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ΜΕΤΑΠΤΥΧΙΑΚΗ ΔΙΑΤΡΙΒΗ

Ο ρόλος της αντίστασης στην ινσουλίνη στην επαγόμενη από σήψη φλεγμονώδη αντίδραση του πνεύμονα.

ΘΕΟΔΩΡΑΚΗΣ ΕΜΜΑΝΟΥΗΛ

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Η ολοκλήρωση της εργασίας αυτής έγινε στο πλαίσιο της υλοποίησης του μεταπτυχιακού προγράμματος το οποίο συγχρηματοδοτήθηκε μέσω της Πράξης «Πρόγραμμα χορήγησης υποτροφιών ΙΚΥ με διαδικασία εξατομικευμένης αξιολόγησης ακαδημαϊκού έτους 2012-2013» από πόρους του Ε.Π. «Εκπαίδευση και Δια Βίου Μάθηση» του Ευρωπαϊκού Κοινωνικού Ταμείου (ΕΚΤ) και του ΕΣΠΑ (2007-2013).

ΠΕΡΙΛΗΨΗ

Σήψη καλείται το σύνολο των μεταβολών στο μεταβολισμό και την αιμοδυναμική που είναι αποτέλεσμα γενικευμένης φλεγμονώδους αντίδρασης του ανθρώπινου οργανισμού σε λοιμώδη παράγοντα (βακτήριο, ιό, μύκητα ή παράσιτο). Η επίπτωση της σήψης είναι υψηλή, ιδιαίτερα στις ηλικιακές ομάδες των βρεφών κι των ηλικιωμένων. Η επιβίωση είναι χαμηλή κι χαρακτηριστικό είναι πως η σήψη συνιστά την κύρια αιτία θανάτου στις Μονάδες Εντατικής Θεραπείας. Ως φλέγον ζήτημα, η σήψη αποτελεί επίκεντρο χρόνιων προσπαθειών κι ερευνών, κλινικών κι μοριακών, για την αποσαφήνιση της παθοφυσιολογίας της με απώτερο σκοπό τον περιορισμό των δεικτών επίπτωσης και θνητότητας.

Η παχυσαρκία σχετίζεται με χαμηλού βαθμού φλεγμονή και αυξημένο κίνδυνο για λοίμωξη. Μολαταύτα, κλινικές μελέτες έχουν αποδείξει ότι παχύσαρκοι σηπτικοί ασθενείς της ΜΕΘ κατέγραψαν χαμηλότερο δείκτη θνητότητας συγκριτικά με ασθενείς κανονικού βάρους, φαινόμενο που χαρακτηρίστηκε ως <<παράδοξο της παχυσαρκίας>>. Η παρατήρηση και ο χαρακτηρισμός του φαινομένου αυτού έστρεψε την έρευνα προς την ανίχνευση της συσχέτισης μεταξύ των μεταβολικών μονοπατιών της ινσουλίνης και της σήψης. Στα πλαίσια αυτά, η παρούσα μελέτη προσπαθεί να χαρακτηρίσει την επαγόμενη από σήψη οξείας πνευμονική βλάβη, σε συνθήκες υπερινσουλιναιμίας και μη. Απόπειρα γίνεται δε, προκειμένου να εξηγηθεί η όποια διαφορά παρατηρείται στη δράση και στην κινητοποίηση συγκεκριμένων φαινοτυπικών μορφών των κυψελιδικών μακροφάγων. Όλα τα ανωτέρω, εξετάστηκαν σε ένα καλώς χαρακτηρισμένο μοντέλο σήψης σε ποντίκι (CLP) κατόπιν χορήγησης διατροφής υψηλής περιεκτικότητας σε λίπος, έτσι ώστε να επιτευχθούν οι συνθήκες υπερινσουλιναιμίας.

Τα αποτελέσματα καταδεικνύουν ότι τα ποντίκια που τρέφονταν με την υψηλή σε λίπος διατροφή, όντως μετέπιπταν σε μία διαφορετική μεταβολική κατάσταση, η οποία χαρακτηρίζονταν από διαταραγμένη ανοχή γλυκόζης και υπερινσουλιναιμία. Το αποτέλεσμα αυτό παρατηρείται κι στα υγιή και στα ποντίκια που έχουν υποβληθεί στο μοντέλο της σήψης. Κατόπιν, η μελέτη της φλεγμονώδους αντίδρασης του πνεύμονα των σηπτικών ποντικών, τονίζει την περιορισμένης έκτασης οξεία πνευμονική βλάβη, στα πλαίσια του ARDS, που ανέπτυσσαν τα ποντίκια που τρέφονταν με υψηλή σε λίπος διατροφή. Επιπρόσθετα, η επιβίωση των σηπτικών ποντικών φαίνεται να είναι βελτιωμένη κατόπιν της ειδικής διατροφής. Επιπροσθέτως, η μελέτη των κυψελιδικών μακροφάγων των σηπτικών ποντικών ανέδειξε ότι κατόπιν χορήγησης της υψηλής σε λίπος διατροφής, τα μακροφάγα αποκτούν διαφορετικό φαινότυπο (M2). Ο συγκεκριμένος φαινότυπος αφορά στον τρόπο ενεργοποίησης των μακροφάγων και σχετίζεται κυρίως με τη μείωση της προφλεγμονώδους δράσης, την λύση της φλεγμονής τοπικά και με την αποκατάσταση των ιστών.

Συνοψίζοντας, τα ανωτέρω ευρήματα καταγράφουν την επαγωγή αντίστασης στην ινσουλίνη στα ποντίκια, ως αποτέλεσμα της χορήγησης υψηλής σε λίπος διατροφής. Επιπλέον, περιγράφεται μία ηπιότερη πνευμονική βλάβη, ως αποτέλεσμα της γενικευμένης

σήψης που προκαλείται στα ποντίκια που ακολουθούν την υψηλή σε λίπος διατροφή. Η καλύτερη αυτή εικόνα των σηπτικών ποντικών μετά από την λήψη της υψηλής σε λίπος διατροφής αναπόφευκτα οδηγεί στον παραλληλισμό με το <<παράδοξο της παχυσαρκίας>> των σηπτικών ασθενών στη ΜΕΘ. Συνεπακόλουθα, πιθανολογείται μια αιτιολογική σχέση μεταξύ της αντίστασης στην ινσουλίνη και του ηπιότερου φαινότυπου πνευμονικής βλάβης. Περεταίρω μελέτη του θέματος, αποπειράται να βρει το μηχανισμό που μπορεί να παίζει κάποιο ρόλο στην προαναφερθείσα σχέση. Η ανευρεθείσα αλλαγή φαινοτύπου των κυψελιδικών μακροφάγων αξιολογείται και εκτιμάται ως ενδιάμεσος κρίκος στην σχέση αντίσταση στην ινσουλίνη – επαγόμενη από σήψη πνευμονική βλάβη.

UNIVERSITY OF CRETE – FACULTY OF MEDICINE

MASTER THESIS

The role of insulin resistance in sepsis induced acute lung injury.

THEODORAKIS EMMANUEL

HERAKLION, NOVEMBER 2013

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ABSTRACT

Sepsis and ARDS are among the most common causes of morbidity and mortality in the ICU. Hyperinsulinaemia and insulin resistance are often present in ICU patients due to stress response, obesity or even due to insulin therapy. Macrophages are central mediators of inflammatory responses in sepsis, and insulin, among other metabolic factors, affects their function. Macrophages can present either with a pro-inflammatory (M1) activation phenotype or with an anti-inflammatory, alternative (M2) activation phenotype. Alternative macrophage activation is protective in animal models of acute inflammation. Our preliminary data imply that insulin affects macrophage phenotype and their responsiveness to pro-inflammatory insults.

The aim of this study is to elucidate the impact of hyperinsulinaemia and HFD-induced insulin resistance on macrophage polarization and its effect on sepsis and secondary ARDS.

Male C57BL/6 WT mice were fed normal diet (ND) or high fat diet (HFD, 60% fat) for 5 days or 8 weeks. Glucose tolerance was tested in vivo by serial glucose measurements after glucose loading (1mg/gr). HFD and ND-fed mice underwent cecal ligation and puncture (CLP) or sham operation. BAL fluid and cells, serum and lungs were collected 6h post-operatively. Protein, IL-6 and MIP2 were measured in BAL and insulin and cytokines were measured in serum. Histological analysis was performed in formalin-fixed paraffin embedded lung sections. Survival rate was monitored for 7 days in HFD and ND-fed mice subjected to CLP, treated with imipenem-cilastatin (500ug every 12 hours). Alveolar cells that were isolated from BAL fluid were stained for M1/M2 markers and were analyzed by FACS. Glucose tolerance was impaired in HFD-fed mice. This was the case, also, regarding insulin. Hyperinsulinemia was recorded after 8 weeks of HFD. Both results implement insulin

resistance. In vivo, CLP-induced mortality was slightly better for HFD-fed mice. BAL and Serum IL-6 and MIP2 were lower in HFD-fed mice than ND-fed mice which suggest a protective role of M2 macrophages in sepsis and ARDS. Lung histological analysis of all CLP mice demonstrated inflammatory cell infiltration and alveolar wall thickening, which were far less in extent in HFD-fed mice. FACS analysis of stained alveolar cells demonstrated an increase of Arg1 expression in HFD-fed mice, which implies M2 macrophages are prevalent in inflammation of HFD-fed mice.

HFD promotes insulin resistance and alternative macrophage activation and reduces systemic and pulmonary inflammatory response to sepsis.

INDEX

A.INTRODUCTION

A.1 Sepsis

Sepsis is a multifactorial life-threatening condition which has left its mark throughout history of medicine. There have been recorded many attempts to define and describe sepsis since Hippocrates' era [4]. Its complexity is highlighted by the large number of research groups around the world, which focus on sepsis. Sepsis is defined as a whole-body inflammation which is caused by systemic response to infectious stimuli. This condition is potentially lethal [5, 6]. Sepsis can continue even after the infection that caused it is gone. Severe sepsis is sepsis complicated by organ dysfunction. The gradually increasing number of researchers putting all their efforts in sepsis as a field highlights its importance itself. However, epidemiology and statistics also confirm this observation. A very accurate study from USA revealed very characteristic numbers regarding the incidence of sepsis. Nearly 3% of patients been admitted to the hospital were recorded with sepsis. Half of them received ICU care. Regarding age, sepsis is recorded mostly in infants and in the elderly, however there is an stable increase through all adulthood [9].

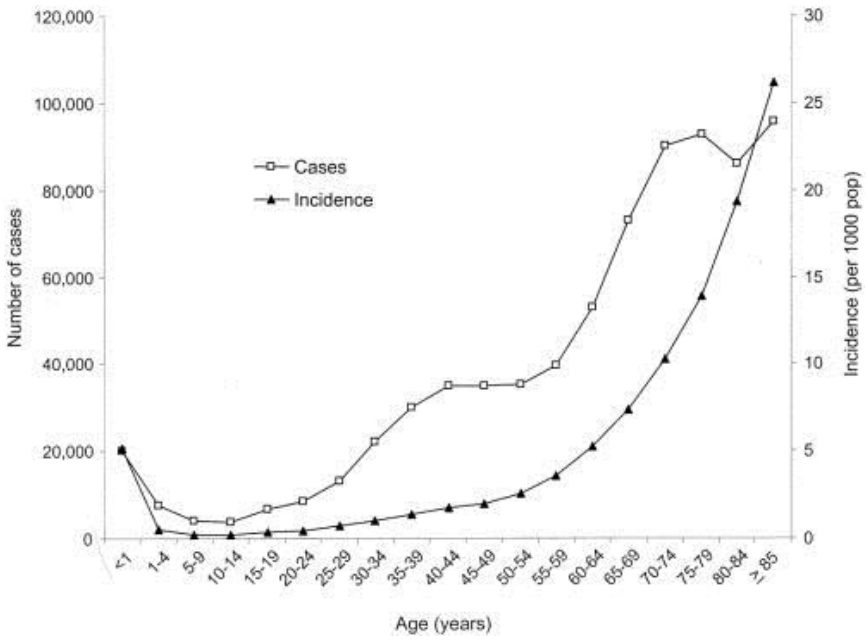

Figure A.1 National age-specific number and incidence of cases of severe sepsis [9].

The overall hospital mortality rate due to sepsis and severe sepsis is 28.6%, which represents 215,000 deaths in the USA [9]. However, the alarming fact is that the mortality rate has increased during the period 2003-2007 (a 35% increase) in the USA [10].
Severe sepsis, 3 million adults in the United States annually and results in substantial morbidity and mortality said Gaieski DF et al [11].

Causes and risk factors

Sepsis is defined as the presence (probable or documented) of infection together with systemic manifestations of infection. The most common primary sources of infection resulting in sepsis are the lungs, the abdomen, and the urinary tract. Pneumonia is the most common infection leading to sepsis, accounting for about half of all cases [59]. No source is found in one third of cases [60]. The infectious agents are usually bacteria but can also be fungi and viruses [60]. Streptococcus pneumoniae and Staphylococcus aureus are the most common gram-positive isolates, while Pseudomonas aeruginosa , Escherichia coli, and klebsiella species are mostly identified among gram-negative isolates. While gram-negative bacteria were previously the most common cause of sepsis, in the last decade, gram-positive bacteria, most commonly staphylococci, are thought to cause more than 50% of cases of sepsis [61]. However, in a more recent study involving 14,000 ICU patients in 75 countries, gram-negative bacteria were isolated in 62% of patients with severe sepsis who had positive cultures, gram-positive bacteria in 47%, and fungi in 19% [59]. Risk factors of sepsis are mainly related to the general condition and health status of each person. The predisposition of a patient to infections, the genetic background, possible chronic diseases, the use of immunosuppressive agents and of course age, sex, and race are all factors that influence the incidence of sepsis [9, 62].

ARDS

Acute respiratory distress syndrome (ARDS), is characterised by a severe inflammatory process causing diffuse alveolar damage and resulting in severe hypoxaemia, and decreased lung compliance [7]. The American-European Consensus Conference (AECC) proposed a definition, which is now widely accepted as a simple diagnostic tool for patient characterisation and research trial conduct. There are 3 mutually exclusive categories of ARDS based on degree of hypoxemia: mild (200 mm Hg < PaO_2/FIO_2 ≤ 300 mm Hg), moderate (100 mm Hg < PaO_2/FIO_2 ≤ 200 mm Hg), and severe (PaO_2/FIO_2 ≤ 100 mm Hg) and 4 ancillary variables for severe ARDS: radiographic severity, respiratory system compliance (≤40 mL/cm H_2O), positive end-expiratory pressure (≥10 cm H_2O), and corrected expired volume per minute (≥10 L/min). [75].

Sepsis from both pulmonary and non-pulmonary origin is the most common cause of ARDS [76]. Non-pulmonary origin ARDS is associated with the highest risk of progression to ARDS [79]. ARDS as a serious complication of sepsis is associated with significant morbidity and mortality up to 80%. Specifically, 6.2% of patients presented with severe sepsis develop ARDS. These patients had 4-fold higher risk of in-hospital mortality [12].

Pathophysiology of sepsis and ARDS

Sepsis occurs as a result of a combination of factors related to the invading organism(s) and the host (pre-disposing illnesses, genetics, and immune system) [14].

Host response

Despite older, simplistic theories which presented all clinical manifestations of sepsis to be a result of the exuberant inflammation due to invading microorganisms, apparently, these are only small part of the whole picture. Sepsis is obviously triggered by a much more complex host response which includes both pro-inflammatory and anti-inflammatory mechanisms [13]. During sepsis, early activation of immune cells—monocyte/macrophages, lymphocytes, and neutrophils—is followed by down-regulation of their activity [15]. Both pro-/anti-inflammatory mechanisms contribute not only to the clearance of the invading microorganisms and the tissue recovery, but also to the organ injury and the secondary infections. Undoubtedly, each organism, based on its characteristics (genetic background, coexisting illnesses), may respond differently to a specific stimulus (Figure 2).

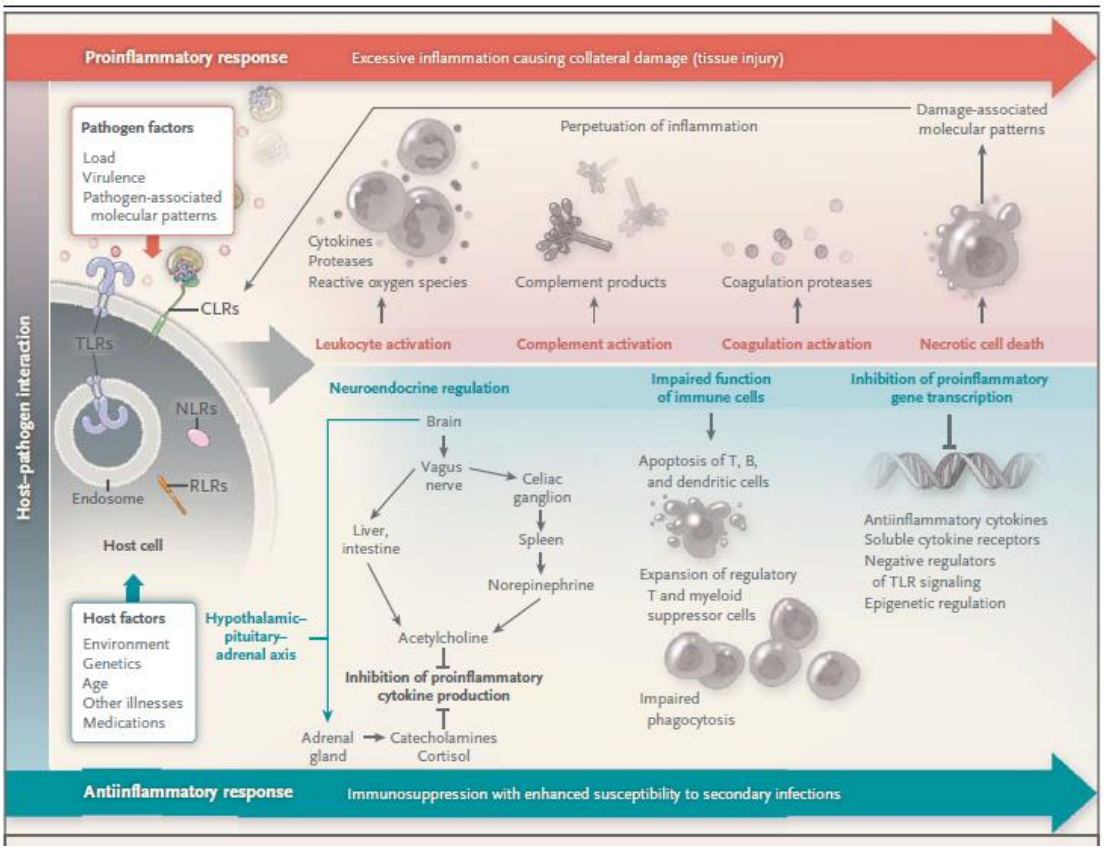

Figure A.2 Pathogen – Host response in severe sepsis [7].
The host response to sepsis is characterized by both proinflammatory responses (top of panel, in red) and antiinflammatory immunosuppressive responses (bottom of panel, in blue). The direction, extent, and duration of these reactions are determined by both host factors (e.g., genetic characteristics, age, coexisting illnesses, and medications) and pathogen factors (e.g., microbial load and virulence). Inflammatory responses are initiated by interaction between pathogen-associated molecular patterns expressed by pathogens and pattern recognition receptors expressed by host cells at the cell surface (toll-like receptors [TLRs] and C-type lectin receptors [CLRs]), in the endosome (TLRs), or in the cytoplasm (retinoic acid inducible gene 1–like receptors [RLRs] and nucleotide-binding oligomerization domain–like receptors [NLRs]). The consequence of exaggerated inflammation is collateral tissue damage and necrotic cell death, which results in the release of damage-associated molecular patterns, so-called danger molecules that perpetuate

inflammation at least in part by acting on the same pattern-recognition receptors that are triggered by pathogens. [7].
Immune and cellular responses

The innate immune system is the major contributor to acute inflammation induced by microbial infection or tissue damage. Pathogens activate immune cells (macrophages, dendritic cells etc.) through an interaction with pattern-recognition receptors. There have been identified 4 main classes (toll-like receptors, C-type lectin receptors, retinoic acid inducible gene 1–like receptors, and nucleotide-binding oligomerization domain–like receptors). The last group partially act in protein complexes called inflammasomes (Figure 3) [16].

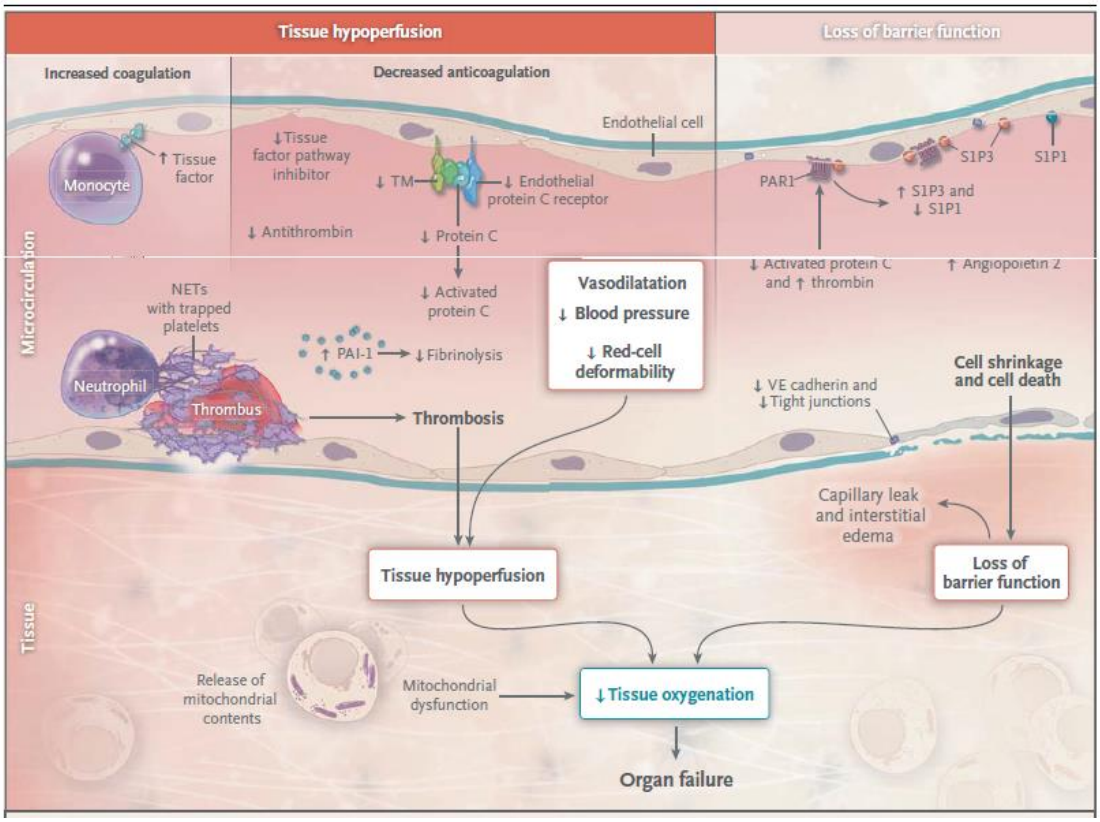

Figure A.3 Organ Failure in severe sepsis and dysfunction of the vascular endothelium and mitochondria [7].

Innate immunity is triggered by the interaction of those receptors with specific structures of the microbial species, named PAMPs (pathogen-associated molecular patterns). The receptors mentioned above, also recognize damage-associated molecular patterns (DAMPs or alarmins) which correspond to endogenous molecules released from damaged cells.

Moreover, sepsis is a condition that is characterized by unregulated coagulation. Normal homeostasis exists as a perfectly balanced machine. Blood remains under strict control and flows within the vessels. The strict regulation of blood flow and clotting is disrupted, when homeostasis is broken. Such case is sepsis. During inflammatory situations such as sepsis, significant alterations occur at multiple levels within both the coagulation system and the cells that regulate this system [24]. Severe sepsis is invariably associated with altered coagulation, frequently leading to disseminated

intravascular coagulation [25]. Coagulation leads to excess fibrin deposition through the action of tissue factor, a transmembrane glycoprotein expressed by various cell types, by impaired anticoagulant mechanisms, including the protein C system and antithrombin and by compromised fibrin removal owing to depression of the fibrinolytic system (Figure 3) [25]. Protease-activated receptors (PARs) form the molecular link between coagulation and inflammation. Among the four subtypes that have been identified, PAR1 in particular is implicated in sepsis. PAR1 causes cytoprotective effects when stimulated by activated protein C or low-dose thrombin but exerts disruptive effects on endothelial- cell barrier function when activated by high-dose thrombin [26]. The protective effect of activated protein C in animal models of sepsis is dependent on its capacity to activate PAR1 and not on its anticoagulant properties [26].

Organ dysfunction

Although organ dysfunction as a result of sepsis has been described several times, the precise mechanism by which sepsis leads to this complication remains unknown. The circulation is clearly affected at both macrocirculatory and microcirculatory levels, compromising tissue perfusion and oxygenation, thus normal organ functioning [17]. Several factors, among which hypotension, reduced red-cell deformability, and microvascular thrombosis, contribute to decreased oxygen delivery in septic shock. Apart from these, the cells themselves may react to a septic insult by modifying their behavior, function, and activity [18] Inflammation can result in dysfunction of the vascular endothelium. This has been shown to be accompanied by cell death and loss of barrier integrity, causing subcutaneous and body-cavity edema. In addition, mitochondrial damage caused by oxidative stress and other mechanisms impairs cellular oxygen use [20]. Moreover, injured mitochondria release alarmins into the extracellular environment, including mitochondria DNA and formyl peptides, which can activate neutrophils and cause further tissue injury[21].

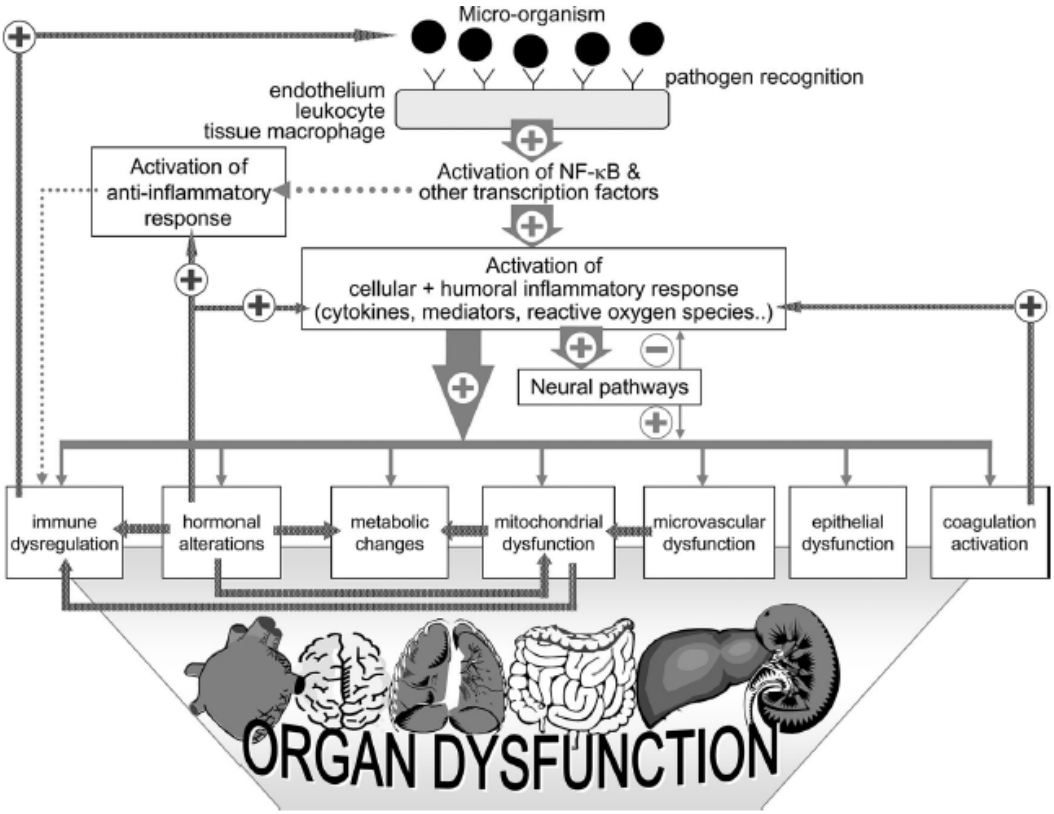

Figure A.4 Systemic pathways contributing to organ dysfunction in sepsis [77].

Sepsis induced organ injury is early expressed with acute lung injury. Acute respiratory distress syndrome (ARDS) is characterized by a disturbance of the alveolar-capillary barrier [35]. Lung inflammatory process includes ingress of activated neutrophils, interstitial edema, loss of surfactant, and fibrinous alveolar exudates [27]. Later, the pathology is characterized by mononuclear cell infiltrates, proliferation of type II pneumocytes, and interstitial fibrosis, leading to fibrosis.

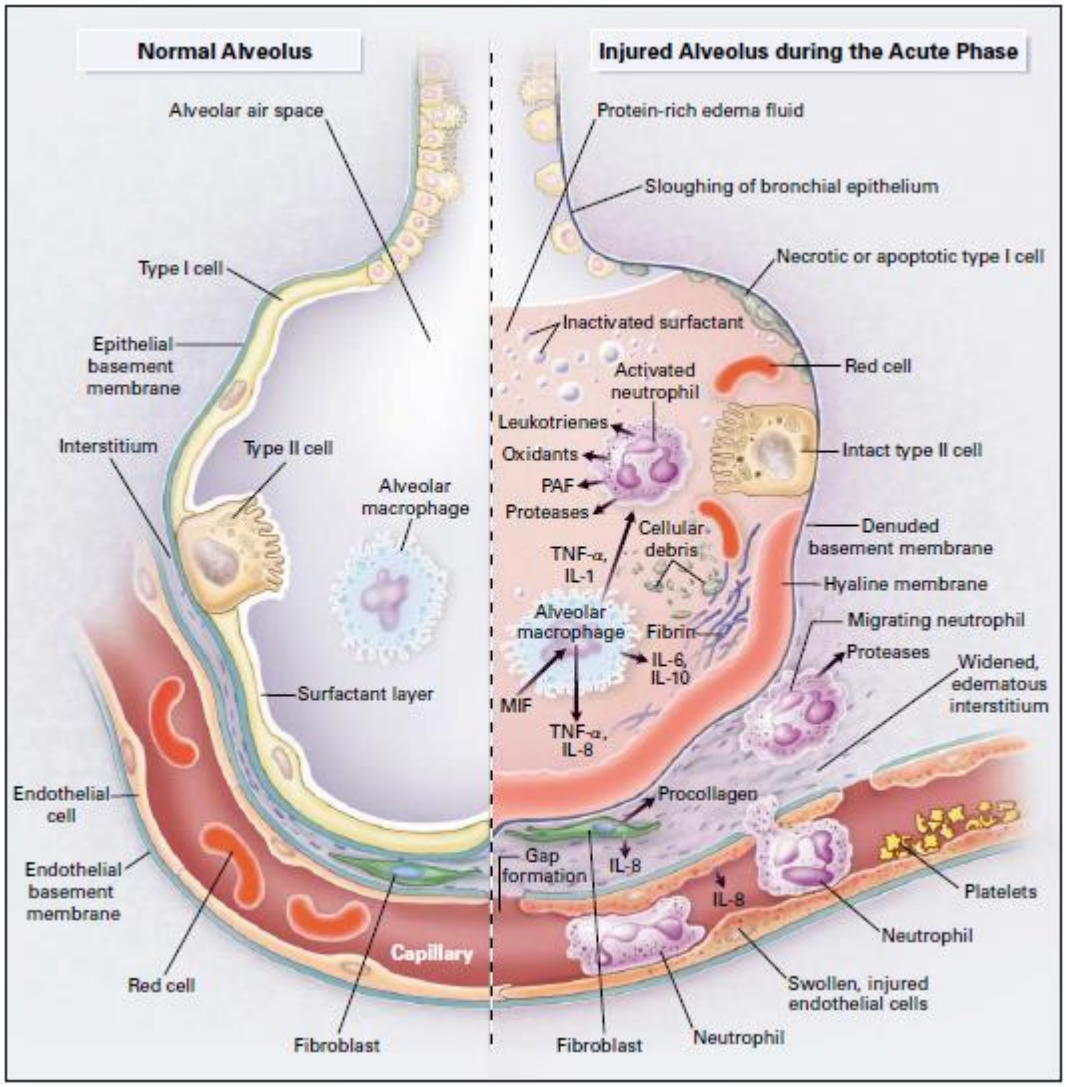

Figure A.5 The normal alveolus (left side) and the injured alveolus in the acute phase of acute lung injury and the Acute Respiratory Distress Syndrome (Right side) [7].

In the acute phase of ARDS both the bronchial and alveolar epithelial cells are sloughed. Neutrophils are shown adhering to the injured capillary endothelium and marginating through the interstitium into the air space. The air space is filled with protein-rich edema fluid. In the air space, an alveloar macrophage is secreting cytokines, interleukin-1, 6, 8, and 10, (IL-1, 6, 8, and 10) and tumor necrosis factor a (TNF-a), which act locally to stimulate chemotaxis and activate neutrophils. Macrophages also secrete other cytokines, including interleukin-1, 6, and 10. Interleukin-1 can also stimulate the production of extracellular matrix by fibroblasts. Neutrophils can release oxidants, proteases, leukotrienes, and other proinflammatory molecules, such as platelet-activating factor (PAF). A number of anti-inflammatory mediators are also present in the alveolar milieu, including interleukin-1–receptor antagonist, soluble tumor necrosis factor receptor, autoantibodies against interleukin-8, and cytokines such as interleukin-10 and 11. The influx of protein-rich edema fluid into the alveolus has led to the inactivation of surfactant. MIF denotes macrophage inhibitory factor [7].

Recently a new idea, regarding pathophysiologic factors of ARDS, was presented. The possibility of genetic predisposition to acute respiratory distress syndrome in patients with severe sepsis was evaluated. Cardinal-Fernández P et al focused to analyze the association between candidate gene polymorphisms and susceptibility to acute respiratory distress syndrome (ARDS) in patients with severe sepsis. They concluded that the presence of the allele D of the ACE gene is associated with ARDS in patients with severe sepsis [78].

Apart from direct lung damage, also iatrogenic influences are taken into account because ventilator-induced lung injury, oxygen toxicity, and the large volumes of fluid used for circulatory resuscitation amplify the degree of lung dysfunction and arguably alter its pathology [28]. Advances in supportive care and more adroit management of mechanical ventilation, use of fluid play an important role in the decreased mortality rates from acute lung injury that have been recorded [29]. Apoptosis, which is present in large extent, can be initiated by receptor-mediated and mitochondrial pathways [30]. In addition, the role of ROS is pathogenic factor involved in in sepsis mediated acite lung injury. Apart from direct cytotoxic effects, ROS have important effects on the inflammatory response mediated via changes in oxidant/antioxidant balance, redox signaling, and iron-mediated catalytic reactions [31, 32]. Iron availability also regulates activity of the nuclear transcription factor hypoxia inducible factor that responds to low oxygen tensions by up-regulating expression of numerous genes, including those encoding for vascular endothelial growth factor, erythropoietin, and inducible heme oxygenase-1 [33]. Catabolism of heme by heme oxygenase-1 produces carbon monoxide, bilirubin, and free iron. Although heme oxygenase-1 is usually considered cytoprotective, it can produce lung injury in animal models relevant to critical illness via mechanisms related to formation of low molecular mass, redox-active iron [34].

A.2 Insulin resistance and sepsis

Too often, metabolic organs display a pathologic reduction in their responsiveness to insulin, that is, insulin resistance (IR). Hyperglycemia and insulin resistance are commonly addressed in patients with critical illnesses and in specific in patients with severe sepsis that are admitted to ICU [36-39]. Several studies provided evidence that strict control of blood glucose improves the outcome of the illness [40-42]. It is considered standard of care to put critical patients into insulin therapy in order to strictly control blood glucose levels, which has been shown to ameliorate the inflammatory response [43] and improve patient outcome [44]. However, the mechanisms mediating these protective effects remain unclear and the overall effect is arguable due to the high risk of severe hypoglycemia. Undoubtedly, insulin signaling in critical ill patients is an issue which creates much controversy and a lot of effort has been put to elucidate the association between stress metabolic response and sepsis [54]. Interestingly, it is shown that the stress response may be considered beneficial by reallocating body's energy resources [45]. Many authors implement that stress induced hyperinsulinemia may be protective in the setting of acute illness [46-48].

A.3 Obesity and sepsis

Obesity is a fast growing epidemic worldwide and is closely associated with morbid conditions including diabetes, cardiovascular and respiratory diseases as well as cancer [49]. Approximately 65% of the United States population is overweight and 30% are obese. The prevalence of obese patients admitted to ICUs is also rising rapidly and poses complex challenges [50, 51]. While obese patients are considered to be difficult to manage, data on outcome, although conflicting, show predominantly either equal or lower mortality rate compared to normal weight patients [52, 53]. This association is referred to as “obesity paradox” [54]. Most of the data on the impact of obesity on septic shock are primarily based on experimental and small clinical studies [55, 56]. Recently, a large cohort study also dealt with the so called “obesity paradox”, pointing, though, that it is unsafe to draw conclusions unless all co-factors (e.g. patient characteristics, therapeutic interventions) are strictly organized [57]. Also, other diseases are associated with “obesity paradox” [89]. Despite the detrimental effects of obesity on coronary heart disease (CHD) and heart failure, obesity is found to be paradoxically associated with improved survival in secondary care of CHD and heart failure [86]. Additional recent studies have raised the issue of “obesity paradox” in patients with T2DM with cardiovascular risk and mortality [87, 88]. Concomitantly, obesity as a metabolic status is also characterized by hyperglycemia, hyperinsulinemia and insulin resistance. Inevitably, the correlation between obesity induced insulin resistance and critical illness outcome is a subject to be examined.

A.4 Macrophages in sepsis

Macrophages perform impressive functions, in health and in response to injury and infection. They are central mediators of inflammatory response [63]. Among other roles, macrophages execute phagocytosis and killing of an array of infectious microorganisms, phagocytose tissue debris, apoptotic parenchymal cells and apoptotic neutrophils, orchestrate the repair of wounded tissue, and link the innate and adaptive parts of immunity. In accordance with their diverse activities, macrophages acquire differential phenotypes, dictated by the form, stage and site of insult [64, 65].

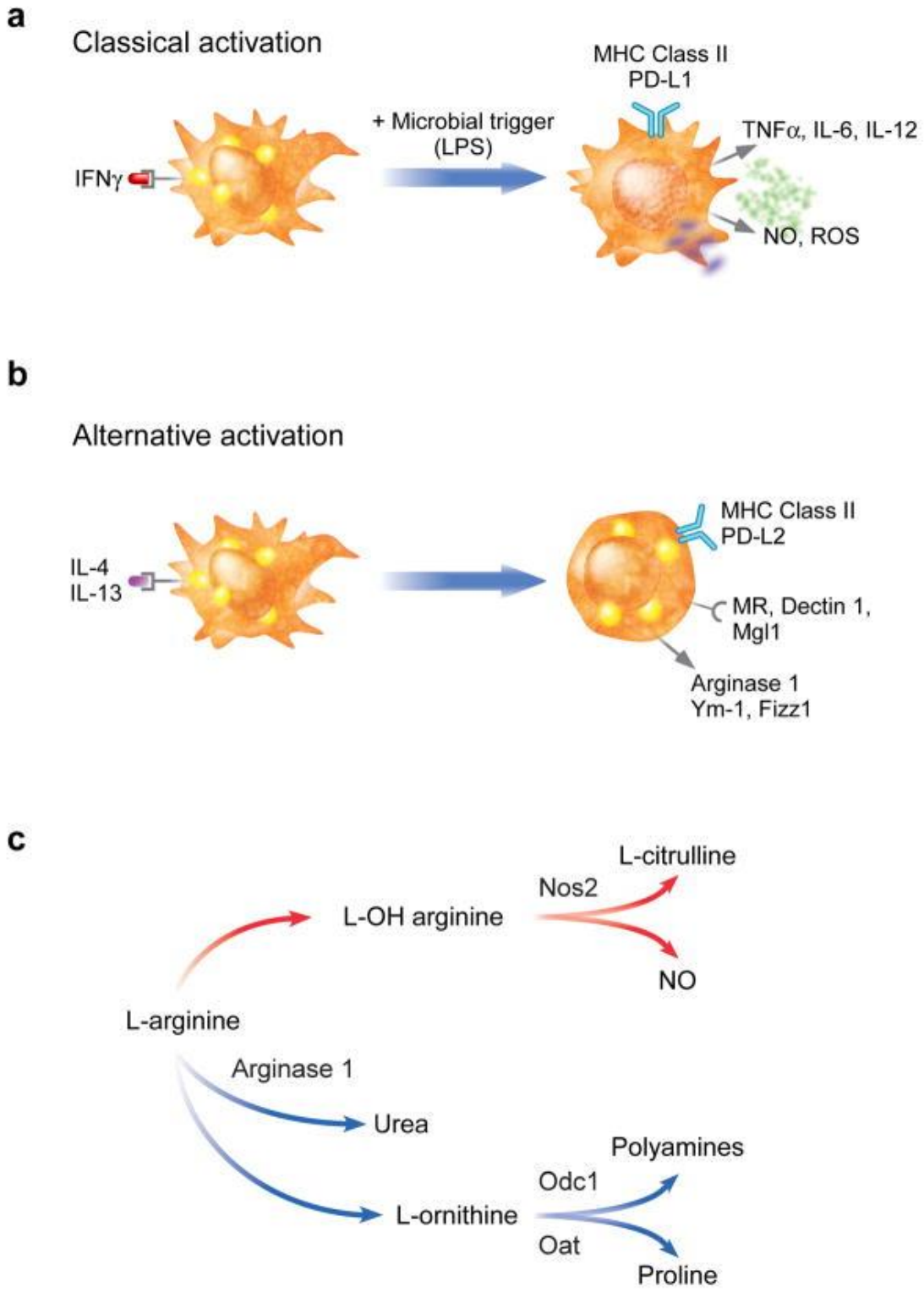

Figure A.6 Classical and alternative macrophage activation [73].

Macrophages activation includes a wide variety of activities which all converge to a specidic response to environmental stimuli. These responses can be separated into two basic patterns: classical, or M1, and alternative, or M2.

a) Classical activation is a pro-inflammatory state purposed for the rapid destruction of bacterial invaders. Macrophages with M1 phenotype act as the first line of defense against intracellular pathogens and they stimulate Th1 polarization of CD4+ lymphocytes. Classically activated macrophages generate reactive oxygen species (ROS) and nitric oxide (NO) for their microbicidal actions, and secrete pro-inflammatory cytokines, such as TNFα and IL-12, to enhance cell mediated immunity.

b) Alternative activation represents a more sustained response such as that typified by infection with parasites. M2 or alternatively-activated macrophages are the counterparts of Th2 lymphocytes and participate in immunity against extracellular parasites, helminthes, and so forth. Interestingly, alternatively activated macrophages display hyporeactivity to M1-type ligands, such as LPS, and are considered to be anti-inflammatory. While the induction of MHC class II and co-stimulatory molecules (PD-L2) indicate these macrophages are activated, they express a distinct repertoire of cell surface receptors (mannose receptor, *Mrc1*; dectin-1, *Clec7a*; and Mgl1, *Clec10A*), and secreted products (Ym-1, *Chi3l3*; and FIZZ1, *Retnla*).

c) Differential metabolism of l-arginine in classically and alternatively activated macrophages by inducible nitric oxide synthase (Nos2) and arginase 1, respectively [73].

Macrophages convert to an M2 phenotype at the later stages of an M1 response. This phenotype switch can take place following the engulfment of apoptotic neutrophils as well as other signals [67]. As a result, M2 macrophages initially downregulate their proinflammatory activity and shift their function towards local resolution of inflammation and tissue repair. M1 macrophages express high levels of inducible nitric oxide synthase (iNOS), and interleukin-12β while M2 macrophages express high levels of arginase-1 (Arg1) and anti-inflammatory cytokines [66].

A.5 Macrophages and insulin

Macrophages are highly related to obesity and insulin signaling [68, 73]. First of all, it is known that obesity, the most common cause of insulin resistance, is accompanied by a low-grade, chronic inflammatory response in the circulation and within metabolic organs. Adipose tissue contains bone marrow–derived macrophages, and the content of these macrophages tracks with the degree of obesity [90]. Some studies report, greater than 40% of the total adipose tissue cell content from obese rodents and humans can be composed of macrophages, compared with ~10% in lean counterparts [91]. Obesity-induced hepatic insulin resistance is also associated with increased expression of inflammatory mediators, which implies that bone marrow-derived macrophage cells of liver (Kupffer cells) promote

insulin resistance. There is also evidence that in high-fat diet (HFD)-induced obesity, increased macrophage accumulation occurs in skeletal muscle. Just as in the adipose tissue, these skeletal muscle pro-inflammatory macrophages are generally positive for F4/80, CD11b, and CD11c and exhibit an M1 macrophage–like phenotype [92]. Pro-inflammatory cytokines secreted by recruited and resident macrophages not only in adipose tissue, but also in liver, and skeletal muscle are a pivotal link between inflammation and abrogation of insulin signaling in adjacent metabolic cells [80]. However, Liu H. et al suggest that initial stage of HFD-induced insulin resistance is independent of inflammation, whereas the more chronic state of insulin resistance in established obesity is largely mediated by macrophage-induced proinflammatory actions [97]. Further studies link macrophages phenotype to the degree of organ-specific and whole-body insulin sensitivity, under normal conditions and in disease. In the lean state, the small numbers of macrophages populating adipose tissue are of the M2 phenotype and are associated with tissue-specific and whole body insulin sensitivity. Obesity, in contrast, is associated with accumulation of M1-type macrophages. The M1-to-M2 ratio is inversely related to tissue-specific and whole body insulin sensitivity [73, 93].

Furthermore, we know that both insulin and insulin signaling play a critical role in obesity-induced insulin resistance [99]. In specific, it is known that basal level of the Akt-dependent classical insulin signaling is increased in B6 mice with obesity and insulin resistance. Thus, the increase of insulin signaling is responsible for the development of insulin resistance induced by HFD [98].

Moreover, insulin signaling is considered to be important in function of macrophages. Akt kinases is a family of three serine/threonine protein kinases (Akt1, Akt2, and Akt3) that regulate a host of cellular functions, including cell survival, proliferation, differentiation, and intermediary metabolism. It is shown that Akt conducts an important role in insulin signaling. In detail, Akt kinase has been proposed to be an intermediate in the signaling pathway by which insulin controls muscle and fat cell glucose uptake as well as hepatic gluconeogenesis [94, 95]. Additionally, Akt2 appears to be enriched in insulin-responsive tissues and has been implicated in the metabolic actions of the insulin [95]. Interestingly, mice deficient in Akt2 are impaired in the ability of insulin to lower blood glucose because of defects in the action of the hormone on liver and skeletal muscle [95]. It is also shown that Akt kinases differentially contribute to macrophage polarization, with Akt1 ablation giving rise to an M1 and Akt2 ablation resulting in an M2 phenotype [81]. Additional data indicate that Akt1 Ablation Promotes, whereas Akt2 ablation protects from, Dextran Sulfate Sodium (DSS)-induced inflammatory bowel disease in mice [81]. Akt2 deficient mice, which are insulin resistant and have M2 macrophages, are also protected from acid-aspiration induced ARDS, according to Vergadi et al (unpublished).

A.6 Study hypothesis

The overall objective of this study is to test the hypothesis that obesity and in the mouse model high fat diet promotes macrophage polarization towards an M2 activation phenotype through development of insulin resistance, which ultimately has a protective effect in sepsis and secondary ARDS.

B. MATERIALS AND METHODS

B.1 Mouse models

WT C57/B6 mice were housed in micro-isolators in pathogen-free facility. All mice that were put into this study were male. Mice were grouped into normal diet-fed mice and high fat diet (HFD)-fed mice. This special diet was purchased from Mucedola srl ™. Mice were fed with either short or long term high fat diet. At the age of 8-9 weeks old mice were fed for 4 days with either high fat diet or normal diet. Long term includes 8 weeks of high fat diet and mice were selected at the age of 3-4 weeks old. Further detail of the special diet is given in the table:

Complete feed for RODENTS
PURIFIED Diet
60% ENERGY FROM FATS

INGREDIENTS:	ADDED per kg:		
Casein powder	**Vitamins:**		
Lard	Vitamin A	I.U.	8400
Maltodextrin	Vitamin D3	I.U.	2000
Sucrose	Vitamin E (α-tocopherol)	mg	157.5
Palm oil	Choline (choline bitartrate)	mg	1500
Cellulose powder			
Soybean oil	**Minerals:**		
Calcium carbonate	Cu (copper carb.basic)	mg	8.26
Potassium phosphate monobasic	Se (sodium selenite)	mg	0.21
L-Cystine			
Sodium chloride			
Mineral dicalcium phosphate	**Analysis**	**%**	
Potassium citrate	**Protein**	**23.00**	
Potassium sulphate	**Fat**	**34.00**	
Magnesium oxide	**Fibre**	**5.00**	
Blue colorant E131	**Ash**	**5.50**	

B.2 Glucose Tolerance Test

Glucose tolerance test is a standard procedure that addresses how quickly exogenous glucose can be cleared from blood. Specifically, uptake of glucose from the blood by cells is regulated by insulin. Impairment of glucose tolerance (i.e, longer time to clear given amount of glucose) indicates problems with maintenance of glucose homeostasis, which could be attributed to insulin resistance [3].

Reagents

- Syringe 1ml
- Dextrose 35% amp (10ml)
- Sterile alcohol prep pads
- Mouse Tailveiner Restrainer (Braintree scientific, inc.)
- Scales
- Blood glucose monitoring system (Glucometer and test strips - Freestyle Freedom Lite®)

Method

All mice that were about to be subjected to glucose tolerance test were previously fasted for 6 hours. Afterwards, the body weight of each mouse was recorded and fasted glucose (0min) was measured. A tailveiner restrainer was used in order to hold mouse stable and to take a blood sample. Then, glucose (1g/kg of body weight) was injected intraperitoneally. Following that, measurements of blood glucose took place at 30, 60 and 120 minutes. Blood sample was collected from tail vein through a small incision. Blood glucose was measured.

B.3 Cecal ligation and puncture (CLP)

Reagents

- C57BL/6 male mice
- Ketamine (80 - 100 mg per kg of body weight) (Ketamin-Actavis injection 10mg/ml)
- Xylazine (5 - 15 mg per kg of body weight) (Xylazine Hydrochloride 20mg/ml, Xylapan® Vetoquinol)
- Sterile alcohol prep pads
- Sterile saline solution

Method
Pre-operative setup

All mice were weighed in order to determine the amount of anesthetics to be used.
Latex gloves, face mask and surgical gown were used as protective equipment both for the human operator and to keep the surgical field reasonably aseptic.
Mice were anesthetized with ketamine (80–100 mg per kg body weight i.p.) and xylazine (5–15 mg per kg body weight i.p.) The intensity of anesthesia was monitored by toe pinch using tweezers. Adequate anesthesia should result in no response of extremity (e.g., no flexion of extremity). When mouse was adequately anesthetized, we disinfected the abdominal area with alcohol prep pads (Fig. 1a). Animals were placed onto Styrofoam pads on their backs, with heads oriented away from the operator. With adequate anesthesia, no restraints are needed.

Exposure

A longitudinal skin midline incision was made with a scalpel. It is important to be careful not to penetrate into the peritoneal cavity. After the initial incision, small scissors was used to extend the incision and to gain entry into the peritoneal cavity (mice, 1.5–2 cm; Fig. 1b). Following that, linea alba (midlines white fascia) of the abdominal musculature was able to be identified and it was dissected for intermuscular incision and incision of fascial and peritoneal layers as shown in Figure 1b. The cecum was then located by using blunt anatomical forceps, it was isolated and exteriorized (Fig. 1c), leaving the remainder of the small and large bowel within the peritoneal cavity. It is critical not to breach or damage the mesenterial blood vessels (Fig. 1c). In the majority of cases, the cecum is found on the left side of the abdomen.
It is of great importance not to damage the cecal branch of the ileocecal artery to avoid severe bleeding complications. The next step was to ligate the cecum at the designated position. It is crucial not to ligate the ileocecal valve so that intestinal continuity is maintained. For sham mice steps of ligation and puncture were not performed. Just Before cecal perforation, cecal contents were pushed gently toward the distal cecum. Next, cecum was perforated by double through-and-through puncture midway between the ligation and the tip of the cecum in a mesenteric-to-antimesenteric direction (Fig. 1h–k). It is important to avoid puncturing blood vessels. After removing the needle, a small amount (droplet) of feces was extruded from both the mesenteric and antimesenteric penetration holes to ensure patency. Then, the cecum was relocated into the abdominal cavity without spreading feces from the cecum onto the abdominal wall wound margins.

Closure and postoperative care

The peritoneum, fasciae, abdominal musculature and skin were closed by applying simple running sutures. All mice were resuscitated by injecting prewarmed normal saline (37 °C; 5 ml per 100g body weight) subcutaneously.

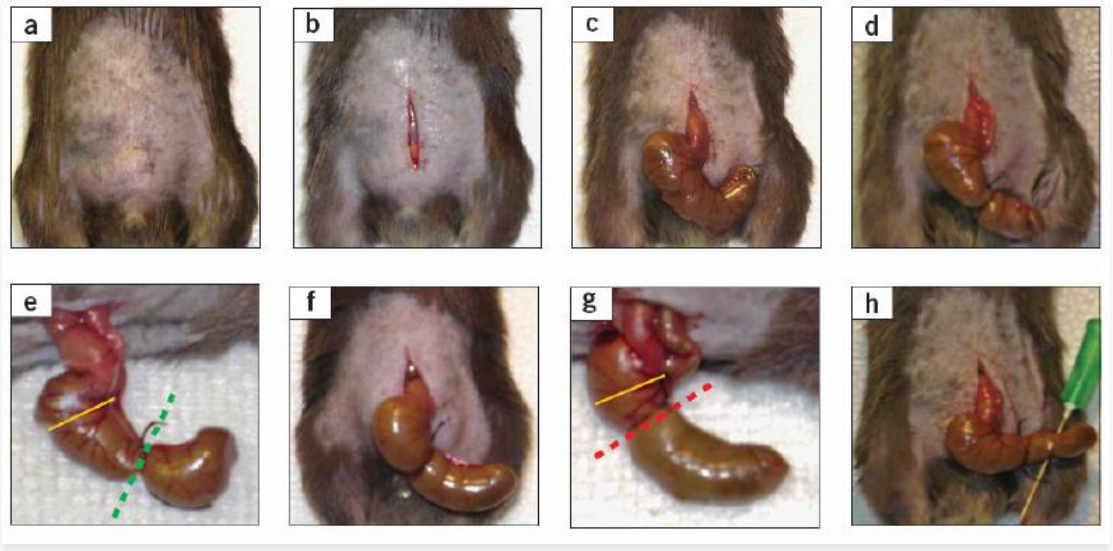

Figure 1

B.4 Sample collection

B.4.1 P-V curve

Mice were anesthetized and ventilated briefly (approximately 1min) on tidal volume VT=10ml/kg, RR=130br/min, PEEP=5 cm H_20 until paralyzed. Then, an inspiratory pressure volume curve was obtained and inspiratory capacity, defined as the volume to inflate the lungs to an airway pressure of 25 cm H_20, was measured [1].

B.4.2 Blood serum

Blood was collected by the right ventricle of the heart using a 1ml syringe. Next, blood samples were left to clot for 30-60 min in room temperature. Afterwards, they were centrifuged at 5000rpm for 10min at 4^{o}C. The upper phase (blood serum) was collected with clean tips and serum samples were immediately stored at -80^{o}C.

B.4.3 Bronchoalveolar lavage (BAL)

Broncoalveolar fluid was obtained by performing BAL. First of all, mice were anesthetized using ketamine and xylazine, as described in CLP. Each mouse was immobilized in a heating pad so that body temperature is 37 ^{o}C. Next, a small incision of the skin was made in the abdominal region using scissors and it was extended till neck was exposed. Neck tissue was removed until trachea was exposed. Following that, a small incision was made in trachea using a18g needle so that a passage for 18g lavage tube (i.v. canula 18g) was secured. Next, the surgical tube was stabilized with surgical sutures. Then, a sterile syringe (1ml) was loaded with 30ml/kg of normal saline (0.9% NaCl). The syringe was then placed into the lavage tube which was in the trachea and the saline was carefully injected into the lungs, trying to avoid any leak. Saline was then aspirated by pulling the barrel of the syringe. The same procedure was repeated for a total of 4 washes, so that as many as possible alveolar cells were collected. All samples acquired were immediately placed on ice. All samples acquired were centrifuged at 450g for 10min at 4 ^{o}C. The supernatant of the first bronchoalveolar wash of each mouse was placed in different tube and stored immediately at -80 ^{o}C for later analysis of protein and cytokines. The remaining pellet, containing alveolar cells, was stored with all other pellets from the total washes of each mouse and was used for staining protocols of flow cytometry.

B.4.4 Lung histology

Firstly, the trachea was canulated, the right lobe was occluded, and then formalinwas instiled in the lungs with a driving pressure of 25cmH2O. Left lung tissue samples were fixed in 10% buffered formalin overnight, embedded in paraffin, sectioned (5 μm), and routinely stained with hematoxylin-eosin for conventional morphological evaluation under light microscopy (Nikon Labophot).

B.4.5 Peritoneal macrophages isolation and LPS stimulation

Mice were divided in 2 groups (n=6/group) according to the diet they were fed (ND/HFD). The 6^{th} day mice were injected with 1ml of 4% thioglycollate intraperitoneally. The 10^{th} day mice were sacrificed and peritoneal cells were harvested by peritoneal lavage with normal saline (0.9% NaCl). Peritoneal cells were enriched by adherence by plating peritoneal cells in FBS-free media for 1 h. Nonadherent cells were removed, replaced with fresh media, and then treated 24 h later with LPS (10 ng/ml) for 6h.

B.5 Survival study

C57/B6 male mice (n=25) at the age of 8–10 weeks old underwent cecal ligation and puncture (CLP). Mice were divided in 4 groups based on the diet and the post-CLP treatment that they received. Thus, we had 2 groups of mice fed with normal diet which received antibiotics or placebo (ND +/-Abx) and 2 groups of mice fed with high fat diet which were treated accordingly (HFD +/- Abx). The antibiotic treatment included imipenem-cilastatin, 500 μg intraperitoneally every 12 hours starting 1h postoperatively. ND- and HFD- mice received normal saline intraperitoneally instead. All mice were housed in the laboratory with unlimited access to food and water and were monitored every 12 hours for 7 days. Every event that happened in each group was recorded. All recorded data were analyzed with Kaplan-Meier analysis, using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com".

B.6 Samples' analysis

B.6.1 Protein assay

Reagents
Pierce® BCA protein assay kit [BCA reagent A and B, Albumin standard ampules, 2mg/mL]

Method

First, of all standards were prepared with a working range of 20-2000 µg/mL. Next, the total number of wells had to be calculated in order to determine the amount of working reagent needed. The calculation formula used was : (# standards + # unknowns) × (# replicates) × (volume of WR per sample) = total volume WR required. In specific, all standards and samples were analyzed in duplicates. 25µL of each sample and standard was added to the respective microplate well (e.g., Thermo Scientific™ Pierce™ 96 -Well Plates, Product No. 15041). Then, 200µL of the working reagent were added to each well and the plate was mixed thoroughly on a plate shaker for 30 seconds. The plate was covered and incubated at 37°C for 30 minutes.

Eventually, the plate was cooled to RT and measured at or near 562 nm on a plate reader.

B.6.2 Enzyme Linked ImmunoSorbent Assay (ELISA)

Interleukin-6

Reagents and Instruments

1. Human IL-6 ELISA Ready-SET-Go! ® [containing 250x Capture Antibody, 250x Detection antibody, 250x Detection enzyme (Avidin-HRP), ELISA/ELISPOT Coating Buffer Powder, 5x concentrated Assay Diluent, Human IL-6 Standard] (eBioscience, Inc., San Diego, California, USA)

2. 1x Tetramethylbenzidine (TMB) Substrate Solution (eBioscience, Inc., San Diego, California, USA)

3. Stop Solution (2N H_2SO_4)

4. Wash Buffer (1x PBS, 0.05% Tween-20)

5. 96-well plate (Nunc C8 White LockWell MaxiSorp) (Thermo Scientific, Waltham, Massachusetts, USA)

6. Model 680 Microplate Reader (BioRad, Hercules, California, USA)

Method

A 96-well plate (Nunc C8 White LockWell MaxiSorp) was coated with 100 µL/well of 1x Capture Antibody in Coating Buffer (prepared by diluting ELISA/ELISPOT Coating Buffer Powder in appropriate volume of distilled water) and left to incubate overnight at 4o C. The plate was washed three times with Wash Buffer and it was blocked with 200 µL/well of 1X Assay Diluent followed by incubation at room temperature for 1 hour. Next, the plate was washed once with Wash Buffer and the samples as well as the standards (diluted according to manufacturer's protocol) were added (100 µL/well) and left to incubate overnight at 4o C on a rocker. After four washes with Wash Buffer, incubation of 100 µL/well of 1x Detection antibody for 1 hour at room temperature, four washes with Wash Buffer and subsequent incubation of 100 µL/well of 1x Detection enzyme (Avidin-HRP) for 1 hour at room temperature, 100 µL/well of 1x Tetramethylbenzidine (TMB) Substrate Solution to each well were added and the plate was left to incubate at room temperature for 15 minutes. The reaction was stopped by adding 50 µL of Stop Solution to each well and the plate was read at 450 nm and 570 nm at a Model 680 microplate reader. Values at 570 nm were subtracted from those of 450 nm and data were analyzed by Graph Pad Prism 5 software.

CXCL2/MIP-2

Reagents
Mouse CXCL2/MIP-2 Duoset by R&D systems®

- Capture Antibody,
- Biotinylated Detection Antibody,
- Standard,
- Streptavidin-HRP
- Wash buffer
- Assay Diluent
- Substrate solution
- 2N H_2SO_4 (Stop solution)

Method

Firtst of all, the Capture Antibody was diluted in PBS to the working concentration (2µg/mL) without carrier protein. Then, a 96-well microplate was coated with 100 µL per well of the diluted Capture Antibody, sealed and incubated overnight at room temperature. Next day, all contents of wells were discarded and the microplate was washed 3 times. Then, wells were blocked with 300µL of assay diluent for 1 hour at room temperature. Following that, 3 washes were performed and 100µL of each standard or sample were added to each well respectively. The plate was incubated for 2 hours at room temperature. Again, one wash step followed. Detection antibody was then added to each well (working concentration of 75ng/mL) and the plate was incubated for 2 hours at room temperature. Next, after a wash step, 100 µL of streptavidin-HRP were added to each well and the plate was incubated for 20min at room temperature in a dark place. A wash step was then repeated and substrate solution was added into each well for 20 min (RT, dark place). Finally, the reaction was stopped by adding 50µL of stop solution to each well and the plate was then placed to a plate reader set to 450 nm.

Insulin

Reagents
ALPCO® Mouse insulin ELISA [Cat. No. 80-INSMS-E01 E10] containing insulin microplate (96 wells), Zero Standard, Standards (A-E)
(0.188, 0.5, 1.25, 3.75, 6.9 ng/mL), Control Levels 2 and 3, 11X Conjugate Stock,
Conjugate Buffer, 21X Wash Buffer Concentrate, TMB Substrate, Stop Solution.

Method
All reagents and microplate strips were equilibrated to room temperature (18-25°C)
prior to use. All reagents were gently mixed before use. A standard curve was performed.
All standards, controls, and samples run in duplicate.
1. 10 µL of each standard, control, and sample were put into their respective wells.
2. Next, Pipette 75 µL of Working Strength Conjugate into each well.
3. Then, microplate was covered with a plate sealer and was incubated for 2 hours at room temperature, shaking at 700-900 rpm on a microplate shaker.
4. The contents of the wells were discarded and the microplate was washed 6 times with 350 µL of Working Strength Wash Buffer per well.
5. 100 µL of TMB Substrate into each well.
6. Again, the microplate was covered with a plate sealer and was incubated for 15 minutes at room temperature, shaking at 700-900 rpm on a microplate shaker.
7. 100 µL of Stop Solution was added into each well.
8. Finally, the microplate was placed in a microplate reader capable of reading the absorbance at 450nm.

B.6.3 Flow Cytometry

Reagents
- Fixation/Permeabilization solution (125 ml)
- BD Perm/Wash™ Buffer, 10 × concentrate containing Fetal Bovine Serum (FBS)
- saponin (dilute 1:10 in distilled H20 prior to use) (100 ml)
- Monoclonal antibodies : PerCP-Cd11c, APC-Arg1, FITC-iNOS

Method

Harvest Cells
Bronhoalveolar fluid cell populations were prepared. The cells were resuspended in staining media, counted and transferred to plastic tubes for immunofluorescent staining. Cells should be protected from light throughout staining and storage.

1. First step was to block Fc receptors. Reagents that block Fc receptors are useful for reducing nonspecific immunofluorescent staining. In the mouse system, purified 2.4G2 antibody, specific for FcγII/III receptors (BD Fc Block™; Cat. No. 553142), can be used to block nonspecific staining by fluorochrome-conjugated antibodies which is mediated by Fc receptors. To block mouse Fc receptors with Fc Block, preincubate

cell suspension with 1 µg BD Fc Block/10^6 cells in 100 µl of Staining Buffer for15 minutes at 4°C. The cells can then be washed and stained with a fluorochrome-conjugated antibody specific for a cell surface antigen of interest which should be diluted appropriately in Staining Buffer.

2. Then, cell surface antigens were stained. Approximately 10^6 cells were stained in 50 µl of Staining Buffer with the appropriate amount of a fluorochrome-conjugated monoclonal antibody (PerCP-Cd11c) (30 min, 4°C).
3. Cells were washed twice with Staining Buffer (e.g. 1 ml/wash for tubes) and pellet by centrifugation (250 × g).
4. Fixation and Permeabilization Protocol
   Cells were resuspended in 100µl of a 4% paraformaldehyde solution at 4°C for 10-20 minutes. Wash cells 2× in Staining Buffer. Cells were resuspend in Staining Buffer for storing. Cells were fixed and stored to continue the intracellular staining the following day.
5. Permeabilizing Fixed Cells.
   Cells were centrifuged and the the staining buffer was discarded. Then, cells were resuspend in BD Perm/Wash™ buffer for 15 minutes. Centrifugation.
6. Stain for intracellular markers Arg1 and iNOS.
   Thoroughly resuspend fixed/permeabilized cells in 50 µl of BD Perm/Wash™ buffer containing a pre-determined optimal concentration of a fluorochrome-conjugated anti-cytokine antibody or appropriate negative control. Cells were incubated at 4°C for 30 minutes in the dark. Then, cells were washed twice with 1× BD Perm/Wash™ buffer (1 ml/wash) and resuspended in Staining Buffer prior to flow cytometric analysis.

B.7 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com and p value < 0.05 was considered as indicative of statistical significance. Furthermore, One-way ANOVA analysis was performed. Tolerance tests were analyzed by Two way ANOVA test with Bonferroni post-test.

C. RESULTS

C.1 Both short and long term high fat diet increased the body weight of mice compared to normal diet-fed mice.

Mice fed with high fat diet for 8 weeks gained significantly more weight compared to standard diet-fed mice. The same effect, in smaller extent, was observed in short term high fat diet-fed mice respectively (Table 1).

Diet	Mean Body Weight in g. +/- SEM
Long term HFD (N=10) *	34.11 ± 1.395
Long term ND (N=8)	27.98 ± 0.4825
Short term HFD (N=14)**	29.02 ± 0.3638
Short term ND (N=14)	27.64 ± 0.4883

Table 1. Body weight of long term ND and HFD-fed mice, * p<0.05 Long HFD vs ND t test, **p<0,05 Short HFD vs ND t test).

C.2 Glucose tolerance was impaired in high fat diet-fed mice

C.2.1 Long term high fat diet

Mice (n=8) were divided in 2 groups (4 per group) and fed either with normal or with high fat (60%) diet for 8 weeks. Following that, mice were fasted for 6 hours and glucose tolerance test was performed for each mouse. It was proved that glucose tolerance was impaired in mice fed with the high fat diet (Figure 1).

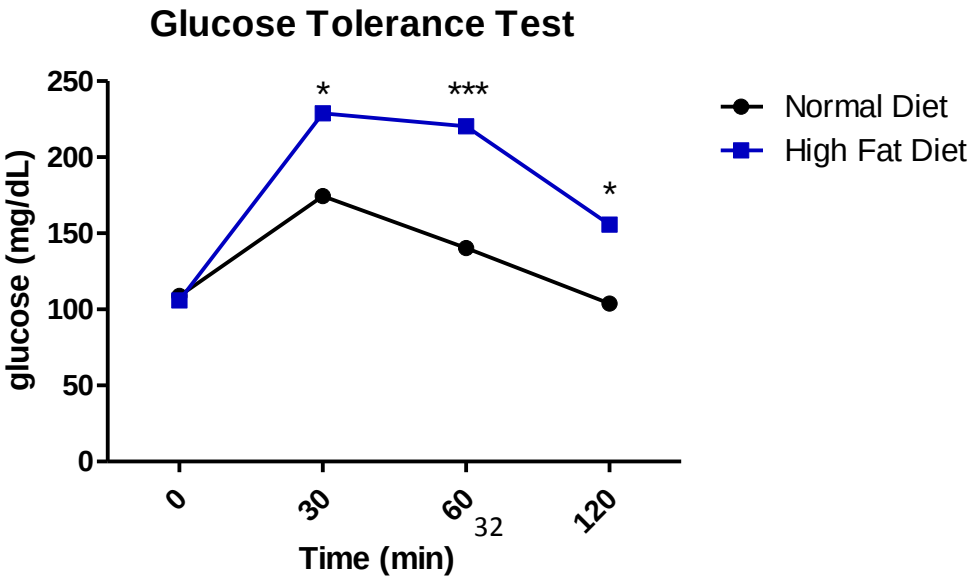

Figure 1 Blood glucose levels in fasted adult mice after 8 weeks of either normal or HF (60%) diet following a glucose injection (1mg/gr of BW) at time 0min. Values represent mean +/- error. (*, p<0.05, ***, p<0.001).

C.2.2 Short term high fat diet

To assess the metabolic effect of high fat diet shown previously, glucose tolerance test was also performed after a short period of the special diet (4 days). As it was expected, due to similar published data [8], the result was in accordance with GTT of long term HFD-fed mice. Mice were glucose intolerant (Figure 2).

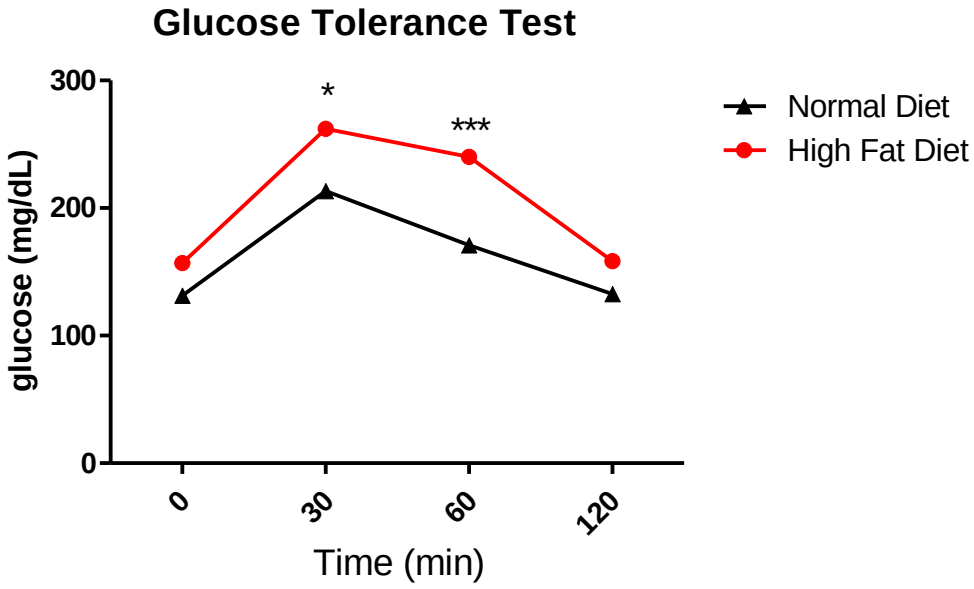

Figure 2 Blood glucose levels in fasted adult mice after 4 days of either normal or HF (60%) diet following a glucose injection (1mg/gr of BW) at time 0min. Values represent mean +/- error (*, p<0.05, ***, p<0.01).

C.3 Insulin resistance

Having already shown that both short and long term HFD causes glucose intorerance, it was examined weather this intolerance is associated with hyperinsulemia. To determine that, blood serum insulin levels were measured in fasted mice.

C.3.1 Long term HFD induces insulin resistance

Mice (n=8) were grouped based on the diet they were fed for 8 weeks. High fat diet-fed mice were found to have significantly increased insulin levels compared to normally-fed mice (Figure 3).

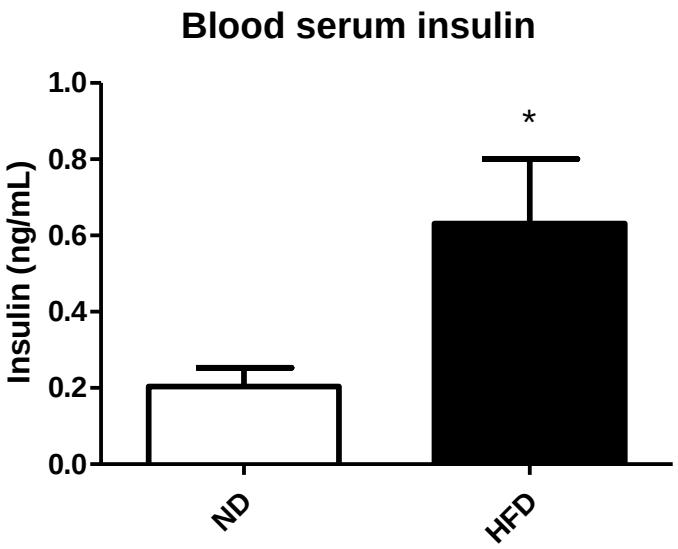

Figure 3

Serum insulin levels of either ND or HFD-fed mice, all fasted for 6h (*, $p<0.05$).

C.3.2 Short term HFD does not induce hyperinsulinemia.

Similarly, there were 2 groups of mice (n=4 per group) regarding the diet they were given. There is no significant difference in the blood serum insulin levels between the 2 groups of mice (figure 4).

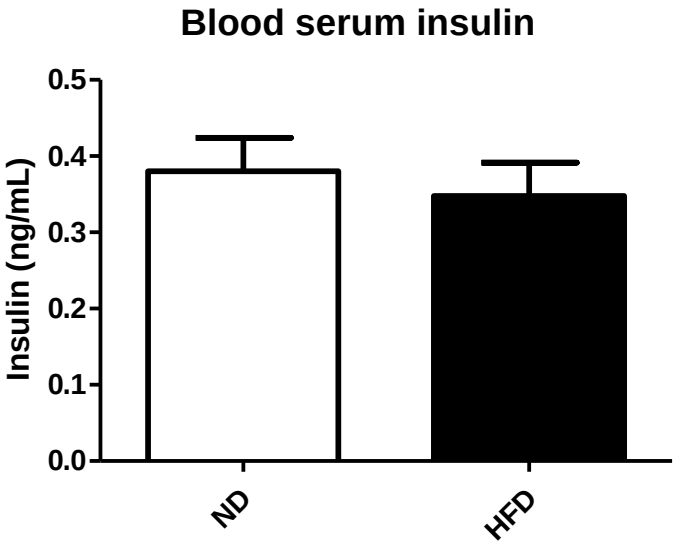

Figure 4 Serum insulin levels of either ND or short term HFD-fed mice, all fasted for 6h.

C.4 Peritoneal macrophages isolated from HFD-fed mice recorded lower values of pro-inflammatory cytokines upon LPS stimulation.

Peritoneal macrophages isolated from HFD-fed mice had decreased response to 6h LPS treatment compared to macrophages acquired from ND-fed mice.

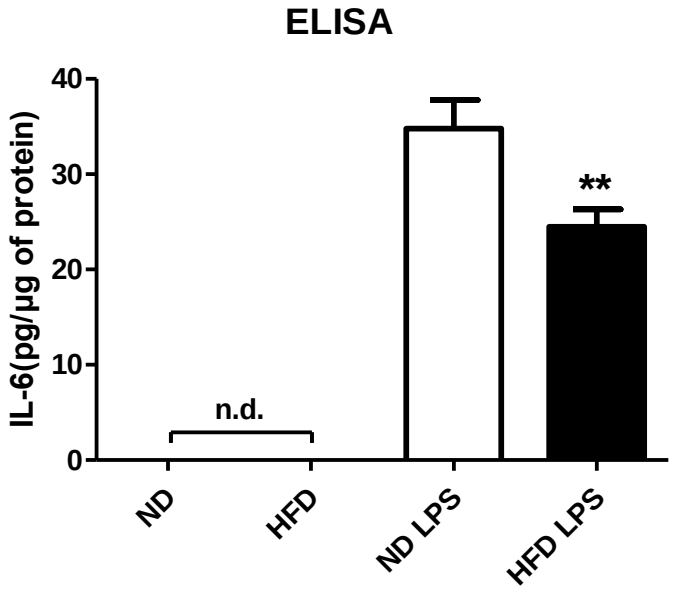

Figure 5 Levels of pro-inflammatory cytokine IL-6 secreted by peritoneal macrophages upon LPS treatment. Macrophages were isolated from mice fed either ND or HFD for short term period (**p<0.05).

C.5 Alveolar macrophages of septic HFD-fed mice showed increased Arg1 expression.

In order to evaluate whether there is an association between the aforementioned protective effect of HFD-induced insulin resistance upon sepsis outcome and macrophage phenotype, alveolar macrophages were analyzed for M1/M2 markers. The FACS analysis showed that macrophages acquired from mice fed with HFD, either sham or CLP, expressed higher levels of Arg1 compared to macrophages isolated from ND-fed mice (Figure 6). iNOS expression seemed to be slightly diminished in alveolar macrophages from HFD-fed mice, but that was not proved to be statistically significant though. This result implements that HFD-induced insulin resistance drives alveolar macrophages towards M2 activation phenotype.

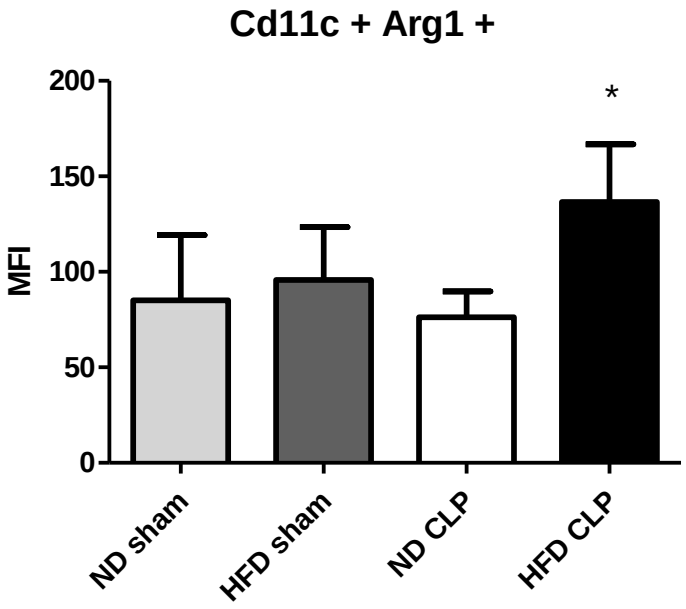

Figure 6 Mean Fluorescent Intensity of Arg1 marker in alveolar macrophages acquired from sham/CLP operated mice that were previously fed with either normal or high fat diet for 8 weeks (vs ND CLP *, $p<0.05$).

C.6 Evaluation of acute lung injury and sepsis in CLP-induced septic mice

Acute lung injury is characterized by high permeability pulmonary edema, indicated by increased concentration of protein in BAL fluid and lung inflammation. To evaluate the presence of injury we measured protein and inflammation as BAL fluid cytokines.

Mice (n=27) were fed either with normal or high fat diet. All these mice underwent cecal ligation and puncture (CLP) or sham operation as a control. 6 hours post-operatively mice were sacrificed and samples were collected.

C.6.1 Protein concentration in BALF was unaltered.

Protein concentration of BAL fluid was measured in bronchoaleolar fluid and it was observed that no significant change in levels of protein occurred neither between CLP and sham operation nor between normal and HFD. The same result arises from both short and long term HFD experiments (Figure 7, 8).

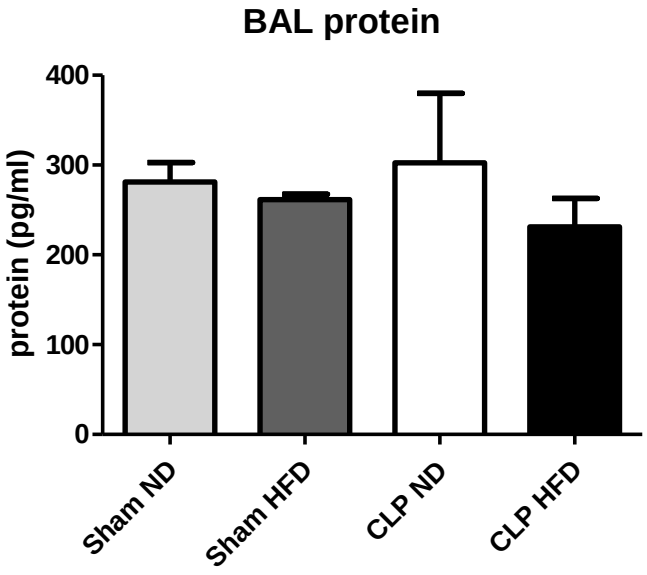

Figure 7 Short term HFD/ND. Protein concentration (pg/ml) of BAL fluid in ND/HFD fed mice that underwent either CLP or sham operation ($p<0.05$).

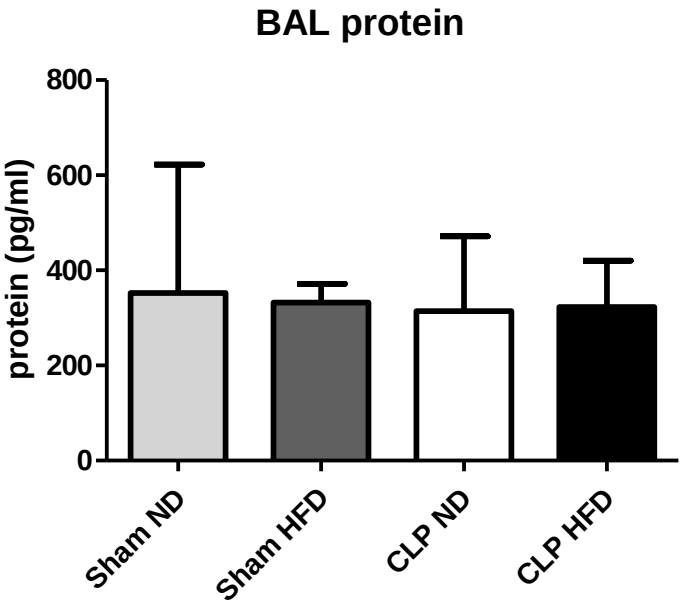

Figure 8 Long term HFD/ND. Protein concentration (pg/ml) of BAL fluid in ND/HFD fed mice that underwent either CLP or sham operation ($p<0.05$).

C.6.2 HFD-fed septic mice showed decreased levels of inflammatory cytokines in BALF.

BALF analysis revealed that HFD-fed mice, which underwent CLP operation, had significantly lower levels of pro-inflammatory cytokine interleukin-6 (IL-6) and macrophage inflammatory protein-2 (MIP-2/CXLC-2) compared to normal diet-fed septic mice (Figure 9, 10).

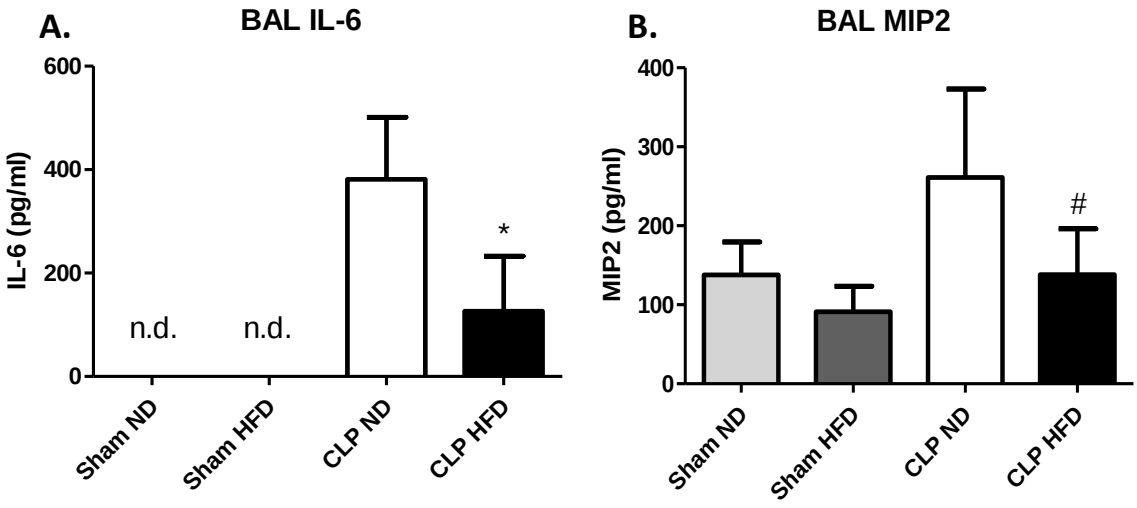

Figure 9 A. Levels of inflammatory cytokines IL-6 and **B.** MIP-2 in BALF collected from short term HFD/ND fed mice which underwent either CLP or sham operation. BALF was collected 6 hours post-operatively (vs CLP ND *, p<0.001, #, p<0.05).

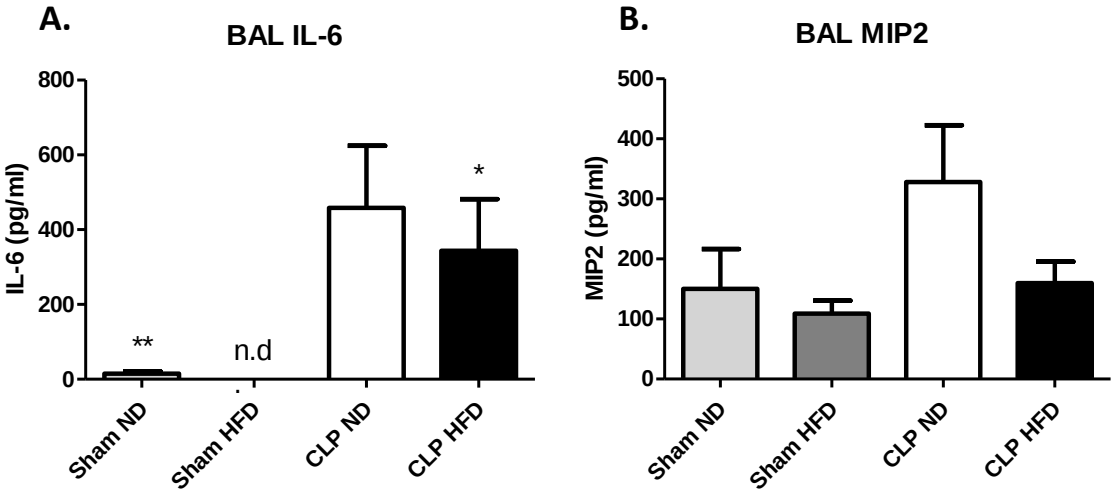

Figure 10 Levels of inflammatory cytokines **A.** IL-6 and **B.** MIP-2 in BALF collected from long term HFD/ND fed mice which underwent either CLP or sham operation. BALF was collected 6 hours post-operatively (vs sham ND*, p<0.05, vs CLP ND**, p<0.05).

C.6.3 HFD-fed septic mice demonstrated lower levels of inflammatory cytokines in blood serum samples.

Aside from acute lung injury, to determine the systemic injury due to sepsis blood samples were analyzed for inflammatory cytokines. 6 hours post-operatively blood samples were acquired from mice which underwent CLP or sham operation. The levels of inflammatory cytokines interleukin-6 and macrophage inflammatory protein-2 (IL-6 & MIP-2) were measured. The analysis revealed that mice which were fed with HFD (either for short or long term period) had diminished levels of both cytokines in blood serum (Figures 11, 12).

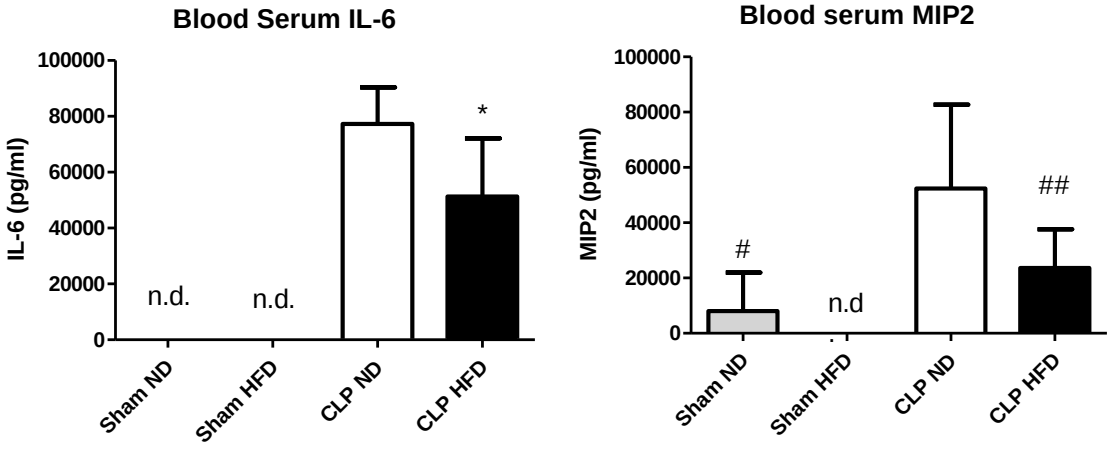

Figure 11 Inflammatory cytokines IL-6 and MIP-2 levels in blood serum samples of mice fed with ND/HFD for 4 days. Mice underwent either CLP or sham operation and blood serum was collected 6 hours post-operatively (vs CLP ND*, p<0.05, #p<0.05, ##, p<0.05).

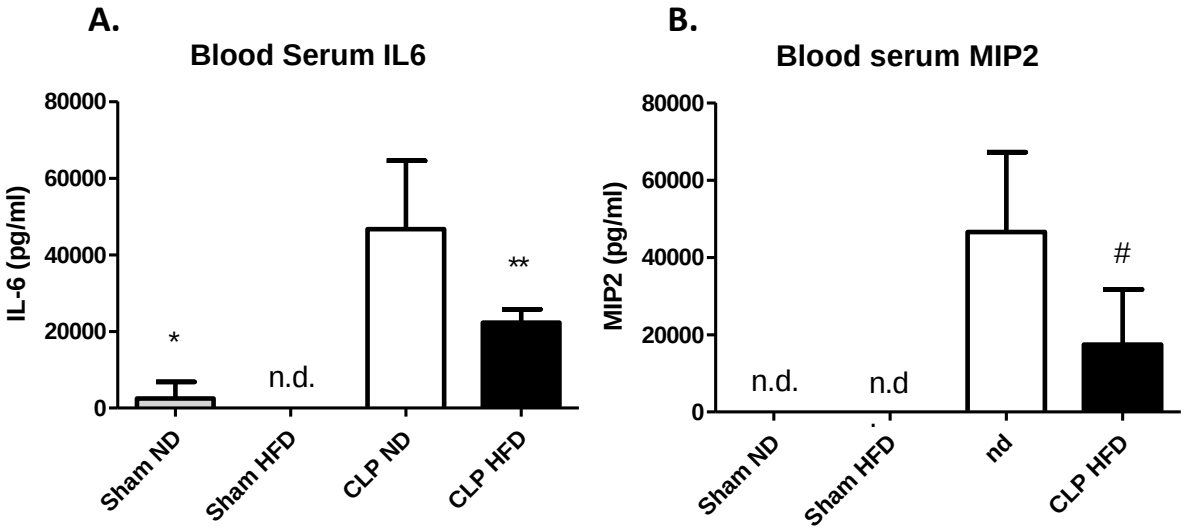

Figure 12 Inflammatory cytokines **A.** IL-6 and **B.** MIP-2 levels in blood serum samples of mice fed with ND/HFD for 8 weeks. Mice underwent either CLP or sham operation and blood serum was collected 6 hours post-operatively (*, p<0.05, **, p<0.01, #, p<0.05).

C.6.4 Histological analysis of lung sections

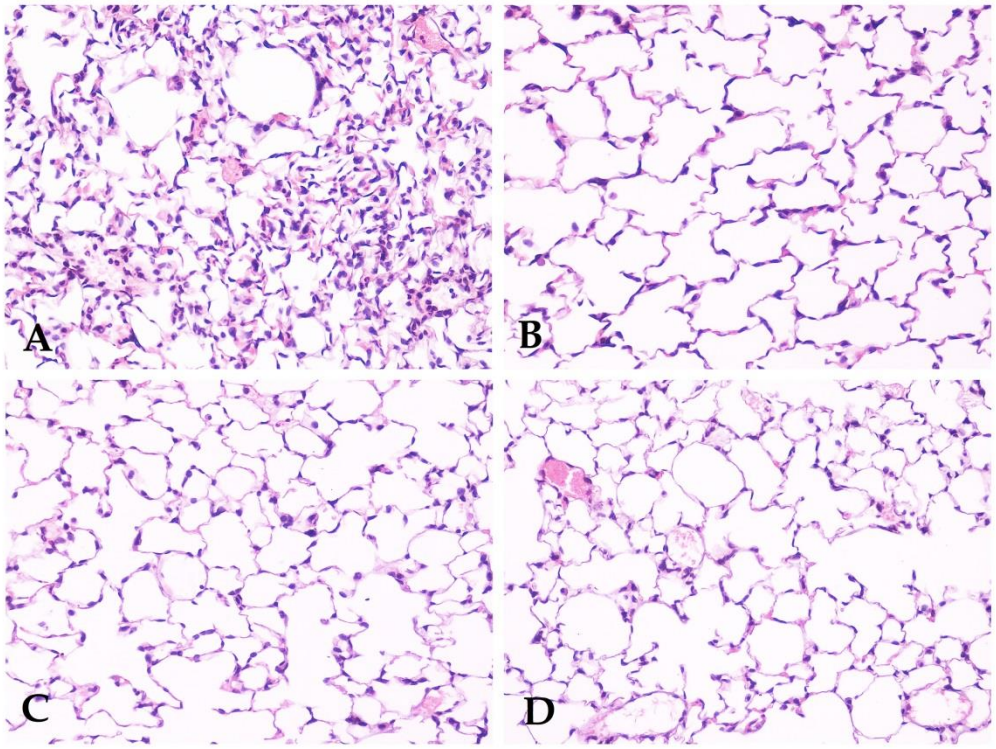

Figure 13 Histopathology of septic mice which followed long term ND/HFD.

On histological examination lungs from CLP induced septic mice subjected to long term High Fat Diet (**C**) displayed a minor lung injury with preservation of the lung architecture, only occasional presence of neutrophils in the alveolar walls and mild increment of alveolar macrophages, while mice subjected to Normal Fat Diet (**A**) displayed severe histopathological damage with accumulation of neutrophilic infiltrates in the alveolar septa and alveoli, proteinaceous debris in the alveolar spaces, thickening of the alveolar walls, focal hyaline membrane formation, capillary congestion and intra-alveolar hemorrhage. Both ND and HFD sham mice (**B** and **D** respectively) displayed no significant histopathological changes. Formalin fixed paraffin embedded lung tissue sections. Magnification X400.

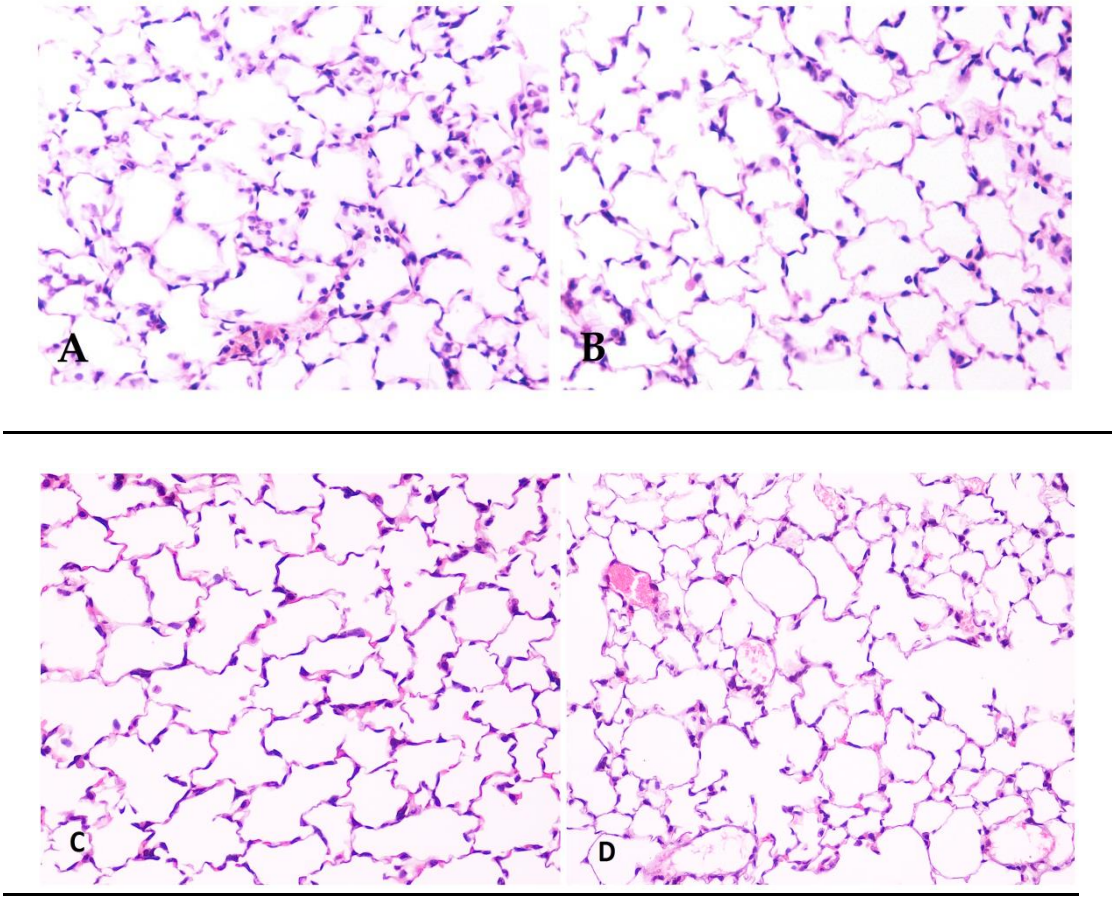

Figure 14 Histopathology of septic mice which followed short term ND/HFD.

Similar discrepancy in the lung injury, but of minor degree and extension, was observed in the short term Normal Fat Diet (**A**) and High Fat Diet fed mice (**B**) subjected to CLP procedure with the former displaying several foci of interstitial and intra-alveolar neutrophilic infiltrations, capillary congestion and proteinaceous debris in the alveoli, while the latter displayed only occasional interstitial and intra-alveolar neutrophilic infiltrates, mild septal thickening and sparse intra-alveolar proteinaceous deb. Both ND and HFD sham mice (**C** and **D** respectively) displayed no significant histopathological changes. Formalin fixed paraffin embedded lung tissue sections. Magnification X400.

C.7 CLP-induced mortality was similar between HFD/ND-fed mice.

All mice underwent CLP procedure and then, the survival was recorded. As it shown in figure 15, there was no statistically significant difference between groups. Mice that were fed with high fat diet showed similar survival rate compared to normal diet-fed mice. The same effect is observed in the presence of antibiotic therapy.

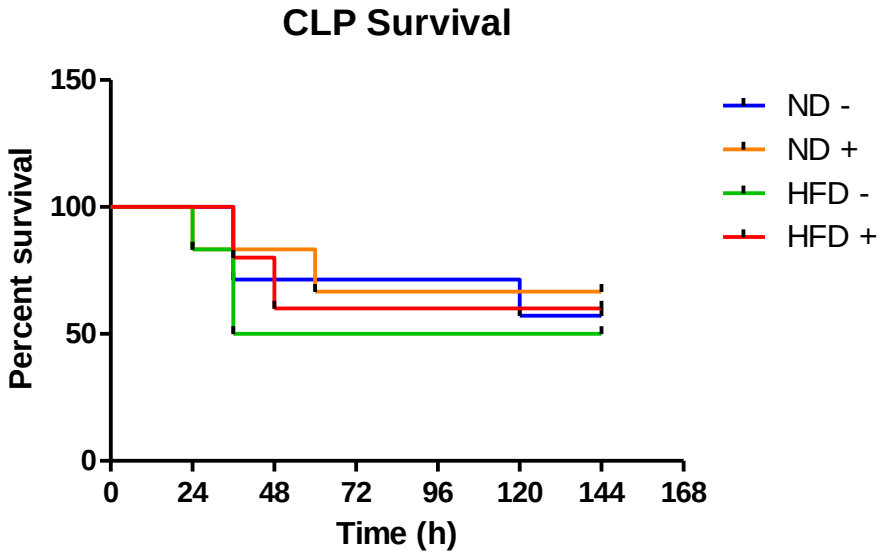

Figure 15 Survival rate of mice fed with HFD/ND after CLP-induced sepsis with antibiotics or placebo.

DISCUSSION

"Obesity paradox", the unexpectedly lower mortality in ICU of obese patients, is still poorly understood. Earlier studies have linked obesity and insulin signaling with macrophage polarization [73, 74]. Concomitantly, it is known that modulation of M1/M2 can affect the severity of inflammatory diseases. In the present study we show that HFD promotes M2 phenotype of alveolar and peritoneal macrophages through insulin resistance. Furthermore, we prove that M2 macrophages mediate a protective effect in sepsis and ARDS in a clinically relevant model of polymicrobial sepsis.

First of all, we recorded that high fat diet induces glucose intolerance and hyperinsulinemia. Both glucose intolerance and hyperinsulinemia imply that insulin resistance is present. This finding was clear in mice fed with long term HFD. Short term HFD also caused impaired glucose tolerance but did not result in overt hyperinsulinemia yet. These results are consistent with other studies. In specific, glucose tolerance and impaired insulin sensitivity were recorded in short term HFD, despite the fact that fasting insulin and glucose levels did not change accordingly [70, 71]. Additionally, preliminary data from our lab showed that peritoneal macrophages isolated from HFD-fed mice had less glucose uptake upon insulin stimulation than ND-fed mice (Ieronymaki et al. unpublished). Therefore, we have a clear picture of both short and long term HFD-induced insulin resistance. However, Liu H. et al suggest that initial stage of HFD-induced insulin resistance is independent of inflammation, whereas the more chronic state of insulin resistance in established obesity is largely mediated by macrophage-induced proinflammatory actions [97].

Furthermore, we know that both insulin and insulin signaling play a critical role in obesity-induced insulin resistance [99]. In specific, it is known that basal level of the Akt-dependent classical insulin signaling is increased in B6 mice with obesity and insulin resistance. Thus, the increase of insulin signaling is responsible for the development of insulin resistance induced by HFD [98].

Moreover, several studies have linked obesity and insulin resistance with macrophage polarization [73, 74]. Other studies show that there is also a connection between insulin signaling in particular and macrophage polarization. In specific, Akt2 is an important player of insulin signaling. Akt2 deficient mice, which are insulin resistant [95], are prone to M2 macrophage polarization [81]. In addition, preliminary data from our lab indicate that macrophages treated with insulin become insulin resistant and further LPS stimulation drives macrophages towards M2 polarization. Apparently, it is both directly and indirectly indicated that obesity and HFD promote M2 polarization of macrophages upon stimulation through insulin resistance.

More preliminary data from our lab suggest that the cytokine production of these insulin pre-treated macrophages upon LPS is reduced. Accordingly, we found that peritoneal macrophages isolated from HFD-fed mice secreted less pro-inflammatory cytokines upon LPS stimulation than macrophages from ND-fed mice. These findings suggest that macrophages not only mediate HFD-induced insulin resistance, but also acquire an insulin resistant status upon stimulation themselves.

Between, it is known that modulating the M1/M2 polarization status of macrophages can affect the severity of inflammatory diseases such as inflammatory bowel disease [96]. Recent study concluded that ablation of Akt2, which promotes M2 macrophage polarization, partially protects mice from DSS-induced inflammatory bowel disease [81]. Meanwhile, Vergadi et al has similarly proved that Akt2-deficient mice, which are known to be insulin resistant and have M2 macrophages [81], are protected from acid-aspiration induced ARDS. In the present study we reported that alveolar macrophages from HFD-fed septic mice have an M2 phenotype. Therefore, we tested the hypothesis that mice fed with HFD would be protected from sepsis and ARDS.

In our study overall better response to sepsis and secondary ARDS was observed in HFD-fed mice post CLP. In detail, we reported lower pulmonary and systemic inflammatory response, expressed as decreased cytokine concentrations in BALF and serum. In addition, lung histological analysis showed significantly less inflammatory lesions in HFD-fed mice. Consequently, HFD-induced M2 macrophages have a protective effect in CLP-induced sepsis and ARDS. This observed phenotype is inducible not only in mice that follow long term HFD, which truly present an obese phenotype, but also in mice that follow short term HFD. This observation suggests that only few days of HFD are enough to establish the obesity-induced changes in insulin signaling. This is very important because short term HFD-induced altered insulin signaling ultimately leads to milder phenotype of sepsis and ARDS.

Furthermore, we tested the survival of CLP-induced septic mice. The survival rate was similar between HFD- and ND-fed mice regardless of antibiotic therapy. There was no statistically significant difference between groups. This observation gives rise to questions about the success of antibiotic therapy. The number of mice studied or the efficacy of the administration of antibiotics or the absorbance of the agent are possible explanations. Apparently, this issue needs to be addressed and other options of administration could be considered.

Former studies exhibited ambiguous results. Kaplan et al demonstrated that, short term high fat feeding increases organ injury and mortality after polymicrobial sepsis [72]. In specific, mice on a HFD had a lower probability of survival and more severe lung injury compared with ND-fed mice following sepsis. However, HFD-fed mice had lower plasma cytokine concentration 3 hours post CLP-induced sepsis, which is in line with our results [72]. Another study with a similar model of sepsis found that mice fed a HFD and infected with *Staphylococcus aureus* or influenza had higher mortality than mice fed a control diet [84, 85]. Such disagreement could be attributed to differences of experimental model.

In summary, we found that HFD-induced insulin resistance does really promote macrophage polarization towards M2 and these M2 macrophages mediate a milder phenotype of sepsis complications.

To conclude, the current study attempts to bridge insulin signaling with inflammatory response and sepsis outcome. The presented data suggest that insulin signaling influences macrophage function and this is part of the mechanism by which obesity-induced insulin resistance ameliorates the outcome in sepsis and secondary ARDS. Certainly, more information is needed in order to characterize and fully understand the

effect of insulin resistance on sepsis outcome. Thus, in the long term this study could lead to an overall picture of pathophysiology of “obesity paradox”, which will hopefully provide useful endpoints for clinical practice.

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