS1981, clone 10H11.E12 (IgG1k) (Rockland) was incubated for 60 min at room temperature followed by an incubation of secondary Ab conjugated with Alexa 488 (Molecular Probes). Fluorescence was measured by flow cytometry, and data analyzed using FlowJo software.

Immunoblotting

Mouse phospho-ATM (4526, Cell Signaling, Danvers, MA, 1:1000 dilution), rabbit total ATM (2873, Cell Signaling, Danvers, MA, 1:1000 dilution), rabbit LC3b (Sigma, 1:1000 dilution) and the rabbit FancD2 (Novus Biologicals, 1:1000 dilution) Ab were used overnight at 4ºC. Primary Ab were detected using peroxidase conjugated secondary Ab (1h; RT) and developed with SuperSignal chemiluminescent detection kit (Pierce, Carcavelos, Portugal).

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Figure Legends

Figure 1 – Epirubicin affords protection against severe sepsis. **(a)** Survival of C57BL/6 wild-type animals subjected to CLP treated with carrier (PBS) or epirubicin (Epi) (0.6μg/g body weight) at the time of procedure and 24 hours later. **(b)** Survival

of C57BL/6 wild-type animals subjected to CLP treated with carrier (PBS), epirubicin (Epi), doxorubicin (Doxo) or daunorubicin (Dauno). Treatment schedule and doses as in (a). **(c)** Survival of NMRI mice subjected to CLP and treated with carrier (PBS) or epirubicin (Epi) as in (a). **(d)** Survival of C57BL/6 wild-type animals following intranasal inoculation of *Klebsiella pneumoniae* and treated with carrier (PBS) or epirubicin (Epi) as in (a). **(e)** Quantification of infectious viral MuHV-4 particles in lung of C57BL/6 wild-type animals previously subjected to mock CLP (S), mock CLP treated with epirubicin (S+E) or CLP treated with epirubicin (C+E). Epirubicin treatment dose and schedule as in (a). Mice were intranasally inoculated with 1000 PFU of MuHV-4 on day 3 post CLP and viral particles quantified by plaque assay at days 6 and 12 post viral infection. Each circle represents individual animals and horizontal lines indicate arithmetic means ± SEM from two independent assays. The dashed horizontal line represents the limit of detection of the assay. ns, not significant; *P<0.05; **P<0.01; ***P<0.001 (log-rank (Mantel-Cox) test for (a) to (d) and Mann-Whitney test for (e)) .

Figure 2 - Epirubicin promotes disease tolerance to severe sepsis. (a) Polymicrobial load (CFUs) in blood, spleen, liver and kidney, at indicated time points, of C57BL/6 animals undergoing CLP and treated with PBS (C+P) or epirubicin (C+E) (0.6μg/g body weight) at the time of procedure and 24 hours later. Each circle represents individual animals. Horizontal lines indicate arithmetic means ± SEM. **(b), (c) and (d)** Epirubicin counteracts tissue damage and inflammation associated with CLP as assessed by (b) LDH, CK, ALT, urea and (c) TNF, IL-1β, IL-6 and HMGB1 plasma concentrations in C57BL/6 wild-type animals 24 hours after mock CLP (S) (n=2) or CLP followed by treatment with PBS (C+P) (n=5) or epirubicin (C+E) (n=7)

as in (a). Results shown represent arithmetic means ± SEM from duplicate (b) or triplicate (c) readings per animal. (d) Representative liver sections immunostained for HMGB1. Original magnification 40X. **(e)** Survival of C57BL/6 wild-type animals following lethal LPS injection and treatment with carrier (PBS) or epirubicin (Epi) as in (a). **(f)** Survival of C57BL/6 wild-type animals subjected to CLP treated with carrier (PBS), meropenem (40µg/g body weight/day) or epirubicin (Epi) as in (a). **(g)** CFUs in blood, at indicated time, of C57BL/6 animals undergoing mock CLP (S) or CLP followed by treatement with PBS (C+P), epirubicin (C+E) or meropenem (C+M) as in (f). Each circle represents individual animals. Horizontal lines indicate arithmetic means ± SEM. **(h)** IL-1β, TNF and HMGB1 plasma concentrations in C57BL/6 wild-type animals 24 hours after CLP followed by treatment with PBS (C+P) (n=4), epirubicin (C+E) (n=5) or meropenem (C+M) (n=5) as in (f). ns, not significant; *P<0.05; **P<0.01; ***P<0.001 (log-rank (Mantel-Cox) test for (e) and (f), Mann-Whitney test for (a) and (g), and unpaired t test for (b), (c) and (h)) .

Figure 3 – The protection afforded by epirubicin against severe sepsis is mediated by ATM (a) Survival of *Atm*$^{+/+}$ and *Atm*$^{-/-}$ C57BL/6 animals subjected to CLP and treated with PBS or epirubicin (Epi) with same schedule and dose as in fig 1. **(b)** LDH, CK, ALT, urea and **(c)** TNF, IL-1β and IL-6 plasma concentrations in *Atm*$^{-/-}$ C57BL/6 animals 24 hours after mock CLP (S) (n=2) or CLP followed by treatment with PBS (C+P) (n=8) or epirubicin (C+E) (n=8) as in (a). Results shown represent arithmetic means ± SEM from triplicate readings per animal. **(d)** Survival of PBS-, etoposide (Eto)-, and epirubicin (Epi)-treated wild-type C57BL/6 animals undergoing CLP. Etoposide dose was 2µg/g body weight. Treatment schedule as in (a). **(e)** FANCD2 and Ub-FANCD2 protein levels by immunoblotting in THP-1 cells

following *E. coli* challenge after a pre-incubation (1 hour) with carrier, epirubicin or KU-55933 as indicated. **(f)** Survival of *Fancd2*$^{+/+}$ and *Fancd2*$^{-/-}$ animals subjected to CLP and treated with PBS or epirubicin (Epi) with same schedule and dose as in (a). ns, not significant; *P<0.05; **P<0.01; ***P<0.001 (log-rank (Mantel-Cox) test for (a), (d) and (f) and unpaired t test for (b) and (c)).

Figure 4 – The ATM-dependent protection of epirubicin against severe sepsis relies on the induction of autophagy. **(a)** Survival of *Lc3b*$^{+/+}$ and *Lc3b*$^{-/-}$ animals subjected to CLP and treated with PBS or epirubicin (Epi) with same schedule and dose as in fig 1. **(b)** LDH, CK, ALT, urea and **(c)** TNF, IL-1β and IL-6 plasma concentrations in *Lc3b*$^{-/-}$ animals 24 hours after mock CLP (S) (n=2) or CLP followed by treatment with PBS (C+P) (n=4) or epirubicin (C+E) (n=7) as in (a). Results shown represent arithmetic means ± SEM from triplicate readings per animal. ns, not significant; *P<0.05; **P<0.01; ***P<0.001 ((Mantel-Cox) test for (a), unpaired t test for (b) and (c))

Figure 5 – The protective effect of epirubicin is dependent on the activation of ATM and the autophagy pathway in the lung. (a) GFP expression in blood monocytes and neutrophils, isolated from transgenic LC3b-GFP animals, 24 hours after mice were subjected to mock CLP (S) or CLP followed by treatment with PBS (C+P) or epirubicin (C+E) (0.6μg/g body weight) at the time of procedure and 24 hours later. Each circle represents individual animals. Horizontal lines indicate arithmetic means ± SEM. **(b)** Survival of *Atg7*$^{loxP/loxP}$ and *Atg7*$^{loxP/loxP}$ *LysM*$^{cre/cre}$ mice subjected to CLP and treated with PBS or epirubicin (Epi) with same schedule and dose as in (a). **(c)** LC3B-I and LC3B-II protein levels by immunoblotting using a

specific antibody against LC3B in lung, liver and kidney, isolated at the indicated times, of naïve C57BL/6 animals (C) or mice subjected to mock CLP (S) or CLP followed by treatment with PBS (C+P) or epirubicin (C+E) as in (a). **(d)** Representative sections of LC3B staining in lungs, isolated at the indicated times, of mice subjected to CLP followed by treatment with PBS (C+P) or epirubicin (C+E) as in (a). **(e)** Survival of wild-type (B6) and $Atg7^{loxP/loxP}$ animals subjected to CLP and treated with PBS or epirubicin (Epi) with same schedule and dose as in (a) 5 days after inhalation of adenoviral vector encoding Cre (Ad^{Cre}). **(f)** Representative sections of H2AX staining and percentage of $H2AX^{+}$ cells per field (right panel) in lungs, isolated 6 hours after mice were subjected to CLP followed by treatment with PBS (C+P) or epirubicin (C+E) as in (a). Results shown represent arithmetic means ± SD from 10 fields. **(g)** Survival of wild-type (B6) and $Atm^{loxP/loxP}$ animals subjected to CLP and treated with PBS or epirubicin (Epi) with same schedule and dose as in (a) 5 days after inhalation of Ad^{Cre}. ns, not significant; *P<0.05; **P<0.01; ***P<0.001 ((Mantel-Cox) test for (b), (e) and (g), and Mann-Whitney test for (a) and (f) (right panel)).

Figure 6 - Epirubicin confers protection against severe sepsis in a therapeutic manner. **(a)** Survival of C57BL/6 wild-type animals subjected to CLP treated with PBS or epirubicin (same dose as in Figure 1) at indicated times in the absence of meropenem; **(b)** with administration of meropenem (40µg/g body weight/day) starting at the time of the procedure or **(c)** with meropenem treatment starting 12 hours after CLP. ns, not significant; *P<0.05; **P<0.01; ***P<0.001 (log-rank (Mantel-Cox)).

Figure S1 – Anthracyclins inhibit the secretion of TNF and IL-1β. (a) Two-dimension plot of TNF and IL-1β production Z scores. The grey square defines the area in which compounds are considered primary hits, i.e., inhibiting both TNF and IL-1β. Black dots identify epirubicin (1), daunorubicin (2) and doxorubicin (3). **(b)** THP1 cell viability upon *E. coli* challenge (4 hours) after a pre-incubation (1 hour) with increasing concentrations of epirubicin (left panel), daunorubicin (middle panel) and doxorubicin (right panel). **(c)** IL-1β and TNF production by E. coli challenged THP-1 cells (4 hours) after a pre-incubation (1 hour) with increasing concentrations of epirubicin (left panel), daunorubicin (middle panel) and doxorubicin (right panel). Results shown represent arithmetic means ± SD from duplicate samples in one of 3 independent assays. ns, not significant; *P<0.05; **P<0.01 ***P<0.001 (Mann-Whitney test for (c)).

Figure S2 - Epirubicin protection against severe sepsis is independent of the route of administration. **(a)** Survival of C57BL/6 wild-type animals subjected to CLP treated with carrier (PBS) or epirubicin (0.6µg/g body weight) intraperitoneally (Epi IP) or intraveneouslly (Epi IV) at the time of procedure and 24 hours later. ns, not significant; *P<0.05; **P<0.01; ***P<0.001 (log-rank (Mantel-Cox)).

Figure S3 – The anti-inflammatory effects of epirubicin are mediated by ATM. **(a)** Two-dimension Z score plot of TNF and IL-1β production by THP-1 cells upon target gene knockdown using a selected group of constructs of the TRC shRNA lentiviral vector library followed by PFA-fixed *E. coli* stimulation for 24 hours. Each dot represents an individual construct. Dotted horizontal and vertical lines define the area in which genes are considered primary hits. Black dots identify ATM (1), ATR

(2) and Check1 (3). **(b) Epirubicin activates ATM** as shown by Flow cytometry analysis of the activated form of ATM, phosphorilated at serine 1981, in THP-1 cells left untreated (C) or treated with epirubicin alone (1μM) (5 hours) (Epi), challenged with PFA-fixed *E. coli* (4 hours) (*E. coli*) or *E. coli* (4 hours) plus epirubicin pre-treatment (1 hour) (*E. coli* + Epi), and **(c)** Immunoblotting of total protein extracts of THP-1 cells untreated or pre-treated with epirubicin (1mM) and challenged with PFA-fixed *E. coli* at indicated timepoints probed for the total and phosphorilated (serine 1981) forms of ATM. **(d)** and **(e)** IL-1β and TNF production by (d) THP-1 cells and (e) BMDM following *E. coli* challenge (4 hours) after a pre-incubation (1 hour) with carrier, epirubicin or KU-55933 as indicated. Results shown represent arithmetic means ± SD from triplicate samples for one of at least 3 independent assays. **(f)** IL-1β and TNF production by $Atm^{+/+}$ and $Atm^{-/-}$ BMDM following *E. coli* challenge and pre-incubation with carrier or epirubicin as in (e). ns, not significant; *P<0.05; **P<0.01 ***P<0.001 (Unpaired t test for (d) to (f)).

Figure S4 – In vivo protective effect of epirubicin is not due to either ROS scavenging, decreased neutrophils or induced miR-146a biogenesis. (a) ROS content in THP-1 cells as assessed by the pan ROS probe CM-H_2DCFDA following *E. coli* challenge (4 hours) after a pre-incubation (1 hour) with carrier, epirubicin or KU-55933 as indicated. Results shown represent arithmetic means ± SEM from 3 independent assays. **(b)** Survival of $Nrf2^{+/+}$ and $Nrf2^{-/-}$ animals subjected to CLP and treated with PBS or epirubicin (0.6μg/g body weight) (Epi) at the time of procedure and 24 hours later. **(c)** Evaluation of apoptosis ($AnnexinV^+$ cells) in total splenocytes of C57BL/6 wild-type animals subjected to CLP and treated with PBS or epirubicin as in (b) at the indicated times. Each circle represents individual animals and horizontal

lines indicate arithmetic means ± SEM from two independent assays. **(c2)** Quantification of (**A**) total cells, (**B**) neutrophils, (**C**) macrophages, (**D**) B cells, (**E**) CD4 T and (**F**) CD8 T lymphocytes in the peritoneal cavity 18 hours post CLP of C57BL/6 wildtype animals treated with PBS or epirubicin (0.6μg/g body weight) at the time of procedure. **(d)** Survival of *miR 146a*$^{+/+}$ and *miR 146a*$^{-/-}$ animals subjected to CLP and treated with PBS or epirubicin as in (b). **(e)** *miR 146a* expression, as assessed by qRT-PCR, in THP1 cells left untreated or pre-treated with epirubicin and challenged with PFA-fixed *E. coli* for the indicated times. ns, not significant; *P<0.05; **P<0.01; ***P<0.001 (unpaired t test for (a) and (c); log-rank (Mantel-Cox) test for (b) and (d)).

Figure 1

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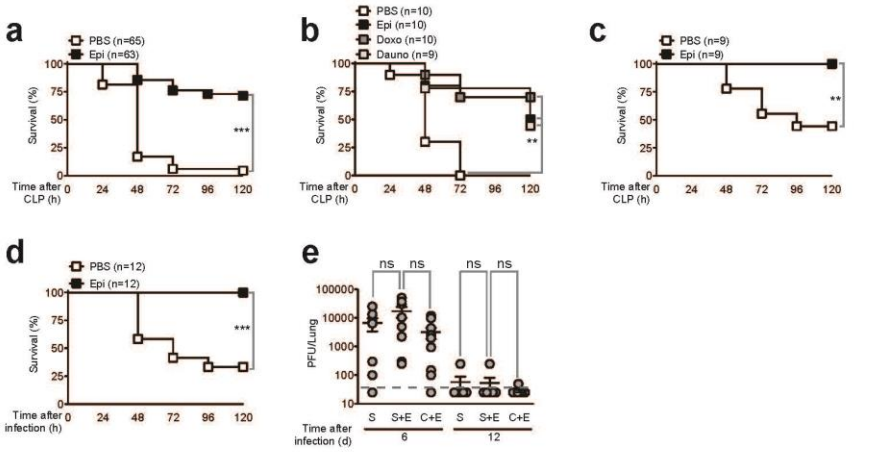

Figure 2
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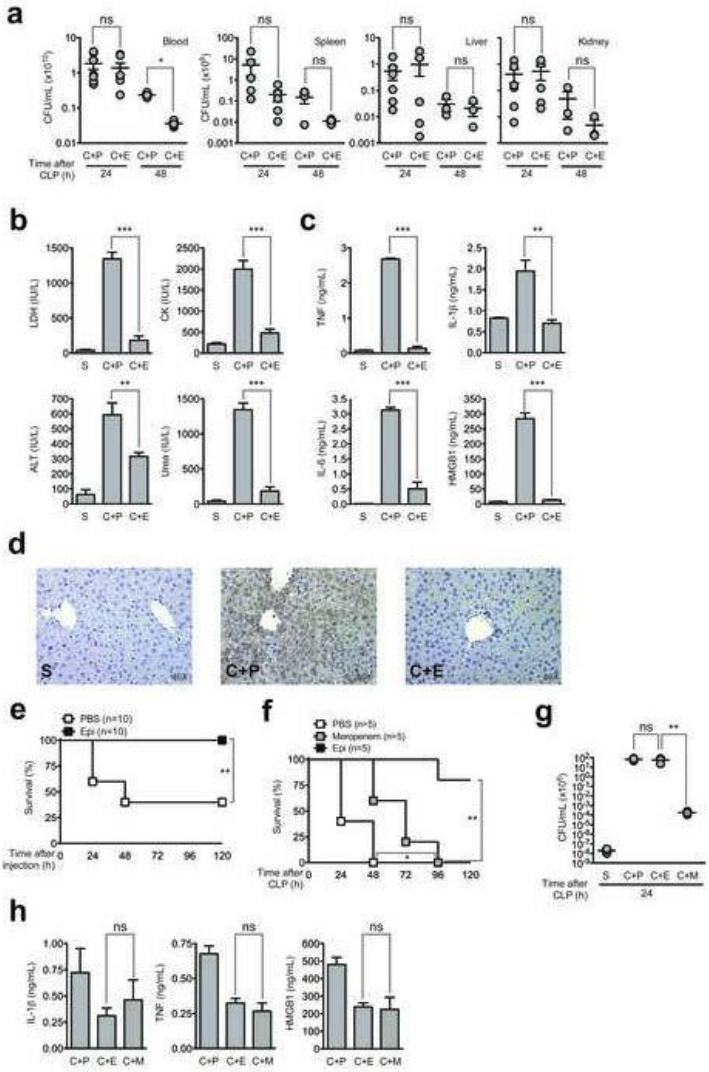

Figure 3

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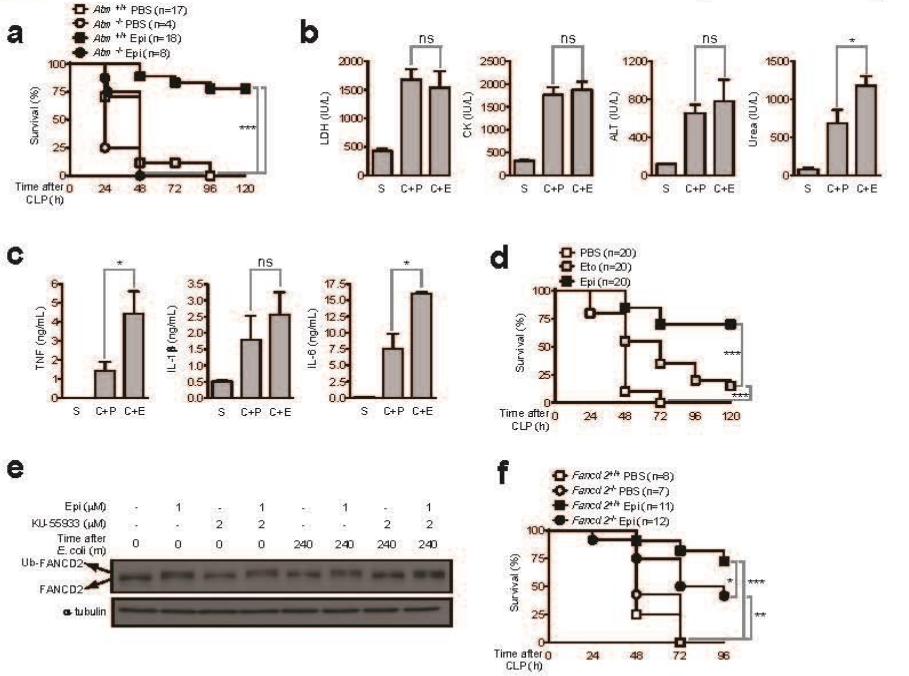

Figure 4

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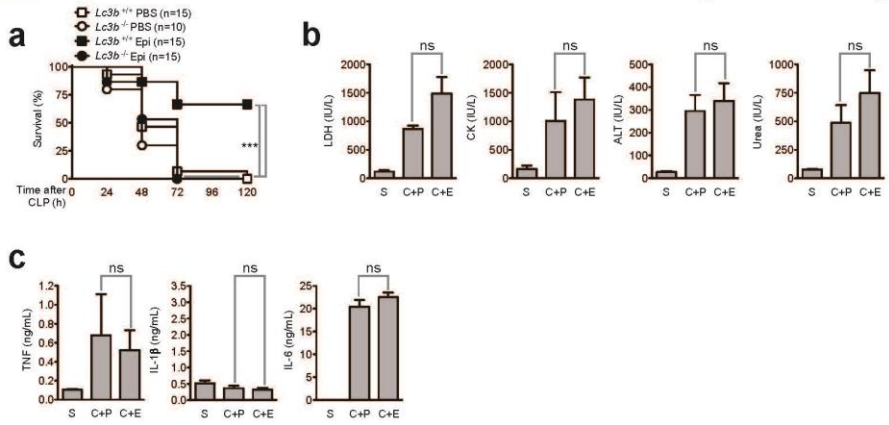

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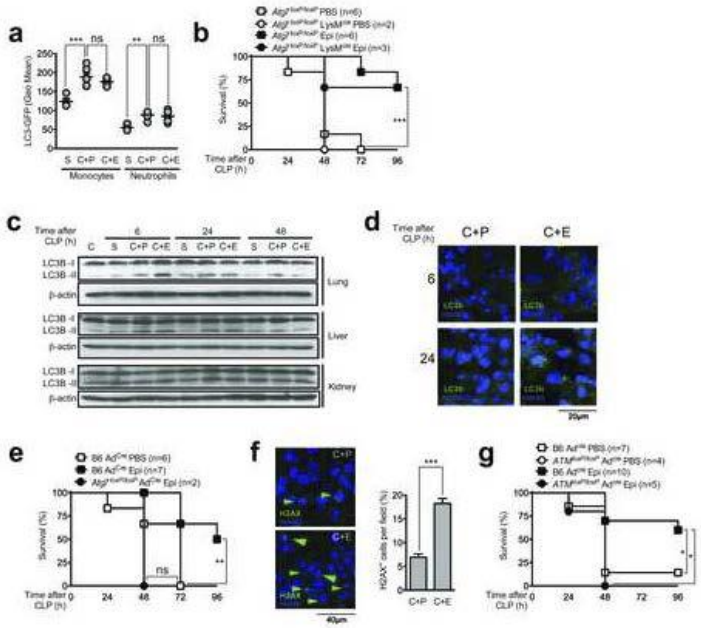

Figure 6

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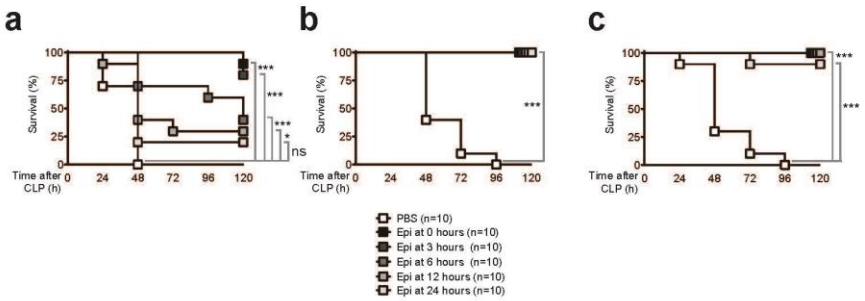

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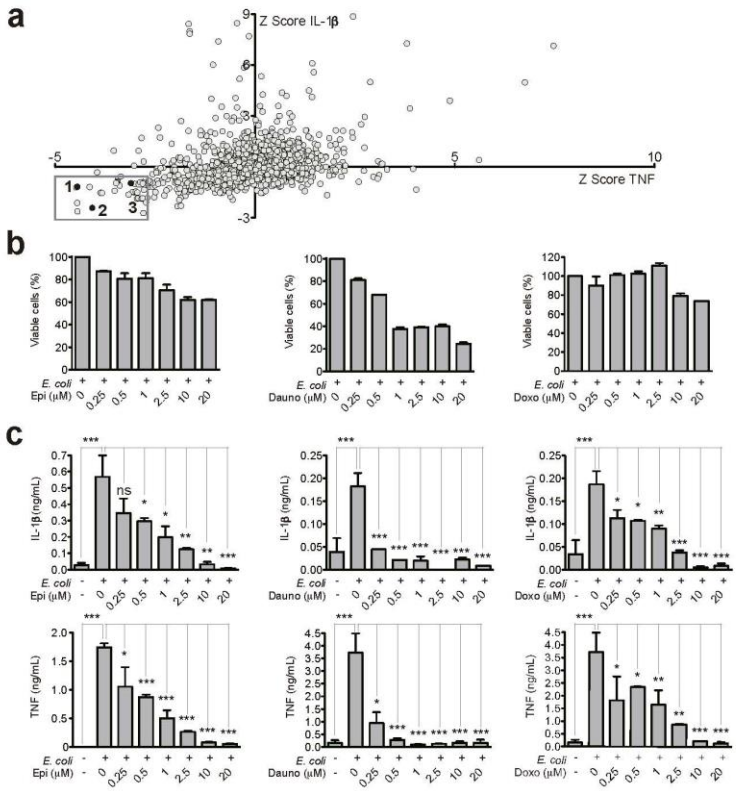
Figueiredo *et al*. Fig S1



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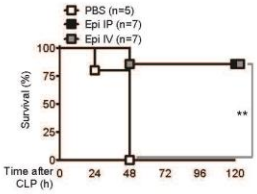

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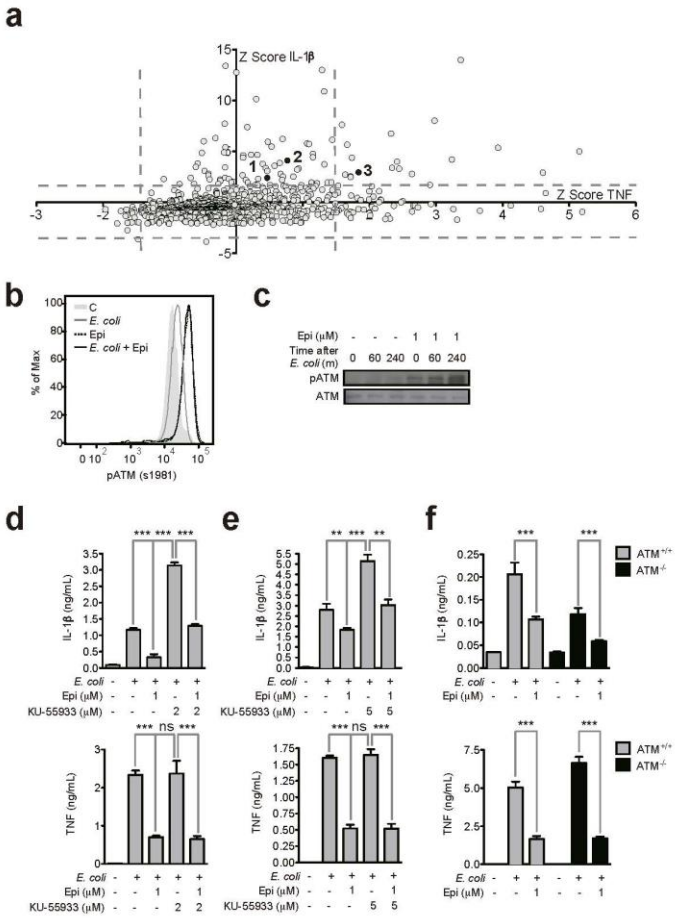

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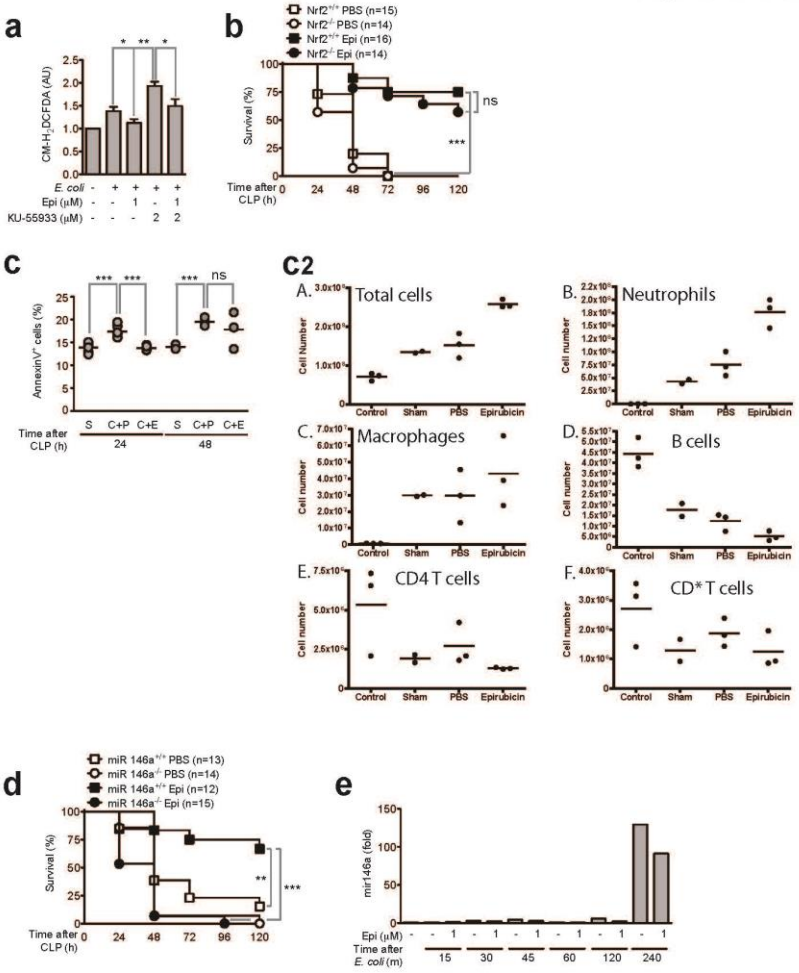

Supplementary Information for Figure S1

Supplementary Experimental Methods for Figure S1 – Chemical Screen

THP-1 human monocytes were plated in 96-well plates at 10^6 cell/ml and incubated with each of the ~2320 compounds included in the Spectrum collection (Microsource Discovery Systems, Gaylordsville, CT) at 10mM for 1 hour. Cells were challenged with 4% PFA-fixed DH5a *E.coli* at a Multiplicity of Infection (MOI) of 20 bacterial cells per THP-1 cell for an additional 24 hours. The cell supernatants were collected and IL-1β and TNF cytokines quantified by DAS-ELISA, using Human IL-1b/IL-1F2 DuoSet® and Human TNF DuoSet® (R&D Systems®), respectively.

Supplementary Information for Figure S3

While epirubicin decreased both IL-1β and TNF secretion in THP-1 cells, only IL-1β, but not TNF, was up-regulated after ATM or ATR silencing (supplementary table II). Similar results were obtained in THP-1 cells using the ATM specific pharmacologic inhibitor KU-55933 (Figure S3d). Similarly, treatment of bone marrow-derived macrophages with epirubicin inhibited IL-1β and TNF secretion (Figure S3e). However, this inhibition was also observed in ATM-deficient bone marrow derived macrophages, suggesting that epirubicin can inhibit IL-1β secretion via a mechanism that is not strictly ATM dependent (Figure S3f).

Supplementary Experimental Methods for Figure S3

The RNAi Consortium Library

Detailed description of the RNAi Consortium (TRC) lentiviral RNAi library used in this study was originally described in (Moffat et al., 2006) (see www.broad.mit.edu/rnai/trc/lib for additional details).

shRNA-based Screen

We generated a working subset of The RNAi Consortium (TRC) shRNA lentiviral vector library (Moffat et al., 2006) that allows for the silencing of most of the genes that are either human kinases or phosphatases. This subset was composed of 1440 individually arrayed lentiviral shRNA vectors targeting ~700 genes, after selecting the most efficient shRNAs (two on average) based on available silencing efficiency data from the Broad Institute of MIT and Harvard. THP-1 cells were plated in 96-well

plates at 10^6 cell/ml and infected with shRNA-expressing lentivirus. 48 hrs later infected cells were selected with puromycin. After the 3 days of selection, plates were duplicated. One of the plates was used to measure the cell number using Alamar Blue® cell viability assay (Invitrogen®). In the other plate, cells were stimulated with 4% PFA-fixed DH5a *E.coli* at a Multiplicity of Infection (MOI) of 20 bacterial cells per THP-1 cell. Twenty-four hours after stimulation, cell supernatants were collected and IL-1β and TNF cytokines quantified by DAS-ELISA. All data values from IL-1b and TNF secretion assays were normalized by dividing the amount of IL-1b and TNF in the conditioned media 24, 12, 8, 6, 4 or 2 hrs after *E. coli* stimulation by the number of cells in each well and then by the average concentration per cell of the plate. Results were logarithmic natural transformed. Scores were sorted in ascending order and graphed. We calculated 1.5 SDEVs above and below the mean to identify the genes that changed IL-1b and TNF secretion when silenced. The same approach was used to identify the compounds that changed IL-1b and TNF secretion. The selected genes were submitted to two or more rounds of phenotypic validation.

Supplementary Information for Figure S4

We explored several additional possible ATM-dependent mechanisms to explain the protective role of epirubicin in sepsis. We found that, *in vitro*, epirubicin is able to counteract the increase in ROS generated by *E. coli* challenge of THP-1 cells in an ATM-dependent manner (Figure S4a). However, mice that are deficient for the nuclear factor (erythroid-derived 2)-like 2 (NRF2), a master regulator of ROS scavenging (Lee et al., 2005), are still protected by epirubicin against mortality due to CLP (Figure S4b). Therefore, epirubicin induces an ATM-dependent ROS scavenging response that is largely dispensable for its protective effect in sepsis.

Increased apoptosis of neutrophils can attenuate sepsis pathogenesis (Garrison et al., 2011). This would be a simple and attractive hypothesis considering that anthracyclines initiate a DDR leading to increased apoptosis if the DNA lesion is too severe for repair (Garrison et al., 2011). However, our data shows that epirubicin treated mice have higher, not lower, numbers of viable neutrophils in the abdomen, excluding an important role for this mechanism (Figure S4c).

The biogenesis of some miRNAs, including miR-146a (a negative regulator of inflammation (Taganov et al., 2007) and a proposed biomarker in sepsis (Wang et al., 2010)), is ATM-dependent (Zhang et al., 2011). We compared the survival of wildtype mice with that of miR-146a –deficient mice in the presence or absence of epirubicin. We conclude that the protection given by this drug is dependent on the presence of miR-146a (Figure S4d). However, our RT-qPCR analysis of miR-146a expression in either RAW cells or THP-1 cells (Figure S4e) does not support a role for epirubicin in the induction of this microRNA. Therefore, direct induction of miR-146a is not the mechanism by which epirubicin protects against the LPS model of septic shock or CLP.

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Effective treatment of rat adjuvant-induced arthritis by celastrol

Cascão R, Vidal B, Raquel H, Neves-Costa A, Figueiredo N, Gupta V, Fonseca JE, Moita LF. **Effective treatment of rat adjuvant-induced arthritis by celastrol**. *Autoimmunity Reviews*. 2012 Oct;11(12):856-62. Epub 2012 Mar 3. PubMed PMID: 22415021.

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Review

Effective treatment of rat adjuvant-induced arthritis by celastrol

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ABSTRACT

We have previously reported an increase in interleukin (IL)-1β and IL-17 levels, and a continuous activation of caspase-1 in early rheumatoid arthritis (RA) patients. These results suggest that drugs targeting IL-1β regulatory pathways, in addition to tumor necrosis factor (TNF), may constitute promising therapeutic agents in early RA. We have recently used a THP-1 macrophage-like cell line to screen 2320 compounds for those that down-regulate both IL-1β and TNF secretion. Celastrol was one of the most promising therapeutic candidates identified in that study. Our main goal in the present work was to investigate whether administration of celastrol is able to attenuate inflammation in a rat model of adjuvant-induced arthritis (AIA). Moreover, since IL-1β is known to play a role in the polarization of Th17 cells, we also investigate whether administration of digoxin, a specific inhibitor of Th17 cells polarization, is able to attenuate inflammation in the same rat model. We found that celastrol administration significantly suppressed joint inflammation. The histological and immunohistochemical evaluation revealed that celastrol-treated rats had a normal joint structure with complete abrogation of the inflammatory infiltrate and cellular proliferation. In contrast, we observed that digoxin administration significantly ameliorated inflammation but only if administrated in the early phase of disease course (after 4 days of disease induction), and it was not efficient at inhibiting the infiltration of immune cells within the joint and in preventing damage. Thus, our results suggest that celastrol has significant anti-inflammatory and anti-proliferative properties and can constitute a potential anti-inflammatory drug with therapeutic efficacy in the treatment of immune-mediated inflammatory diseases such as RA. Furthermore, we find that early inhibition of Th17 cells polarization ameliorates arthritis but it is not as effective as celastrol.

Contents

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1. Introduction

Rheumatoid arthritis (RA) is a chronic systemic immune-mediated inflammatory disease characterized by synovial hyperplasia caused by a large proliferative cellular infiltrate of immune cells, high expression of proinflammatory cytokines and consequent erosion and remodelling of joint cartilage and bone. RA management strategy has been revolutionized in the last decade with the discovery of specific treatments targeted against cytokines, such as tumor necrosis factor (TNF), and immune (B and T) cells. The current treatment goal for RA is to achieve a state of disease remission but, despite all available therapeutic approaches, RA remains an incurable, progressive, debilitating and destructive disease with only 20% of patients reaching remission [1]. Moreover, these novel treatment strategies are only effective in around 70% of the patients, with many of them eventually loosing response to the drugs or being forced to interrupt drug administration due to adverse effects. Anti-TNF treatment has been shown to be more effective when introduced early in the disease course [2,3]. However, still a relatively large proportion of patients fail to respond in these optimal conditions.

We have previously reported increased levels of interleukin (IL)-1β in very recent onset arthritis and in the synovial fluid of established RA patients [4]. This observation may be explained by activation of caspase-1, the pro-inflammatory enzyme which is activated by the inflammasome and which is responsible for the processing of pro-IL-1β, which we have also observed to be increased both in early and established RA patients [4]. Interestingly, the role of the inflammasomes has been recently addressed in the context of RA. In fact, it has been reported that polymorphisms in NLRP3 and CARD8 inflammasome genes are associated with anti-citrullinated protein antibodies (ACPA)-positive RA, an increased susceptibility for the disease and a worse prognosis for these patients [5–7]. Inflammasomes are activated by several different foreign and self-antigens and recent evidences suggest that these multiprotein complexes may participate in the development of the new syndrome termed ASIA, 'Autoimmune (Auto-inflammatory) Syndrome [8], which is induced by adjuvants and assembles a spectrum of immune-mediated diseases triggered by an adjuvant stimulus [9,10].

The importance of IL-1β in the early phase of RA is further highlighted by reports of its ability to promote the differentiation of Th17 cells [11,12] through the induction of the transcription factors IFR4 and RORγt expression [11]. These cells are characterized by the production of IL-17, a cytokine that is also up-regulated in the early phase of RA [13]. Interestingly, IL-17 serum levels and Th17 frequency are decreased in Cryopyrin-associated periodic syndromes (CAPS) patients following *in vivo* IL-1β blockage [14]. Therefore, it is possible that IL-1β plays an important role in early rather than late stages of the disease and that pathways regulating this cytokine and TNF, such as the inflammasome/caspase-1 and NF-kB, can potentially constitute promising combined therapeutic targets. Based on this background and on the results of a recent drug screen performed in our laboratory for compounds that simultaneously inhibit IL-1β and TNF secretion (Figueiredo et al., *unpublished*), we have identified celastrol as a promising therapeutic candidate for arthritis. Celastrol, a pentacyclic-triterpene extract from *Trypterigium wilfordii Hook*, is used in traditional Chinese medicine and was recently shown to possess anti-tumor [15,16] and anti-inflammatory [17] effects. Our aim in this study was to investigate whether celastrol administration is able to attenuate inflammation in a rat model of adjuvant-induced arthritis (AIA) and which mechanisms might be important for its protective effect. Moreover, since IL-1β is known to play a role in the polarization of Th17 cells, we have also analyzed the anti-inflammatory and anti-proliferative properties of digoxin, a specific inhibitor of RORγt transcriptional activity and consequently inhibitor of Th17 cells polarization [18], in the same rat model. We found that celastrol, in contrast to digoxin, has significant anti-inflammatory and anti-proliferative properties and can putatively constitute an anti-inflammatory drug with therapeutic efficacy in the treatment of immune-mediated inflammatory diseases such as RA.

2. Methods

2.1. Compounds

Celastrol and digoxin were purchased from Sigma (Missouri, USA).

2.2. IL-1β and TNF secretion assay

THP-1 cells were stimulated with 4% PFA-fixed DH5 *Escherichia coli* (*E. coli*) at a Multiplicity of Infection (MOI) of 20 bacterial cells per THP-1 cell, 1 hour after incubation with celastrol. Cell supernatants were collected and IL-1β and TNF cytokines quantified by enzyme linked immunosorbent assay (ELISA) technique (R&D systems, Minnesota, USA) according to the provider's instructions.

2.3. Cell culture

THP-1 (ATCC TIB-202) macrophage-like cell line and THP-1/NF-kB reporter cell line were cultured in R10 - RPMI media 1640 supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin-streptomycin, 1% (v/v) pyruvate, 1% (v/v) L-glutamine, 1% (v/v) non-essential aminoacids, 1% (v/v) hepes buffer and 2-mercaptoethanol to a final concentration of 0.05 M, as recommended by the American Tissue Culture Collection (ATCC). Cells were cultured at 250.000 cells/mL, incubated with 10 μM of celastrol for 1 h at 37 °C 5% CO_2, and then stimulated with PFA-fixed *E. coli* (20 *E. coli* per cell) for 8 h and 24 h at 37 °C 5% CO_2. Simultaneously, non-stimulated negative control cells were also cultured at the same density as the stimulated population for comparison. Caspase-1 activity was measured in THP-1 macrophage-like cell line using the Carboxyfluorescein FLICA Detection kit for Caspase Assay (Immunochemistry Technologies, LLC, Minnesota, USA) following the reagent instructions. Briefly, cells from the different assays were protected from light exposure while incubated for 1 hour at 37 °C with 30X FLICA solution at a 1:30 ratio.

NF-kB activity was measured in THP-1/NF-kB reporter cell line. Lentiviral particles carrying a NF-kB-responsive GFP-expressing reporter gene (Cignal Lenti Reporters, SABiosciences, Maryland, USA) were used to infect THP-1 cells and to establish a stable cell line. All samples were analyzed by flow cytometry using a FACS Calibur (BD biosciences, New Jersey, USA). The data collected were further analyzed using FlowJo software (Tree Star Inc, Oregon, USA).

2.4. Animal experimental design

Wistar AIA rats were purchased from Charles River Laboratories International (Massachusetts, USA). Female Wistar AIA rats weighing 125–150 g were maintained under specific pathogen free (SPF) conditions and all experiments were approved by the Animal User and Ethical Committees at the Instituto de Medicina Molecular, according to the Portuguese law and the European recommendations. Celastrol and digoxin were administrated at a dose of 1 μg/g and 2 μg/g body weight every day, respectively [18,19]. Drugs and vehicle control were dissolved in normal saline solution and injected intraperitoneally to AIA rats (N = 5–10 animals per group) after 4 days (early treatment group) and after 11 days (late treatment group) of disease induction, when arthritis was already present. The inflammatory score, ankle perimeter and body weight were measured during the period of treatment. Inflammatory signs were evaluated by counting the score of each joint in a scale of 0–3 (0 — absence; 1 — erythema; 2 — erythema and swelling; 3 — deformities and functional impairment) [20]. The total score of each animal was defined as the sum of the partial scores of each affected joint. Rats were sacrificed after 19 days of disease evolution and paw samples were collected for histological and immunohistochemical evaluation.

2.5. Histological and immunohistochemical evaluation

For histopathological observation, paw, lung, liver, kidney and pancreas samples were collected at the time of sacrifice. Samples were fixed immediately in 10% neutral buffered formalin solution and then dehydrated with increasing ethanol concentrations (70%, 96% and 100%). Paw samples, after being fixed, were also decalcified in 10% formic acid. Samples were next embedded in paraffin, sectioned and stained with hematoxylin and eosin for morphological examination. Paws were also used for immunohistological staining with Ki67 antibody, a cellular proliferation marker. Tissue sections were incubated with primary antibody against rat polyclonal Ki67 (Abcam, Cambridge, UK) and with EnVision + (Dako, Glostrup, Denmark). Colour was developed in solution containing diaminobenzadine-tetrahydrochloride (Sigma, Missouri, USA), 0.5% H_2O_2 in phosphate-buffered saline buffer (pH 7.6). Slides were counterstained with hematoxylin and mounted. All images were acquired using a Leica DM 2500 (Leica microsystems, Wetzlar, Germany) microscope equipped with a colour camera. Data regarding the degree of proliferation of synovial cells was scored from 0–3 (0 — fewer than three layers; 1 — three to four layers; 2 — five to six layers; 3 — more than six layers). Lymphoid cell infiltration was scored from 0–3 (0 — none to diffuse infiltration; 1 — lymphoid cell aggregate; 2 — lymphoid follicles; 3 — lymphoid follicles with germinal center formation) [21].

2.6. Intracellular IL-17 staining

Spleen cells were cultured in complete cell culture media at 37 °C 5% CO_2. Cells were stimulated for 3 h with brefeldin A (0.01 mg/ml) (Epicenter Technologies, Nebraska, USA), phorbol 12-myristate 13-acetate (PMA) (50 ng/ml) (Sigma, Missouri, USA) and ionomycin (500 ng/ml) (Calbiochem, Darmstadt, Germany). Next, cells were permeabilised using saponin (Sigma, Missouri, USA) and stained with anti-IL17 FITC (Biolegend, California, USA) to detect intracellular IL-17. Lastly, cells were acquired with a FACS LSR Fortessa (BD biosciences, New Jersey, USA) and data collected were further analyzed using FlowJo software (Tree Star Inc, Oregon, USA).

2.7. Statistical analysis

Statistical differences were determined with non-parametric Kruskal-Wallis and Mann–Whitney tests using GraphPad Prism (GraphPad, California, USA). Differences were considered statistically significant for $p<0.05$.

3. Results

3.1. Celastrol decreases IL-1β and TNF secretion

Based on the hypothesis that drugs which block the secretion of both IL-1β and TNF might be particularly effective at decreasing early disease activity in RA, we have recently used the human THP-1 macrophage-like cell line to screen for drugs that can simultaneously down-regulate the secretion of both cytokines. Among the 2320 tested drugs included in the Spectrum collection (Microsource Discovery Systems, Connecticut, USA), we found 45 that significantly decrease the levels of both IL-1β and TNF secretion (Figueiredo et al., *unpublished*). We further narrowed the selection by taking into account the possible human tolerability to chronic exposure and other biological properties that could be of interest in the context of RA. Celastrol was thus selected for testing in a rat model of adjuvant-induced arthritis (AIA), due to its prior human use in traditional Chinese medicine and due to its concomitant alleged anti-proliferative effects [15,16]. We started by validating the effect of celastrol in the inhibition of IL-1β and TNF secretion in a human THP-1 macrophage-like cell line, by using increasing concentrations of celastrol for 1 hour before challenging them with PFA-fixed *E. coli* for 6 hours. The conditioned media was then probed for the secretion of either IL-1β or TNF using ELISA technique. Celastrol was very effective at inhibiting the secretion of both cytokines over a wide range of tested concentrations (Fig. 1A).

3.2. Celastrol inhibits the activation of NF-kB and caspase-1

The sequence of events culminating in IL-1β secretion is complex, but it can be summarized in two steps: induction of pro-IL-1β and its processing by activated caspase-1 (reviewed in [22]). Both pro-IL-1β and TNF depend on NF-kB activation for the transcription of their respective mRNAs. We therefore tested the effect of celastrol on these key pathways. To investigate its effect in the activation of NF-kB, we used an NF-kB reporter cell line made by stably infecting THP-1 cells with a commercial lentiviral GFP reporter under the control of a minimal CMV promoter and tandem repeats of the NF-kB transcriptional response element (TRE). We found that celastrol was able to suppress NF-kB reporter activation upon *E. coli* stimulation in comparison with cells that were also stimulated but did not receive treatment (Fig. 1B). To test the effect of this drug in caspase-1 processing and activation we used a caspase-1 fluorescent substrate and measured the relative active caspase-1 levels using FACS. Also in this case, celastrol administration decreased the activation of caspase-1 (Fig. 1B). We can thus conclude that celastrol inhibits NF-kB and caspase-1 activation.

3.3. Celastrol is able to suppress inflammation in Wistar rat adjuvant-induced arthritis

To study the anti-inflammatory properties of celastrol *in vivo*, AIA rats were treated daily with this drug after the disease had already become symptomatic. We started the treatment after 4 days of disease induction (early treatment group) and after 11 days of disease induction (late treatment group). The inflammatory score and ankle

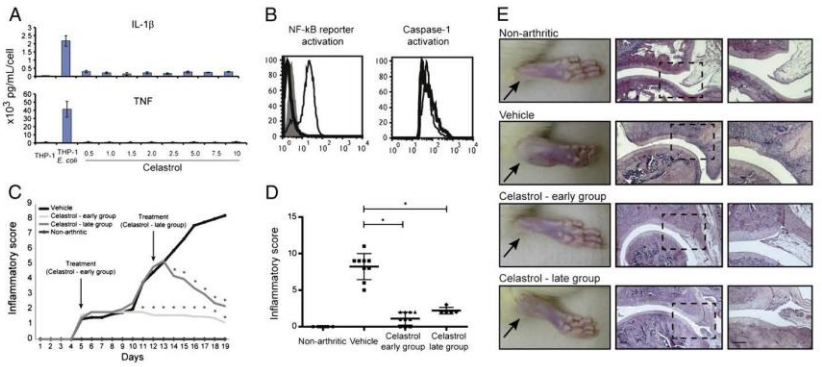

Fig. 1. (A) *IL-1β and TNF secretion are inhibited by celastrol treatment.* Conditioned media samples from human THP-1 macrophage-like cell line cultured with growing concentrations of celastrol were analyzed by ELISA technique. Differences were considered statistically significant for p values<0.05. (B) *The activation of NF-kB reporter and caspase-1 is decreased with celastrol treatment.* NF-kB expression was measured by flow cytometry in a THP-1/NF-kB reporter cell line incubated with celastrol and then stimulated for 24 h with *E. coli*. Each thin line in the histogram corresponds to untreated but *E. coli* stimulated cells, the shaded area corresponds to drug-treated and *E. coli* stimulated cells and the thick line corresponds to untreated non-stimulated cells, used as a control. Caspase-1 activation was measured using flow cytometry in a THP-1 cell line incubated with celastrol and then stimulated for 8 h with *E. coli*. Each thin line in the histogram corresponds to untreated but *E. coli* stimulated cells used as control and the thick line corresponds to drug-treated and *E. coli* stimulated cells. (C) *Celastrol is able to suppress inflammation throughout time.* Notice that after 6 days of treatment the vehicle injected group increased the inflammatory manifestations sharply, whereas in celastrol-treated rats there was minimal inflammatory activity or even complete abrogation of arthritis manifestations. Arrows indicate the beginning of treatment after 4 and 11 days of disease induction. Differences were considered statistically significant for p values<0.05. (D) *Celastrol possesses anti-inflammatory properties.* Inflammation score in celastrol-treated AIA rats is maintained significantly diminished in comparison with vehicle-treated rats after treatment. Differences were considered statistically significant for p values<0.05. (E) *Histological evaluation of joints after celastrol treatment.* Notice that celastrol has completely prevented immune cellular infiltration and bone and cartilage invasion, allowing for a normal joint structure comparable to non-arthritic rats in both early and late treatment groups. Magnification 50× and 100×. Bars: 100 μm.

perimeter were evaluated during the period of treatment. As shown in Fig. 1C, all animals already presented arthritis by the fourth day of disease induction, which corresponds to the first day of treatment. After 6 days of treatment the vehicle-injected group increased the inflammatory manifestations sharply, while in early celastrol-treated rats there was minimal inflammatory activity or even complete abrogation of arthritis manifestations. In the late treatment group, drug administration was started after 11 days of disease evolution, when animals presented a mean inflammatory score of 5. Also in this group, by the second day of treatment with celastrol the inflammatory manifestations started to significantly decrease over time. This result shows that this drug has anti-inflammatory effects even when administrated in a later phase of arthritis. After 15 (early treatment group) and 8 (late treatment group) days of treatment, celastrol showed significant anti-inflammatory effects, as assessed by the evaluation of the inflammatory score shown in Fig. 1D and also by the evaluation of ankle perimeter ($p=0.007$ in early and late treatment groups vs. untreated animals).

3.4. Celastrol prevents joint immune cells infiltration and proliferation as well as cartilage and bone erosions

To evaluate the infiltration of immune cells within joints of AIA rats, joint tissue sections stained with hematoxylin and eosin were performed. The histological evaluation shown in Fig. 1E revealed that rats treated with celastrol had a normal joint structure with complete abrogation of the inflammatory infiltrate ($p<0.0001$ in early and $p=0.006$ in late treatment group vs. untreated animals). We also studied cell proliferation by staining joint tissue sections with Ki67. The immunohistochemical results revealed that rats treated with celastrol presented a reduced level of immune cells proliferation in the early treatment group ($p=0.0009$ vs. untreated animals). The late treatment group showed a less effective proliferation decrease ($p=0.046$ vs. untreated animals), even though the drug successfully diminished the inflammatory score. Early and late treatment with celastrol prevented cartilage and bone damage (Fig. 1E). We have not observed significant differences in body weight or any other side effects in treated rats, as revealed during autopsy and histological analysis made in lung, liver, kidney and pancreas (*data not shown*).

3.5. Digoxin delayed the course and reduced the severity of arthritis

To study the *in vivo* anti-inflammatory properties of digoxin, AIA rats were treated daily with this drug using the same experimental setup used for celastrol. As shown in Fig. 2A, after 11 days of treatment, we observed a delay in the course of arthritis in the early digoxin-treated rats, with a reduction in the severity of inflammatory signs in comparison with the vehicle-injected group. After 19 days of disease induction, digoxin showed significant anti-inflammatory effects, as assessed by the evaluation of the inflammatory score (Fig. 2B) and also by the evaluation of ankle perimeter ($p=0.007$ in early vs. untreated animals). In the late treatment group, there were no statistically significant differences in the inflammatory score when compared with the vehicle-treated rats (Fig. 2B). This result suggests that digoxin has anti-inflammatory effects only when administrated in the early phase of arthritis. Additionally, we observed that the percentage of IL-17-producing T cells in digoxin-treated rats was reduced as compared to vehicle-treated rats ($p=0.0286$ in early and $p=0.0286$ in late treatment group vs. untreated animals).

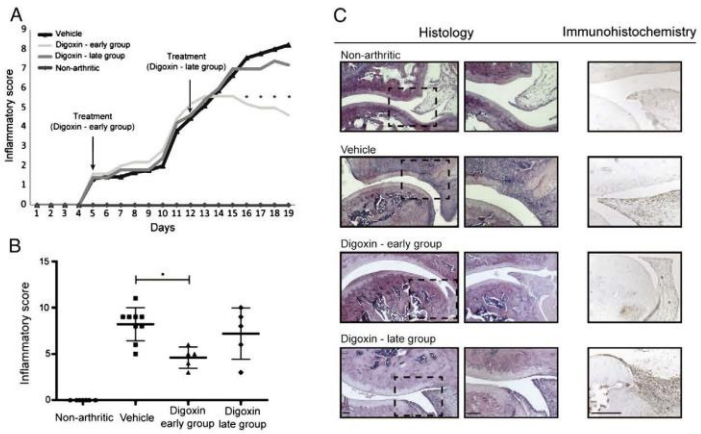

Fig. 2. (A) *Digoxin is able to reduce the severity of inflammation throughout time.* After 11 days of treatment, the digoxin-injected group started to progressively reduce inflammatory manifestations. Arrow indicates the beginning of treatment after 4 and 11 days of disease induction. (B) *Digoxin possesses anti-inflammatory properties.* Inflammation score in early digoxin-treated AIA rats is significantly diminished in comparison with vehicle-treated rats after treatment. Of note, when treatment begins in the later phase of inflammation it has no effect in reducing the inflammatory process. Differences were considered statistically significant for p values <0.05. (C) *Histological and immunohistochemical evaluation of joints after 15 days of treatment.* Notice that digoxin reduced immune cellular proliferation only if treatment administration started in the early phase of arthritis but had no effect in immune cell infiltration within joints. Magnification 50× and 100× in histological images and a magnification 200× in immunohistochemical images. Bars: 100 μm.

3.6. Digoxin prevents proliferation but not infiltration of immune cells within joints

The histological evaluation shown in Fig. 2C revealed that digoxin was not able to suppress the infiltration of immune cells within joints ($p=0.1201$ in early and $p=0.3475$ in late treatment group vs. untreated animals). Furthermore, the immunohistochemical results revealed that rats treated with digoxin presented a reduced level of immune cell proliferation in the early treatment group ($p=0.0042$ vs. untreated animals), in contrast with the late treatment group which showed no effect in immune cell proliferation ($p=0.4100$ vs. untreated animals). Minimal cartilage and bone damage was present both in early and late digoxin treated animals (Fig. 2C). Also in the case of digoxin, we have not observed significant differences in body weight or any other side effects in treated rats, as revealed during autopsy and histological results (*data not shown*).

4. Discussion

In the present study, we demonstrated that AIA can be effectively treated through a possible inhibitory effect over IL-1β and TNF secretion induced by celastrol. The effect of this compound was profound as it induced a complete abrogation of joint immune cellular infiltration and proliferation, preventing cartilage and bone damage.

Celastrol is a novel compound that has been shown to inhibit cancer progression and NF-kB activity [15,16,23]. Our results reveal that the anti-inflammatory properties of this drug might not only be related with its ability to inhibit the activation of NF-kB but also with its capacity to inhibit caspase-1 activation. Celastrol has also been reported to abrogate the release of IL-1β in LPS-stimulated human peripheral mononuclear cells [24] and to exert anti-inflammatory properties in animal models [17,25]. Interestingly, Pinna et al. described that celastrol inhibited pro-inflammatory cytokine secretion from mucosal inflammatory biopsies from Crohn's patients, possibly due to the abrogation of cytokine gene transcription [26]. Of note, tripterine isolated from *Tripterygium wilfordii Hook F* has previously been shown to be effective on adjuvant [27] and collagen-induced arthritis [25] in rats, supporting the 2002 report showing that an ethanol/ethyl acetate extract of *Tripterygium wilfordii Hook F* shows therapeutic benefit in patients with refractory RA [28]. Furthermore, in an *in vivo* model of metastatic bone disease associated with breast cancer, celastrol inhibited bone resorption, consistent with the inhibitory effect on osteoclast formation and survival observed in *in vitro* experiments [19]. Of interest, these data support our findings that celastrol suppresses synovial immune cells infiltration and proliferation, preventing bone erosions. Importantly, celastrol treatment is effective when administrated both in the early and more established phase of arthritis which is relevant for the possible clinical implications of our findings. In RA the infiltration of immune cells and the proliferation of joint lining synovial fibroblasts lead to the formation of the tumor-like pannus tissue, which invades and destroys joint cartilage and bone. The cell proliferation inhibitory effect of celastrol may thus prove to be of interest to prevent and treat this complication of established RA.

Additionally, we also found that digoxin is able to ameliorate inflammatory signs in the same AIA rat model of arthritis. This is in agreement with the recent report from Huh et al. in which it was shown that digoxin was able to delay the onset and reduce disease severity in an experimental autoimmune encephalomyelitis (EAE) mice model, through the inhibition of RORγt transcriptional activity and, consequently, of Th17 cells differentiation [18]. However, despite our observation that digoxin was able to suppress the severity of inflammatory signs, we also found that it was not able to efficiently reduce the infiltration of immune cells within the joints. Importantly, we observed that digoxin treatment was only effective if the drug was administrated in the early phase of arthritis development, in contrast to what we found in the case of celastrol. In fact, blocking IL-1β and

TNF simultaneously results in a significant inhibitory effect in arthritis progression and severity, even when administrated in a later phase of disease course, with a complete abrogation of the inflammatory score, infiltration and proliferation of immune cells within joints and prevention of structural damage. In contrast, we observed that digoxin had a slower and less efficient effect on disease progression. These data indicate that IL-1β, in the context of arthritis, might play a role independent from Th17/IL-17. Besides inducing Th17 cell polarization, IL-1β also directly stimulates the influx of neutrophils and macrophages into the damaged site. These cells in turn can destroy the tissue by the release of proteases and reactive oxygen species [29], and also by the formation of osteoclasts [30] leading to tissue damage and consequent functional disability characteristic of RA patients. IL-1β, together with IL-6 and TNF, also has a potent capacity to induce the receptor activator of nuclear factor kappa-B ligand (RANKL) expression on synovial fibroblasts/osteoblasts and to facilitate RANK signaling, thus directly contributing to the bone destruction process. In contrast, IL-17 seems to have a more limited effect on inflammatory cell influx and consequent inflammatory symptoms, as we observed in this study. Moreover, in agreement with this fact, a recent phase II study testing an anti-IL-17A drug in RA did not achieve its primary end point [31]. This does not preclude the important involvement of Th17 cells in driving the innate immune inflammation towards the adaptive (auto)immune chronic inflammation in RA, involving several other cytokines apart from IL-17A [32].

Despite this apparent crucial role of IL-1β signaling in RA, clinical benefits after IL-1β inhibition have been modest compared to anti-TNF drugs, at least in moderate to severe long established RA. Further, in 2004 a study which tested the efficacy of combination therapy using anakinra and etanercept in 244 long-standing and very active RA patients who have been treated unsuccessfully with MTX showed that concomitant IL-1β and TNF inhibition provides no added benefit and increased infections as compared to etanercept alone [33]. Possibly, in the context of RA inhibiting the IL-1β pathway at the receptor level is not an effective strategy but an upstream inhibition might work better, at least, in animal models. On top of that, downregulating TNF and IL-1β production might be safer than inhibiting completely TNF and IL-1β.

5. Conclusions

In conclusion, celastrol can putatively constitute an anti-inflammatory and anti-proliferative drug with therapeutic efficacy in the treatment of immune-mediated inflammatory diseases such as RA, possibly through the down-regulation of caspase-1 and inhibition of NF-kB activation (Fig. 3). Its anti-proliferative effects might be of additional value in RA to counteract the formation of the characteristic pannus leading to the destruction of cartilage and bone. In addition, we have shown that despite the ability to decrease the severity of early inflammatory signs in AIA, digoxin is not effective in reducing the infiltration of immune cells within the joints. We thus suggest that the isolated inhibition of Th17 polarization might be a strategy with limited efficacy, at least in established RA. Further animal experimentation is required to determine the real efficacy and safety of celastrol for arthritis treatment but these results suggest that it might be worth of entering into phase I clinical trials. Simultaneously, this study highlights the need for more research on the role of the inflammasome/caspase-1 and NF-kB pathways in the etiopathology of RA.

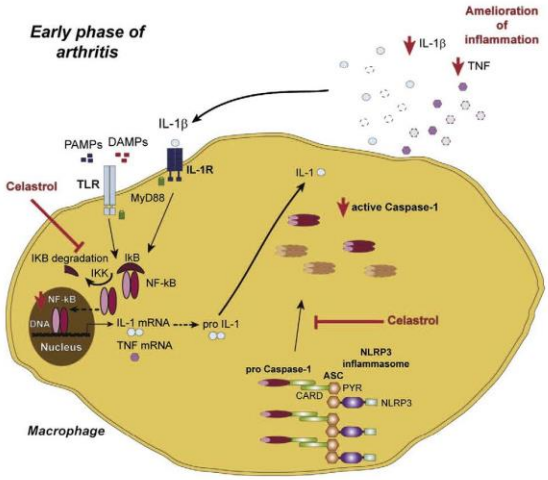

Fig. 3. Proposed mechanism for celastrol anti-inflammatory effects. Celastrol showed anti-inflammatory and anti-proliferative properties *in vivo*, promoting a complete suppression of arthritis development and abrogation of joint immune cellular infiltration and proliferation, preventing cartilage and bone damage. This compound induced a down-regulation of caspase-1 and NF-kB activation and, consequently, lead to a decrease in IL-1β and TNF secretion. ASC — adaptor molecule apoptosis associated speck-like protein containing a caspase recruitment domain, CARD — caspase recruitment domain, DAMP — danger associated molecular pattern, IL — interleukin, IL-1R — IL-1 receptor, NF-kB — nuclear factor kappa-light-chain-enhancer of activated B cells, NLRP — NOD-like receptor protein, PAMP — pathogen associated molecular pattern, PYR — pyrin domain, TLR — toll-like receptor, TNF — tumor necrosis factor.

List of abbreviations

RA	rheumatoid arthritis
TNF	tumor necrosis factor
IL	interleukin
ACPA	anti-citrullinated protein antibodies
AIA	adjuvant-induced arthritis
CAPS	cryopyrin-associated periodic syndromes
MOI	multiplicity of infection
ELISA	enzyme linked immunosorbent assay
SPF	specific pathogen free
TRE	transcriptional response element
EAE	experimental autoimmune encephalomyelitis
RANKL	receptor activator of nuclear factor kappa-B ligand

Conflict of interests

The authors declare that they have no competing interests.

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Take-home messages

- Celastrol has significant anti-inflammatory and anti-proliferative properties.
- Digoxin is not as effective as celastrol treatment for AIA.
- Blocking IL-1β and TNF (upstream to their receptors) is effective in arthritis.
- Therapies targeting IL-1β pathway should be re-evaluated in RA patients.

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Gambogic acid is a potent anti-inflammatory and anti-proliferative drug in a rat model of antigen-induced arthritis

Manuscript in preparation.

PLoS ONE

GAMBOGIC ACID IS A POTENT ANTI-INFLAMMATORY AND ANTI-PROLIFERATIVE DRUG IN A RAT MODEL OF ANTIGEN-INDUCED ARTHRITIS

--Manuscript Draft--

Manuscript Number:	
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Full Title:	GAMBOGIC ACID IS A POTENT ANTI-INFLAMMATORY AND ANTI-PROLIFERATIVE DRUG IN A RAT MODEL OF ANTIGEN-INDUCED ARTHRITIS
Short Title:	GAMBOGIC ACID IS A POTENT ANTI-INFLAMMATORY DRUG
Corresponding Author:	Rita Cascao Instituto de Medicina Molecular Lisbon, PORTUGAL
Keywords:	Gambogic acid; Wistar AIA rat model; IL-1beta; caspase-1; NF-kB.
Abstract:	Background: In a previous study, we have reported a continuous activation of caspase-1 in rheumatoid arthritis (RA) patients in the early phase of the disease, as well as increased levels of interleukin (IL)-1beta. These observations raised the hypothesis that drugs targeting IL-1beta regulatory pathways, as well as tumor necrosis factor (TNF), may be particularly effective for the treatment of early RA. We have recently identified gambogic acid as one of the most promising therapeutic candidates to simultaneously block the secretion of IL-1beta and TNF after screening ~2320 compounds, using a THP1 macrophage-like cell line challenged with E. coli. Our main goal here is to investigate whether administration of gambogic acid is able to attenuate inflammation in a rat model of antigen-induced arthritis (AIA). Methodology/Principle Findings: Gambogic acid was administered to AIA rats in the early phase of arthritis (4 days after disease induction) for a period of 15 days. The inflammatory score, paw perimeter and body weight were evaluated during the time of treatment. Rats were sacrificed after 19 days of disease progression and paw samples were collected for histological and immunohistochemical evaluation. We found that inflammation in joints was significantly suppressed with administration of gambogic acid. Histological and immunohistochemical evaluation of treated rats revealed normal joint structures with complete abrogation of the inflammatory infiltrate and cellular proliferation. Conclusions/Significance: Our results suggest that gambogic acid has significant anti-inflammatory properties and can putatively constitute an anti-inflammatory drug with therapeutic efficacy in the treatment of inflammatory diseases such as RA, possibly through the down-regulation of IL-1beta and TNF secretion.
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Cover Letter

GAMBOGIC ACID IS A POTENT ANTI-INFLAMMATORY AND ANTI-PROLIFERATIVE DRUG IN A RAT MODEL OF ANTIGEN-INDUCED ARTHRITIS

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The authors declare that the present research article "GAMBOGIC ACID IS A POTENT ANTI-INFLAMMATORY AND ANTI-PROLIFERATIVE DRUG IN A RAT MODEL OF ANTIGEN-INDUCED ARTHRITIS" has been seen and approved by all the authors, that they have given necessary attention to ensure the integrity of the work and there is no conflict of interest. To the best of our knowledge, this study constitutes the first analysis of the anti-inflammatory and anti-proliferative effect of gambogic acid in Wistar AIA rat model. We would like to propose as Academic Editor to consider our submission Prof.

Collin Stultz. We are submitting this manuscript to Plos One because we consider that its content can contribute to the rationale of future new early targeted therapies.

We hope that you find this paper suitable for publication in your journal.

Yours sincerely,

João Eurico Fonseca and Luis Ferreira Moita

GAMBOGIC ACID IS A POTENT ANTI-INFLAMMATORY AND ANTI-PROLIFERATIVE DRUG IN A RAT MODEL OF ANTIGEN-INDUCED ARTHRITIS

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ABSTRACT

Background: In a previous study, we have reported a continuous activation of caspase-1 in rheumatoid arthritis (RA) patients in the early phase of the disease, as well as increased levels of interleukin (IL)-1β. These observations raised the hypothesis that drugs targeting IL-1β regulatory pathways, as well as tumor necrosis factor (TNF), may be particularly effective for the treatment of early RA. We have recently identified gambogic acid as one of the most promising therapeutic candidates to simultaneously block the secretion of IL-1β and TNF after screening ~2320 compounds, using a THP1 macrophage-like cell line challenged with *E. coli*. Our main goal here is to investigate whether administration of gambogic acid is able to attenuate inflammation in a rat model of antigen-induced arthritis (AIA). **Methodology/Principle Findings:** Gambogic acid was administered to AIA rats in the early phase of arthritis (4 days after disease induction) for a period of 15 days. The inflammatory score, paw perimeter and body weight were evaluated during the time of treatment. Rats were sacrificed after 19 days of disease progression and paw samples were collected for histological and immunohistochemical evaluation. We found that inflammation in joints was significantly suppressed with administration of gambogic acid. Histological and immunohistochemical evaluation of treated rats revealed normal joint structures with complete abrogation of the inflammatory infiltrate and cellular proliferation. **Conclusions/Significance:** Our results suggest that gambogic acid has significant anti-inflammatory properties and can putatively constitute an anti-inflammatory drug with therapeutic efficacy in the treatment of inflammatory diseases such as RA, possibly through the down-regulation of IL-1β and TNF secretion.

INTRODUCTION

Rheumatoid arthritis (RA), which afflicts about 1% of the world population, is the most common of the inflammatory joint diseases. This disease can have a very aggressive course and poor outcome as inferred by the analysis of its social impact (after 10 years, more than 50% of the RA patients are too impaired to perform professional activities) [1] and life expectancy diminishes 10 years due to disease activity and associated comorbidities [2]. RA is a chronic systemic inflammatory disease characterized by synovial hyperplasia caused by a large proliferative cellular infiltrate of leukocytes, high expression levels of proinflammatory cytokines and consequent erosion of joint cartilage and bone. The therapeutic approach of RA has been revolutionized in the last decade with the discovery of specific targeted treatments. However, despite all available therapeutic options, RA remains a progressive, destructive and debilitating disease with only 20% of patients reaching remission [3]. Anakinra, an antagonist of interleukin (IL)-1, was approved for RA treatment in the last decade. However, the real impact on disease activity has been shown in practice to be lower than what was anticipated from clinical trial results, casting doubts on the role of IL-1β as a therapeutic target [4]. Nonetheless, we have previously reported increased levels of IL-1β in very recent onset arthritis and in the synovial fluid of established RA patients [5]. This observation could be explained by the activation of caspase-1 that we also have observed both in early and established RA patients [6]. Therefore, it is possible that IL-1β plays an important role in early rather than late stages of the disease and that pathways regulating this cytokine, such as caspase-1 and NF-kB activation, can potentially constitute promising therapeutic targets for specific drugs. The effect might be further boosted if an inhibitory effect on tumor necrosis factor (TNF) can also be achieved. Based on the

results of a recent drug screen for compounds that simultaneously inhibit IL-1β and TNF secretion (Figueiredo et al., *unpublished*), we chose gambogic acid as a promising therapeutic candidate for the treatment of arthritis. Gambogic acid is a polyprenylated xanthone abundant in resin derived from *Garcinia hanburyi* and *G. Morella* and is used in Southeast Asia complementary and alternative medicine [7]. Recent studies showed that gambogic acid could inhibit the growth of a wide range of tumor cells [8]. Our aim in this study is to investigate whether gambogic acid administration is able to attenuate inflammation in a rat model of antigen-induced arthritis (AIA).

METHODS

Ethics statement

All experiments were approved by the Animal User and Ethical Committees at the Instituto de Medicina Molecular, according to the Portuguese law and the European recommendations.

Compounds

Gambogic acid was purchased from Santa Cruz Biotechnology (Santa Cruz, USA).

IL-1β and TNF quantification

THP-1 cells were stimulated with 4% PFA-fixed DH5 *Escherichia coli* (*E. coli*) at a Multiplicity of Infection (MOI) of 20 bacterial cells per THP-1 cell, 1 hour after incubation with gambogic acid. Cell supernatants were collected and IL-1β and TNF cytokines quantified by enzyme linked immunosorbent assay (ELISA) (R&D systems, USA) according to the provider's instructions.

AIA rat model and assessment of arthritis

Female Wistar AIA rats were purchased from Charles River Laboratories International (Massachusetts, USA) and maintained under specific pathogen free (SPF) conditions. Gambogic acid was administrated at a dose of 4μg/g body weight every day. Drug and vehicle control were dissolved in normal saline solution and injected intraperitoneally to AIA rats (N=5-10 animals per group) after 4 days of disease induction, when arthritis was already present, and then for a period of 15 days. The inflammatory score, paw perimeter and body weight were measured during the time of treatment. Inflammatory signs were evaluated trough the counting of the score of each joint in a scale of 0-3 (0 – absence; 1 – erythema; 2 – erythema and swelling; 3 – deformities and functional impairment). The total score of each animal was defined as the sum of the partial scores of each affected joint [9]. Rats were sacrificed after 19 days of disease evolution and paw samples were collected for histological and immunohistochemical evaluation.

Histology and Immunohistochemistry

For histopathological observation, paws, lungs, livers, kidneys and pancreas samples were collected at the time of sacrifice. Samples were fixed immediately in 10% neutral buffered formalin solution and then dehydrated using increased ethanol concentrations (70%, 96% and 100%). Paw samples, after being fixed, were also decalcified in 10% formic acid. Samples were next embedded in paraffin, sectioned and stained with hematoxylin and eosin for morphological examination. Paws were also used for immunohistochemical staining with Ki67 antibody, a cellular proliferation marker. Tissue sections were incubated with primary antibody against rat polyclonal Ki67 (Abcam, UK) and with EnVision+ (Dako, Denmark). Colour was developed in solution containing diaminobenzadine-tetrahydrochloride (Sigma, USA), 0.5% H_2O_2 in

phosphate-buffered saline buffer (pH 7.6). Slides were counterstained with hematoxylin and mounted. All images were acquired using a Leica DM 2500 (Leica microsystems, Germany) microscope equipped with a colour camera. Data regarding the degree of proliferation of synovial cells was scored from 0-3 (0 – fewer than three layers; 1 – three to four layers; 2 – five to six layers; 3 – more than six layers). Lymphoid cell infiltration was scored from 0-3 (0 – none to diffuse infiltration; 1 – lymphoid cell aggregate; 2 – lymphoid follicles; 3 – lymphoid follicles with germinal center formation) [10].

Caspase-1 and NF-kB assay

THP1 (ATCC TIB-202) macrophage-like cell line and THP1/NF-kB reporter cell line were cultured in R10 - RPMI media 1640 supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin-streptomycin, 1% (v/v) pyruvate, 1% (v/v) L-glutamine, 1% (v/v) non-essential aminoacids, 1% (v/v) hepes buffer and 2-mercaptoethanol to a final concentration of 0.05M, as recommended by the American Tissue Culture Collection (ATCC). Cells were cultured at 250.000 cells/mL, incubated with 10µM of gambogic acid for 1h at 37ºC 5% CO_2, and then stimulated with PFA-fixed *E. coli* (20 *E. coli* per cell) for 8h and 24h at 37ºC 5% CO_2. Simultaneously, non-stimulated negative control cells were also cultured at the same density as the stimulated population for comparison. Caspase-1 activity was measured in THP1 macrophage-like cell line using the Carboxyfluorescein FLICA Detection kit for Caspase Assay (Immunochemistry Technologies, LLC) following the reagent instructions. Briefly, cells from the different assays were protected from light exposure while incubated for 1 hour at 37ºC with 30X FLICA solution at a 1:30 ratio. NF-kB activity was measured in THP1/NF-kB reporter cell line. Lentiviral particles carrying a NF-kB-responsive GFP-expressing reporter gene

(Cignal Lenti Reporters, SABiosciences, USA) were used to infect THP-1 cells and to establish a stable cell line. All samples were analyzed by flow cytometry using a FACS Calibur (BD biosciences, USA). The data collected were further analyzed using FlowJo software (Tree Star Inc, USA).

Statistical analysis

Statistical differences were determined with non-parametric Kruskal-Wallis and Mann-Whitney tests using GraphPad Prism (GraphPad, USA). Differences were considered statistically significant for $p < 0.05$.

RESULTS

Gambogic acid reduces IL-1β and TNF production

To study the effect of this drug in the inhibition of IL-1β and TNF secretion we treated the human THP-1 macrophage-like cell line with growing concentrations of gambogic acid for 1 hour before challenging them with PFA-fixed *E. coli* for 6 hours. The conditioned media was then probed for the secretion of either IL-1β or TNF using ELISA. Gambogic acid significantly inhibited the secretion of both cytokines over a wide range of concentrations (Fig. 1), confirming the previously reported effect of gambogic acid in blocking the secretion of these cytokines [11] and validating our earlier finding (Figueiredo et al., *unpublished*).

Gambogic acid inhibits the activation of NF-kB and Caspase-1

Pro-IL-1β and TNF both depend on NF-kB activation for the transcription of their respective mRNAs. Pro-IL-1β processing is further dependent on the activation of caspase-1. We therefore tested the effect of gambogic acid on these key pathways. To

investigate the effect of this drug in the activation of NF-kB, we used an NF-kB reporter cell line created by stably infecting THP-1 cells with a commercial lentiviral GFP reporter under the control of a minimal CMV promoter and tandem repeats of the NF-kB transcriptional response element (TRE). Gambogic acid was able to suppress NF-kB reporter activation upon *E. coli* stimulation in comparison with cells that were also stimulated but did not receive treatment (Fig. 2A). To test the effect of this drug in caspase-1 processing and activation we used a caspase-1 fluorescent substrate, and measured relative active caspase-1 levels using FACS. Also in this case, gambogic acid significantly decreased the activation of caspase-1 (Fig. 2B).

Gambogic acid is able to suppress inflammation in Wistar rat antigen-induced arthritis

To study the *in vivo* anti-inflammatory properties of gambogic acid, AIA rats were treated daily with this drug after disease had already started to be symptomatic and for a period of 15 days. The inflammatory score and paw perimeter were evaluated during the period of treatment. As shown in Fig. 3, by the 4th day all induced animals already presented arthritis. All induced animals received either vehicle or gambogic acid at that time point. After 6 days of treatment the vehicle injected group increased sharply the inflammatory manifestations, whereas in gambogic acid-treated rats there was minimal inflammatory activity or even complete abrogation of arthritis manifestations. After 15 days of treatment, gambogic acid showed an anti-inflammatory effect as assessed by the evaluation of the inflammatory score (Fig. 4) and paw perimeter (p=0.007 vs untreated animals).

Gambogic acid prevents joint inflammatory infiltration and proliferation

To evaluate the infiltration of immune cells within joints in AIA rats, joint tissue sections were stained with hematoxylin and eosin. The histological evaluation shown in Fig. 5 revealed that rats treated with gambogic acid had a normal joint structure with complete abrogation of the inflammatory infiltrate ($p<0.0001$ vs untreated animals). In contrast, vehicle-treated rats exhibited infiltration of inflammatory cells, bone invasion and erosions (Fig. 5). We have not observed significant differences in body weight or any other side effects in treated rats, as revealed during autopsy and histological analysis made in lung, liver, kidney and pancreas (*data not shown*). We also studied the levels of proliferation of immune cells, by staining joint tissue sections with Ki67. The immunohistochemical results revealed that rats treated with gambogic acid presented reduced proliferation of immune cells within joints ($p=0.0098$ vs untreated animals).

DISCUSSION

Our results demonstrated that treatment with gambogic acid protected Wistar AIA rats from arthritis development with a complete abrogation of joint immune cellular infiltration and proliferation, preventing cartilage and bone damage.

Previous reports have already demonstrated that gambogic acid can inhibit the growth of a wide variety of tumor cell lines, possibly due to its ability to induce apoptosis [12] via the transferrin receptor (TfR1) [13]. Additionally, recent data have shown that this drug can inhibit NF-kB signalling pathway in human leukemia cancer cells [8] and in a non-cancerigenous macrophagic cell line [14] also via TfR1. Therefore, the anti-inflammatory effects of gambogic acid appear to be mediated by the inhibition of NF-kB activation pathway which in turn leads to the silencing of most of the inflammatory genes. In our study we demonstrated that the anti-inflammatory properties of this drug in AIA rats might not only be related with its ability to suppress the activation of NF-kB

but also to its effect on inhibiting caspase-1 activation. Importantly, in RA the inflamed synovium expands into and destroys the underlying cartilage and bone, resulting in irreversible erosion of the bone and in the loss of normal joint architecture leading to disability [15]. The inhibitory effect of gambogic acid in cellular proliferation can thus prove relevant for the management of RA course.

In conclusion, gambogic acid constitutes an effective drug in a rat model of RA, possibly by its ability to down-regulate caspase-1 and NF-kB activation and by blocking synovial hyperplasia due to its anti-proliferative properties. Further animal experimentation is required to explore the safety of this compound for the treatment of inflammatory diseases, such as RA, but these results hold the promise that gambogic acid could be worth of entering into phase I clinical trials.

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Authors' contributions

RC performed all the laboratorial work, data collection, statistical analysis and writing of the paper. BV contributed in animal manipulation, histological and immunohistochemical experiments. HR, ANC and NF participated in some laboratory experiments. VG contributed with the drugs used in the primary screening. LFM and JEF as senior authors conceived the study, participated in its design and coordination, and contributed in the draft of the manuscript with important intellectual input. All authors read and approved the final manuscript.

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FIGURE LEGENDS

Figure 1. *IL-1β and TNF secretion are inhibited by gambogic acid treatment.* Media samples from human THP-1 macrophage-like cell line cultured with growing concentrations of gambogic acid were analyzed by ELISA technique. Differences were considered statistically significant for p values < 0.05.

Figure 2. (A) *NF-kB reporter activation is suppressed by gambogic acid treatment.* NF-kB expression was measured by flow cytometry in a THP-1/NF-kB reporter cell line incubated with gambogic acid and then stimulated for 24h with *E. coli*. Each thin line in the histogram corresponds to untreated but *E. coli* stimulated cells, the shaded area corresponds to drug-treated and *E. coli* stimulated cells and the thick line corresponds to untreated non-stimulated cells as a control. *Caspase-1 activation is decreased with gambogic acid treatment.* **(B)** Caspase-1 activation was measured using flow cytometry in a THP-1 cell line incubated with gambogic acid and then stimulated for 8h with *E. coli*. Each thin line in the histogram corresponds to untreated but *E. coli* stimulated cells used as control and the thick line corresponds to drug-treated and *E. coli* stimulated cells.

Figure 3. *Gambogic acid is able to suppress inflammation throughout time.* After 6 days of treatment the vehicle injected group increased inflammatory manifestations, whereas in gambogic acid-treated rats there was a significantly reduction in the inflammatory activity. Arrow indicates the beginning of treatment after 4 of disease induction. Differences were considered statistically significant for p values < 0.05.

Figure 4. *Gambogic acid possess anti-inflammatory properties.* Inflammation score in gambogic acid-treated AIA rats is significantly diminished in comparison with vehicle-treated rats after treatment. Differences were considered statistically significant for p values < 0.05.

Figure 5. *Histological and immunohistochemical evaluation of joints after 15 days of treatment.* Non-arthritic control **(A)**, vehicle-treated **(B)** and gambogic acid-treated **(C)** AIA rats (magnification 50x and 100x in histological images and a magnification 200x in immunohistochemical images). Notice that gambogic acid has completely prevented immune cellular infiltration, proliferation and bone invasion.

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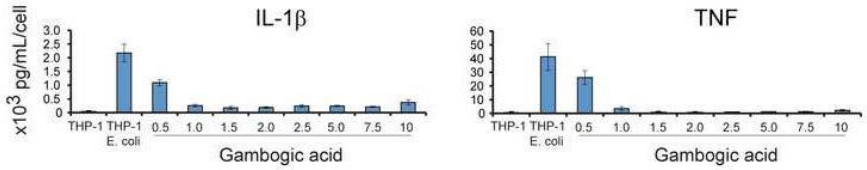

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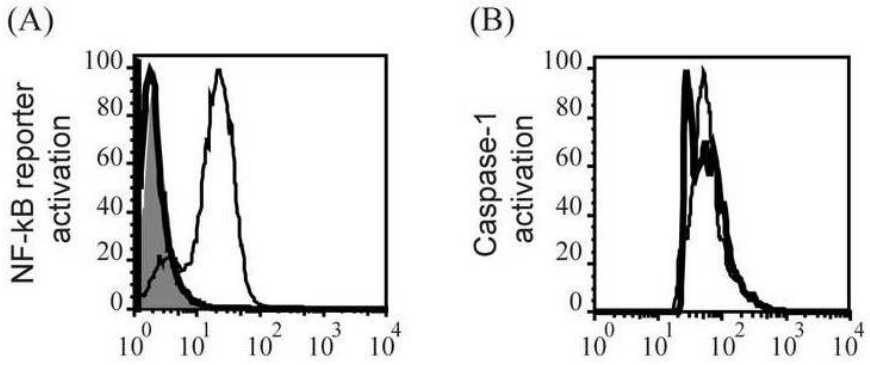

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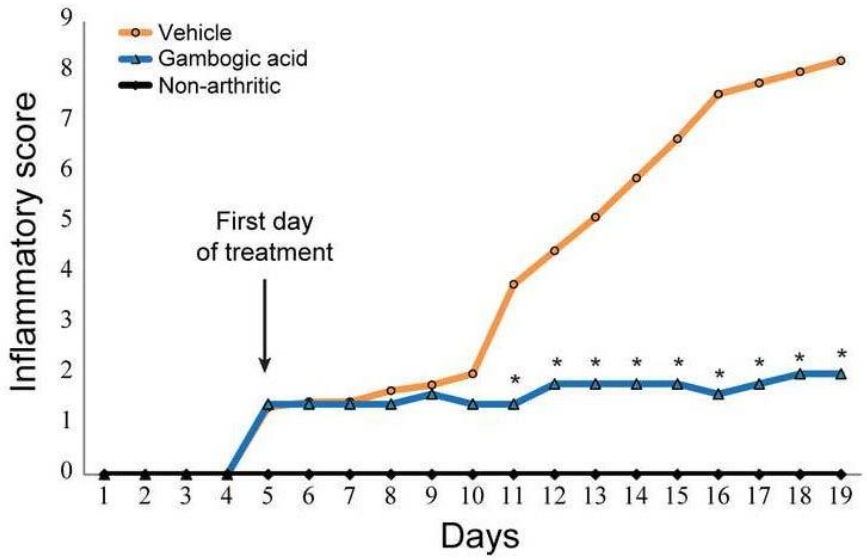

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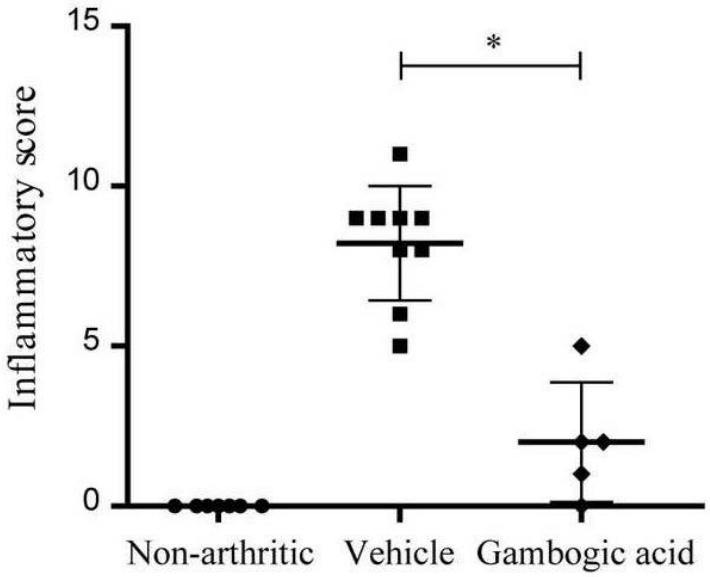

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