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Mitochondrial functions in the modulation of immunometabolism: physiological and pathological aspects

Tutor

Prof. Nazzareno Capitanio

PhD Student

Dr. Marta Menga

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Sommario

Abstract

Recent studies on the metabolism of immune cells have highlighted the tight link between metabolic state and phenotype of these cells. An emerging concept is that switches in metabolic profile not only sustain the immune response as a consequence of alterations in cellular signalling, but also feed back and alter signalling to drive immune-cell phenotype.

On this basis, since the interplay of cellular bioenergetics, metabolism and inflammation occurs in both innate and adaptive immune responses, targeting mitochondrial metabolism is becoming an attractive therapeutic strategy for modulation of the immune response in inflammatory pathological states. In this study we used two *in vitro*-models of physically/biologically-stimulated myeloid cells to determine metabolic check-points responsible for the linkage between inflammation and metabolism, in order to identify metabolic pharmacological targets to hamper the pro-inflammatory metabolic priming of immune cells.

In the first part of this study we used the murine macrophage RAW 264.7 cell line as an *in vitro* model to investigate the consequences of lipopolysaccharide (LPS)-mediated activation on mitochondrial metabolism and the effect on this of p-hydroxyphenylpyruvate (pHPP), an intermediate of the phe/tyr catabolic pathway that in addition to provide intermediates of the TCA cycle (i.e. fumarate and acetylCoA) proved to exhibit antioxidant and anti-inflammatory activities. Treatment of RAW 264.7 cell line with a concentration of LPS comparable with that detected in sera of septic patients resulted in up-regulation of the inducible nitric oxide synthase (iNOS) expression and consequently of nitric oxide (NO) production and in increased production and release of the pro-inflammatory cytokine Interleukine-6 (IL-6). Respirometric and metabolic flux analysis of LPS-treated RAW 264.7 cells highlighted a marked metabolic shift consisting in downregulation of the mitochondrial oxidative phosphorylation (OxPhos) and upregulation of aerobic glycolysis. Indeed we found a significant 50% inhibition of the mitochondrial respiration in intact LPS-challenged RAW 264.7 and also of the specific activities of Complex I (CxI) and Complex IV (CxIV) of the respiratory chain. The LPS-mediated decrease of the OxPhos activity was accompanied with compensatory up-regulation of the aerobic glycolysis and enhanced production of reactive oxygen species. Inhibition of the respiratory activity was also observed following incubation of human neonatal fibroblasts (NHDF-neo) with sera from septic patients. pHPP inhibited all the above described alterations both in LPS-

stimulated RAW 264.7 and in septic sera-treated-NHDF-neo. The effect of pHPP likely results from a combination of direct antioxidant and mitochondrial "bioenergizing" activities.

As regardes the second part of this research project, we used human monocyte-derived immature dendritic cells (MoDCs) challenged with a relatively short-time (3h) thermal shock (39°C) to mimic a fever-like condition, deepen and investigate its direct effect on dendritic cell metabolism. Fever-like temperature is known to stimulate innate and adaptive immune responses by inducing specific immunophenotypes and gene expression profiles. Considering that mitochondrial metabolism is now recognized to play a pivotal role in the contest of the immune response both as energy supplier and as signaling platform, we asked if mimiking fevrile conditions in MoDCs could cause alterations in the mitochondrial oxidative metabolism. The results obtained clearly show that the mild hyperthermic stress resulted in enhanced TNF-α-release by dendritic cells. This immune stimulation was accompanied with rewiring of the oxidative metabolism characterized by decrease of the mitochondrial respiratory activity, enhanced production of reactive oxygen and nitrogen species and accumulation of mitochondrial Ca^{2+}. The hyperthermia-induced impairment of the mitochondrial OxPhos is irreversible, since the re-conditioning of cells to normothermia did not recover the respiratory chain activity. Moreover, this inhibitory effect is mimicked by exposing normothermic cells to the conditioned medium of the hyperthermia-challenged cells, suggesting the involvement of released mediators and it is largely prevented by the inhibitor of the mitochondrial calcium porter Ruthenium Red. These observations combined with gene expression analysis support a model based on an autocrine signaling of heat shock proteins via toll-like receptors and thermal activation of transient receptor potential cation channels.

In conclusion the results presented in this study support the emerging notion that the immunological response elicited either by physical and biological stimuli involves and requires specific metabolic reprogramming of immune-competent cells. In cases of dysregulated immune response, modulation of the metabolic rewiring at specific check-points could represent an effective pharmacological strategy to prevent the pro-inflammatory metabolic priming of immune cells, attenuating certain symptoms induced by endotoxemia as well as by febrile temperatures.

Abbreviations

Normal Human Dermal Fibroblasts (NHDF), Murine Macrophages (RAW 264.7). Human Monocyte-derived Dendritic Cells (MoDCs). Lipopolysaccharide (LPS), Complex I, II, III, IV of the mitochondrial respiratory chain (CxI, CxII, CxIII, CxIV), Oxygen Consumption Rate (OCR), Extracellular Acidification Rate (ECAR), Reactive Oxygen Species (ROS), Reactive Nitrogen Species (RNS), Nitric Oxide (NO), Hypoxia Induced Factor (HIF), Toll like receptors (TLRs), Para-hydroxyphenyl Pyruvate (pHPP), Ethyl Pyruvate (EP).

Introduction

Recent studies on the metabolism of immune cells have highlighted the tight link between metabolic state and phenotype of these cells. Immune cells are highly dynamic in terms of their growth, proliferation and effector functions and they can adopt distinct metabolic configurations that allow the cell to balance its requirements for energy, molecular biosynthesis and longevity [1]. However, in addition to drive immune cell activity in response to alterations in cellular signalling, it is now becoming clear that cellular metabolism has a direct role in regulating immune cell function. Emerging data, in fact, support that switches in bioenergetic profile inextricably link metabolism with inflammation and immunity to protect cells and organisms and to restore homeostasis [2].

1.1 Sepsis

Sepsis is a systemic illness that originates from a break of integrity of the host barrier, either physical or immunological, and direct penetration of the pathogen into the bloodstream, creating the septic state. It is as an infection-induced syndrome characterized by a generalized inflammatory state and it represents a frequent complication in surgical patients. Sepsis develops when the initial, appropriate host response to an infection becomes amplified and is followed by dysregulation. This becomes “severe sepsis” when it is accompanied by evidence of hypoperfusion or dysfunction of at least one organ system, whereas the term “septic shock” is used if severe sepsis is associated to hypotension or need for vasopressors, despite adequate fluid resuscitation. Increasing severity correlates with increasing mortality, which rises from 25-30% for severe sepsis up to 40-70% for septic shock [3]. Usually a potent immunological cascade ensures a protective response to microbial invasion, while in septic shock it may damage the host through a dysregulated release of endogenous inflammatory compounds due to an excessive immune response to the offending organism (Gram-negative or Gram-positive bacteria, fungi, viruses or microbial toxins). The mechanisms involved in the pathogenesis of septic shock include the release of cytokines, the activation of neutrophils, monocytes and microvascular endothelial cells, as well as the activation of neuroendocrine reflexes and plasma protein cascade systems, such as the complement system, the intrinsic (contact system) and extrinsic pathways of coagulation and the fibrinolytic system [4]. Severe sepsis caused by Gram-negative invasive infection is associated to endotoxemia because of the

presence of circulating LPS (Lipopolysaccharide), an important component of the Cell wall of Gram-negative bacteria, responsible for severe sepsis [5]. High levels of endotoxin can cause changes in the expression of more than 300 genes in activated macrophages, neutrophils, dendritic and endothelial cells and give rise to the coagulation cascade [6].

1.2 Sepsis and Innate Immune Response

The inflammatory response is partly mediated by innate immune cells, such as macrophages, neutrophils, Dendritic Cells and natural killer T cells, which can enhance or suppress host inflammation by producing pro-inflammatory cytokines or inhibitory cytokines [7, 8]. The innate immune system is an evolutionarily conserved system acting as a first-line of defense against invading microbial pathogens and other potential threats to the host. "Innate" immunity refers to immune responses that are present from birth and not learned, adapted, or permanently heightened as a result of exposure to microorganisms, in contrast to the responses of T and B lymphocytes in the adaptive immune system [9].

A range of pattern recognition receptors (PRRs) recognize specific pathogen-associated molecular patterns (PAMPs) exclusively present on microbes such as viruses, bacteria, parasites and fungi. In addition, PRRs are involved in sensing endogenous 'danger' signals by recognizing danger-associated molecular patterns (DAMPs). The PRR family of the innate immune system comprises: the Toll-like receptors (TLRs), C-type lectin receptors, retinoid acid-inducible gene-1-like receptors, and NOD-like receptors (NLRs; nucleotide-binding oligomerization domain receptors) [10]. Toll-like receptors (TLRs) are a family of surface receptors usually located on plasma-membrane of innate immune cells, that can specifically recognize molecules shared by a variety of microbial components. Based on the TLR cellular localization and PAMP ligands, this family is divided into two groups. The first members (TLR3, TLR7, TLR8, and TLR9) are located in the endoplasmic reticulum, endosomes and lysosomes; the second (TLR1, TLR2, TLR4, TLR5, and TLR6) are expressed on cell membranes and sense different PAMPs, including fungal cell wall components, LPS, bacterial flagella and peptidoglycan [11]. Activation of PRRs by microorganisms, toxins, chemical compounds, cytoplasmic PAMPs and/or endogenous DAMPS triggers intracellular signalling leading to inflammasome generation. For example, upon LPS recognition, TLR-4 undergoes oligomerization and recruits its downstream adaptors through interactions with the TIR (Toll-interleukin-1 receptor)

domains. There are five TIR domain-containing adaptor proteins: MyD88 (myeloid differentiation primary response gene 88), TIRAP (TIR domain-containing adaptor protein, also known as Mal, MyD88-adapter-like), TRIF (TIR domain-containing adaptor inducing IFN-b), TRAM (TRIF-related adaptor molecule), and SARM (sterile a and HEAT-Armadillo motifs-containing protein). LPS/TLR-4 signalling can be separated into MyD88-dependent and MyD88 independent pathways (Fig.1), which mediate the activation of proinflammatory cytokines and Type I interferon genes [12].

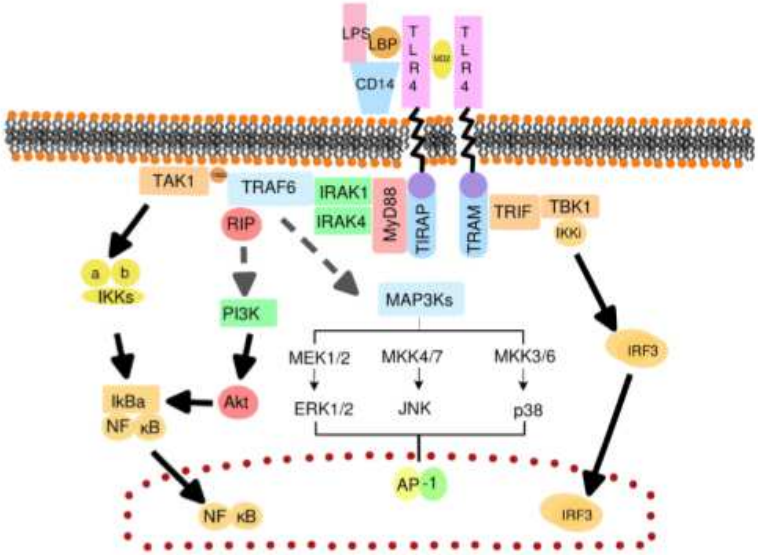

Fig.1 TLR4 Signalling.

So the innate immune cells activates a generalized response pattern, characterized by the release of secretory proteins or cytokines and reactive Oxygen and Nitrogen species (ROS and RNS), in order to enhance their phagocytic and bactericidal potential [13]. However, because of a widespread activation and dysfunction of the innate immune system, many of its components normally related to host defences against infection, might, under some circumstances, cause cell and tissue damage and result in multiple organ dysfunction syndrome (MODS) or even multiple organ failure (MOF) [14, 15].

1.3 Metabolic reprogramming in Myeloid Cells

Mature myeloid cells such as activated M1 macrophages, Dendritic cells (DCs), and granulocytes are all highly glycolytic with little or no flux through OxPhos [16], because they tend to be non proliferative and so have substantially different metabolic requirements if compared with lymphocytes.

In this cells aerobic glycolysis is induced upon activation and this early glycolytic reprogramming provides a surge of ATP for anabolic processes, which is generated under normoxia conditions, simulating the Warburg effect typical of many cancer cells. "Aerobic glycolysis" is a common feature of pro-inflammatory immune cells that use glucose not just as a fuel to generate energy but also as a source of carbon for biosynthetic processes [17], providing immune cells with the components needed to facilitate proliferation and synthesis of inflammatory molecules. So metabolic reprogramming to aerobic glycolysis gives cells enhanced biosynthetic capacity and at the same time it allows cells to adapt and survive in metabolically restrictive conditions, such as hypoxia. In fact, while hypoxia prevents efficient ATP synthesis through OxPhos, high rates of glycolysis can generate enough ATP to maintain energy homeostasis [1].

1.4 Dendritic cell activation

Although originally defined as an apparently homogeneous population of adherent stellate cells in the spleen [18], Dendritic cells (DCs) include several subsets within all lymphoid organs and the majority of peripheral tissues. Dendritic cells are the most potent and versatile antigen-presenting cells, with a unique ability to induce specific immune responses as well as tolerance [19]. In peripheral tissues they reside in an immature state waiting for incoming antigens. They act as a bridge between innate and adaptive immunity, presenting antigens and inducing a primary immune response in resting naïve T lymphocytes. There are four major subsets of DCs: conventional DCs (cDCs), Langerhans cells, monocyte-derived DCs (MoDCs) and plasmacytoid DCs (pDCs). DCs that have the characteristic ability to respond to pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) and/or to cytokines by pattern recognition receptors (PRRs). Activated DCs show large changes in metabolism and gene expression that allow them to produce mediators such as chemokines and cytokines and to degrade proteins in order to present peptide epitopes in the context of MHC I or II, to stimulate T cells in draining lymphonodes. This transition from resting cell to activated cell is accompanied by a marked change in their morphological appearance due to the fact that they become more secretory and more interactive with other cells [20], modifying their functions and migratory properties [21]. In general, DCs express CD11c and MHC class II, but once activated, they increase their expression levels of surface MHC–peptide complexes and of costimulatory molecules (CD80 and CD86), and this allows

them to activate T cells [22]. Moreover DCs activation in response to TLR agonists causes a marked increase in glucose consumption and although increased glucose uptake by DCs during the early stages after activation is accompanied by lactate production, this does not reflect a commitment to Warburg metabolism, because ATP is however provided by OXPHOS [23]. In this case glycolysis fulfills a need of citrate [23], which is than exported from mitochondria into the cytoplasm through the citrate transporter SLC25A for fueling fatty acid synthesis [20]. Increased glycolytic flux also induces considerable alterations in pentose phosphate pathway intermediates, providing NADPH, the essential cofactor for fatty acid biosynthesis. Transmission electron microscopy experiments revealed that de novo synthesis of fatty-acids promotes ER and Golgi expansion, whereas inhibition of either glycolysis or fatty acid synthesis blocks this phenomenon [23]. Thus, glycolysis drives lipogenesis to support the expansion of Endoplasmic Reticulum (ER) and Golgi apparatus and to increase the biosynthetic capacity of cytokines that is essential for mature DC function. The enlargement of ER and Golgi apparatus occurs simultaneously with increased gene expression downstream of TLRs and is also controlled by the Tbk1-IKKε complex downstream the TLRs. This complex can induce Akt-dependent phosphorylation of hexokinase II (HK-II) [23], promoting its association with voltage-dependent anion channels (VDACs) located in the outer mitochondrial membrane, to expose HK-II to high ATP concentrations, enhancing its enzymatic activity to drive glycolysis. Moreover this metabolic shift is also important in regulating DC-induced T cell responses, because it impacts upon DC lifespan and thus the duration over which DC can activate T cells [24].

Dendritic cells can also express receptors like G protein– coupled receptors (GPCRs), that allow them to respond to extracellular metabolites. For example, DCs recognize extracellular succinate through the succinate receptor GPR91, and the increase in intracellular Ca^{2+} downstream of this event promotes DC activation and their migratory ability synergistically with TLR3 or TLR7 signalling [25]. DCs can also recognize, by specific GPCRs, the short chain fatty acid butyrate [26], which is a product of commensal bacteria, adenosine/ATP [27], and lactic acid [28]. In contrast to succinate, these metabolites generally promote IL-10 production by DCs and increase their tolerogenicity.

1.5 Macrophage activation

More than 40 years ago G.C. Hard showed that activated murine peritoneal macrophages, called M1 macrophages, had lower levels of oxygen consumption and higher levels of glycolysis than resting ones [29]. M1 macrophages are part of the first line of defence of the innate immune system and they are classically activated by bacterial-derived products such as LPS, as well as by signals associated to infection, like IFNγ, which potently induce macrophages toward a highly inflammatory phenotype with high phagocytic and bactericidal potential. Following classical activation, M1 macrophages show an increase in Glut1 expression and a switch at the transcriptional level in the expression of 6-phosphofructose-2-kinase/fructose-2,6-bisphosphatase (PFK2) isoforms from the liver-form (L-PFK2) to the more active ubiquitous form (u-PFK2), leading to fructose-2,6-bisphosphaste accumulation, which pushes the glycolytic flux [30]. Glycolysis also activates the pentose shunt to provide NADPH, the essential cofactor for anabolic processes and to kill bacteria by reactive oxygen species (ROS) produced by NADPH oxidase-1 (NOX-1), a membrane-bound enzyme complex that faces the extracellular space and generates superoxide anion by transferring electrons from NADPH to molecular O_2.

At the same time, the activity of the respiratory chain is attenuated by pyruvate dehydrogenase kinase PDHK, which deactivates mitochondrial-located pyruvate dehydrogenase PDH, blocking mitochondrial acetyl-CoA production from cytosolic pyruvate [31]. As a consequence, activation of macrophages results in production of ROS and increased levels of Krebs cycle intermediates such as succinate, that was shown to drive IL1β production through HIF-1α (hypoxia-induced factor1α). Hypoxia-induced factors are heterodimeric transcription factors consisting of a constitutively expressed β subunit, and an α subunit, which is constitutively transcribed but immediately degraded in normoxia. This is achieved by the hydroxylation of proline residues by the three prolyl-hydroxylases (PHDs), which depend on oxygen, 2-oxoglutarate, Fe^{+2} and ascorbate as substrates and cofactors for their activity. Hydroxylation recruits the von Hippel Lindau protein (VHL), which forms a complex together with elongin B, elongin C, cullin-2 and ring-box1 that has an E3-ubiquitin ligase activity. This complex ubiquinates HIF1-α subunits and targets them for proteosomal degradation [32]. Although HIF-1α subunits are typically induced in hypoxia, they can also be expressed in normoxia upon inflammatory stimulation where HIF-1α is stabilized by inactivating prolyl

hydroxylase activity and transactivated by the NF-kB pathway [33], resulting in the activation of the gene encoding the PHDK [34] and in the production of pro-inflammatory cytokines, glycolytic enzymes and glucose transporters. HIF-1α pathway is essential for M1 macrophages survival and immune function, considering that they are mainly found in hypoxic environments and therefore have to rely on glycolysis for their ATP production.

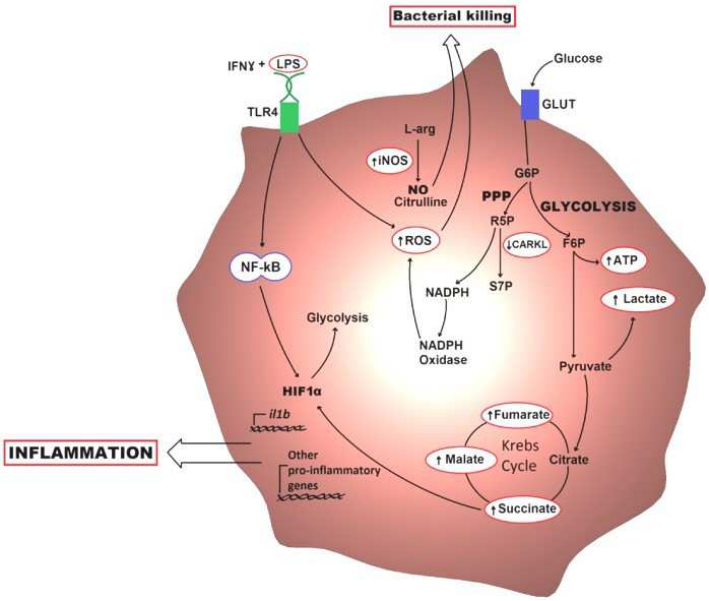

Fig.2 Metabolic profile of an M1 activated macrophage.

Another key mediator of inflammation regulated by HIF1α is the M1 marker nitric oxide synthase iNOS. In an M1 macrophage iNOS is upregulated, resulting in a rise of the arginine catabolism to citrulline and nitric oxide, that plays a key role in the intracellular killing of pathogens. Under hypoxic conditions, NO production through iNOS is HIF1α-dependent, thus implicating HIF1α in bacterial clearance [35]. Nevertheless, superoxide production during the respiratory burst, which is also required for bacterial clearance, seems to be a HIF1α-independent event [36]. Bacterial products such as lipopolysaccharide (LPS) selectively induce the expression and activation of oxidative enzymes and decrease the expression of antioxidative enzymes [37] leading to the generation and accumulation of ROS and consequent tissue damage. NOX-1, the primary NADPH oxidase in macrophages, can be both transcriptionally induced and post-transcriptionally activated by LPS. IRAK-1 is one of many intracellular signalling components downstream of the LPS receptor (TLR4) [38] and it is required for LPS-induced expression of NOX-1. IRAK-1 further contributes to LPS-induced ROS formation by suppressing the expression of

antioxidases such as GPX3 and catalase by decreasing the levels of nuclear receptors such as PPAR and PGC-1 [39].

All together, these metabolic events can provide the cell with rapid energy and reducing equivalents, which are required for bactericidal activity. Anyway, as the innate immune system activates severely enough, the host response itself can drive the patient to manifest Systemic Inflammatory Response Syndrome (SIRS) or even shock and multiple organ dysfunction syndrome (MODS) or multiple organ failure (MOF) MODS/MOF. For example, in severe human sepsis and septic shock, secondary hemophagocytic-lymphohistiocytic syndrome can develop, followed by macrophage activation syndrome (MAS), characterized by excessive activation of macrophages and increased cytokine secretion (such as IFN-γ, IL-12, IL-18) that feedback and promote macrophage activation [40].

1.6 Role and induction of fever

The fever response is a characteristic symptom of infection and inflammatory disease retained through hundreds of millions of years of natural selection. In ~30 BC Celsus described febrile temperatures (calor) as one of the four cardinal signs of inflammation, along with pain (dolor), redness (rubor), and swelling (tumour) [41].

During infection, ectothermic vertebrates raise their core temperature through behavioural regulation [42], whereas in endothermic animals, the induction and maintenance of fever involves a specific interplay between the innate immune system and neuronal circuitry within both the central and peripheral nervous systems. Immune sensing of infection begins from innate immune cell populations, including macrophages, neutrophils and dendritic cells, where PRRs activation stimulates the synthesis of pyrogenic cytokines, like IL-1, tumour necrosis factor TNFα and IL-6 both at the site of infection and within the brain [38] [43]. These pyrogenic cytokines stimulate the synthesis of cyclooxygenase (COX2) in the brain vascular endothelial cells in order to produce prostaglandin E2 (PGE2) by oxidizing arachidonic acid. After that, PGE2 produced by brain can bind to EP3 prostaglandin receptors expressed by thermoregulatory neurons in the median preoptic nucleus within the hypothalamus [44], determing the release of norepinephrine, which increases thermogenesis in brown adipose tissue and induces vasoconstriction in the extremities [45], and acetylcholine which stimulates the musculature to convert stored chemical energy into thermal energy increasing overall metabolic rates [46] (fig 3).

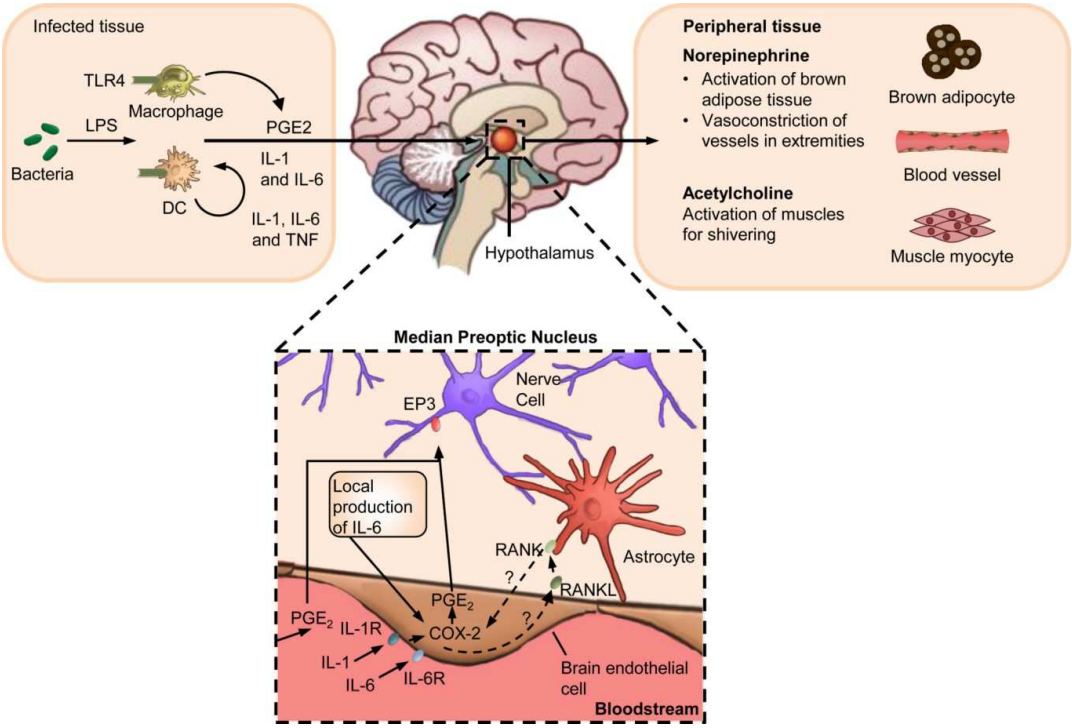

Fig 3: Induction of fever.

Fever is a preserved symptom because febrile temperatures confer a survival advantage. In fact, the increase of 1 to 4°C in core body temperature is associated with improved survival and resolution of many infections. One mechanism involves direct effects of febrile temperatures on the infectious potential of pathogens [47].

However, fever is not universally beneficial and it is associated with worse outcomes in cases of extreme inflammation like in patients with sepsis or neurological injuries, where treatments that induce hypothermia can have a clinical benefit [48] [49].

1.7 Fever and Dendritic cell functions

Several studies demonstrate that elevated temperatures can stimulate the innate immune response, enhance the phagocytic potential of Dendritic cells (DCs) and increase interferon-α (IFNα) production in response to viral infection [50] [51].

Fever enhances DC cross-presenting functions by increasing DC expression of MHC class I and class II molecules and co-stimulatory molecules, like CD80 and CD86, and stimulates the secretion of the IL-12 and TNFα [52].

Furthermore fever-range thermal stress augments the migration of antigen-presenting cells (APCs) to draining lymph nodes [53], increases the proliferation of naïve CD4+ T cells as well as their differentiation towards a Th1 cell phenotype [54] and also enhances the cytotoxicity of effector T cells thanks to the heat-induced upregulation of both MHC class I and HSP70 expression in mature DCs [55]. Moreover, heating of immature DCs also upregulates their expression of TLR2 and TLR4, enhancing pathogen sensing [56].

1.8 Inflammation and ion-channels

Heat is the primordial defense mechanism conserved in fishes, amphibians, reptiles, birds, and mammals and as a consequence is tightly linked to the immune system. Although it is widely recognized that temperature sensing in neurons is mediated by several transient receptor potential (TRP) channels, it is not known how immune cells sense cold or hot temperatures.

Recent studies have identified intracellular pathways linking immune and inflammatory response to ion channels [10]. Among ion channels, transient receptor potential (TRP) channels are a major family of non-selective cation permeable channels that function as polymodal sensors in different physiological [57] and pathological processes [58]. About 30 different mammalian TRP channels are identified and classified into six subfamilies on the basis of sequence homology: TRP canonical (TRPC; TRPC1-7), TRP vanilloid (TRPV; TRPV1-6), TRP melastatin (TRPM; TRPM1-8), TRP polycystin (TRPP; TRPP2,TRPP3,TRPP5), TRP mucolipin (TRPML; TRPML1-3), and TRP ankyrin (TRPA; TRPA1) [59]. These channels are involved in various physiological responses, such as neuronal responses associated with sensory functions [60] and they show distinct cation selectivity and gating mechanisms [61], since they are thermo- and chemo-sensitive channels. For example, TRPV1 can be activated by membrane depolarization, noxious heat, vanilloids, endocannabinoids, extracellular protons and inflammatory mediators [62]. In general, the Ca^{2+} signalling in immune cells is important for several regulatory functions like differentiation of immune cells, gene transcription and effector functions [63]. Intracellular Ca^{2+} participates in TLR4-dependent signalling and several studies have indicated that TLR-mediated immune responses are associated with TRP channel-dependent Ca^{2+} signalling. Recently it has been shown that in RAW 264 macrophages TRPV2 - mediated intracellular Ca^{2+} mobilization is involved in LPS-induced expression of inflammatory cytokines (TNF-α and IL-6) in a NF-κB-dependent manner

[64]. TRPV2 is also fundamental for the early phases of phagocytosis in innate immune cells [65] and it is expressed in CD19+ B lymphocytes where it regulates Ca^{2+} release during B cell development and activation [66]. As regards TRPV1, it plays a pivotal role in pain and neurogenic inflammation [67]. The thermosensitivity and Ca^{2+} permeability in general suggests that this group of ion channels can be ideal candidates to act as molecular sensors of immune cells detecting changes in body temperature. In conclusion, temperature can influence antimicrobial immune defenses orchestrating cell activation, antigen presentation, cytokines and ROS production required for optimal host defense, but in case of disregulated inflammation it appears to participate in the pathogenesis of sepsis stimulating an excessive immune response to the offending organism.

1.9 The Energy Crisis Model

Sepsis is the leading cause of death in the intensive care unit, and most of these deaths are attributed to the multiple organ dysfunction syndrome (MODS) sepsis dependent. Uncontrolled systemic inflammation and inadequate tissue perfusion are the putative mechanisms through which sepsis induces organ failures. However, precise mechanisms through which organ dysfunction develops remain unknown, in fact inhibition of inflammatory responses or optimization of tissue perfusion have little or no benefit in septic patients. Moreover, a growing body of evidence indicates that sepsis-induced organ failures are associated with fundamental alterations of cellular metabolism and that tissue hypoxia does not play a central role in this process. In this regard, several groups independently observed tissue acidosis in the condition of elevated tissue oxygenation in septic animals and reduced oxygen consumption in liver tissue extracted from endotoxin-treated animals [68], indicating abnormal tissue oxygen utilization [69]. This finding suggests that the predominant defect might lie in cellular oxygen use (tissue dysoxia) rather than in oxygen delivery. Considering that Mitochondria are the main oxygen users, mitochondrial dysfunction could be a key feature of septic shock that contributes to the development of sepsis related organ dysfunction. Indeed it is characterized by a variable reduction of the respiratory chain (RC) activities, altered mitochondrial morphology and reactive oxygen species (ROS) production [70]. The mechanisms of such mitochondrial dysfunction are extremely complex and involve an excessive production of ROS, such as superoxide, nitric oxide (NO) and peroxynitrite ($ONOO^-$), directly inhibiting the respiratory chain complexes activities [71]. NADH-ubiquinone

oxidoreductase (complex I) and cytochrome c oxidase (complex IV), in fact, can be inhibited by reactive oxygen and nitrogen species such as nitric oxide [72]. These reactive species are produced in substantial excess during sepsis and are also generated by the mitochondria. The metabolism of all metabolic substrates is altered during severe sepsis, and mitochondria contribute significantly to this phenomenon. Increased protein catabolism, impaired β-oxidation of long-chain fatty acids and inhibition of ketogenesis are well-documented complications of severe sepsis [73], where indeed the expression of rate-controlling enzymes of the urea cycle is significantly increased, which has implications for protein catabolism and excessive nitric oxide production. Conversely, the expression of acyl-coenzyme A (CoA) dehydrogenase and hydroxymethylglutaryl (HMG)-CoA synthase, were reduced [74]. At the tissue level a resistance to substrates such as carbohydrates and lipids develops and energy production at the cellular level is compromised because of pyruvate dehydrogenase inhibition. This creates a discrepancy between the availability of O_2 and ATP production. In fact, absolute muscle ATP concentrations and ATP:ADP ratios are significantly lower in non-surviving septic patients than survivors. The rise in AMP (and a lower adenine nucleotide pool) suggests an imbalance in ATP turnover, which could be a result of decreased ATP production, although an increase in ATP use might also be contributory [75].

Assuming that the development of mitochondrial dysfunction is among the main causes of MODS, preventing or reversing mitochondrial dysfunction and cellular energetic failure may represent an effective therapeutic strategy in the treatment of sepsis [76].

1.10 A possible solution for Energy Crisis Model: Para Hydroxyphenyl Pyruvate

Pyruvic acid is a simple three-carbon alpha-keto monocarboxylic acid. Under physiological conditions, pyruvic acid largely exists in cells and extracellular fluids as its conjugate anion, pyruvate. Being the final product of glycolysis and the starting substrate for the tricarboxylic acid cycle, pyruvate plays a central role in metabolism and it also functions as endogenous antioxidant. Recognition that pyruvate is an effective scavenger of reactive oxygen species (ROS) prompted investigators to use it as therapeutic agent for various pathological conditions that are mediated by redox dependent phenomena. Despite these promising findings, the usefulness of pyruvate as

therapeutic agent is limited by its poor stability in solution, where it undergoes an aldol-like condensation reaction.

Para-hydroxyphenyl Pyruvate (pHPP) and Ethyl Pyruvate (EP) belong to the class of enone containing compounds characterized by the presence of an alpha cheto oxy-acid or oxy-ester. Ethyl pyruvate is more effective and safer than equimolar doses of Sodium Pyruvate and proved to exhibit anti-inflammatory in vitro properties but with limited efficacy in clinical trials. The farmacological basis for the anti-inflammatory effects of EP remains to be explained, but it is plausible that EP inhibits NF-kB activation and secretion of NO and pro-inflammatory cytokines [77]. In general, the anti-inflammatory features of enone containing molecules is linked to their direct antioxidant activities which, depending on the cheto-enol tautomer involved, can scavenge either H_2O_2 and $ONOO^-$ or OH and NO. Of note, pHPP is characterized by a relatively more stable enol tautomer and showed more effective anti-inflamatory effects than equimolar doses of EP and other compounds containing the enon group. Moreover, pHPP seems to be a bio-energizer, since it is able to stimulate cellular respiration.

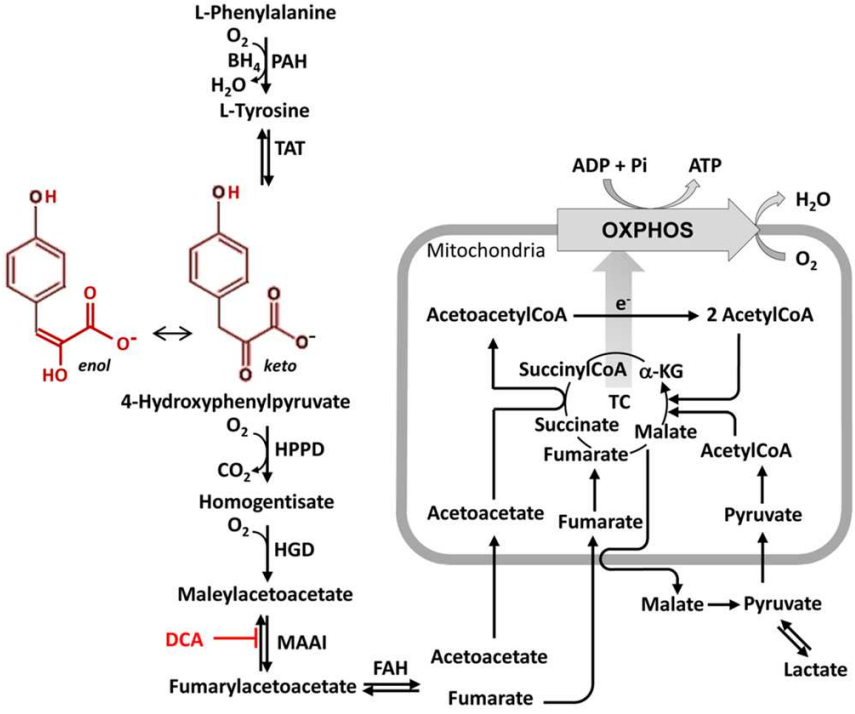

Fig.4. Catabolic pathway of Phenylalanine/Tyrosine.

The effect of pHPP on mitochondrial bioenergetics has been recently demonstrated by experiments in vivo and in vitro on endothelial cells involved in hemorrhagic shock and its bio-energizing role can be explained considering

that it is an intermediate in the phe/tyr catabolic pathway. The end-products of pHPP catabolism in the cytoplasm are Fumarate and Acetoacetate and both of these compounds can enter into mitochondria and support the tricarboxylic acid cycle either as intermediates or substrates (see scheme in Fig. 4). Importantly, it was demonstrated that the enhanced delivery of reducing equivalents to the mitochondrial respiratory chain is fully coupled to the synthesis of ATP. For this reason pHPP appears to be an efficient bio-energizer providing extra ATP to support adaptation to stressing conditions or to foster normal cell growth [78].

Research Objectives

Since the interplay of cellular bioenergetics, metabolism and inflammation occurs in innate and adaptive immune responses, manipulating cellular bioenergetics is becoming an attractive therapeutic strategy to treat inflammatory and immune diseases.

Considering the importance of mitochondrial dysfunction in sepsis-dependent MODS development, we hypothesized that an efficient antioxidant and bio-energizer compound, like pHPP, could represent an effective pharmacological strategy to cope with the tissue energy crisis due to sepsis, by the stimulation of oxidative phosphorylation (OxPhos) and the prevention of oxidative damages. So the first aim of this research was to test the impact on this of p-hydroxyphenylpyruvate (pHPP) on metabolic activity, cytokine production, ROS and RNS production in an *in vitro* model of sepsis.

As regardes the second part of this research project, it was dedicated to deepen the direct effect of fever on dendritic cell metabolism, starting from the assumption that elevated temperatures can stimulate the innate immune response and that, at the same time, inflammation and immune function are closely linked to switches in bioenergetic profile. In this perspectives, the modulation of the metabolic shift could lead to an attenuation of certain pro inflammatory symptoms induced by febrile temperatures, expecially in cases of extreme inflammation, like sepsis, where fever is associated with neurological injuries.

Materials and Methods

2.1 Materials

Cell lines: Normal Human Dermal Fibroblasts (NHDF) Murine Macrophages RAW 264.7 and Human Monocyte-derived Dendritic Cells (MoDCs).

Pyruvate, Malate, Succinate, decil-Ubiquinol, decyl-Ubiquinone, Ascorbate, N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD), Malonate, Rotenone, Antimycin A, Myxothiazol, Sodium Azide, KCN, Oligomycin, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), 4-Hydroxyphenylpyruvic acid (pHPP), Etil Piruvate (EP), Lipopolysaccharides from Escherichia Coli (LPS), 7-dichlorofluorescin diacetate (DCF), 4-amino-5-methylamino-20,70-difluorofluorescein (DAF-FM), dichloroacetic acid (DCA), X-Rhod-1AM, Ruthenium Red (RR), L-NAME, Seahorse XF Glycolysis Stress Test, Seahorse XF Mitochondrial Stress Test, Quantikine ELISA Immunoassay Kit for Human TNF-α (R&D Systems, Minneapolis, MN), Quantikine Immunoassay Kit for IL-6 (R&D Systems, Minneapolis, MN). Lymphoprep^{R}.

2.2 Cell Cultures

We carried out experiments related to this study on three different cell lines: Normal Human Dermal Fibroblasts (NHDF), Murine Macrophages RAW 264.7 and Human Monocyte-derived Dendritic Cells (MoDCs).

NHDF and RAW 264.7 were maintained in a humified 5% CO_2 incubator at 37 °C, in Petri dishes (100mm), in Dulbecco modified Eagle medium DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% HEPES, 1% L-Glutamine.

RAW 264.7 cells were incubated 18 h with 10 ng/ml LPS in the absence and in the presence of 3mM pHPP, 3mM EP, 2,5mM DCA, 1mM L-NAME, while parallel controls were treated with vehicle.

NHDF cells were incubated for 18h with media supplemented with 1/10 volume of sera isolated from either healthy or septic patients without or with 3mM pHPP.

2.3 Monocyte-Derived Dendritic Cells Isolation

Peripheral blood mononuclear cells were isolated from buffy coats (obtained through the courtesy of the local blood bank, O.O.R.R., Foggia) by

standard Lymphoprep^R gradient centrifugation. After over night incubation at 37°C with 5% CO_2 in T25 flasks, non-adherent cells were removed while adherent cells were incubated in AIM-V supplemented with 50ng/ml GM-CSF and 1,000 U/ml IL-4, for five days in a humified 5% CO_2 incubator at 37 °C.

MoDCs were incubated 3h with 5μM Ruthenium Red, 1mM L-NAME at 39°C and 37°C 5% CO_2, while parallel controls were treated with vehicle at 39°C and 37°C 5% CO_2.

2.4 Polarographic Measurements

Polarographic measurements in intact cells*:* Oxygen consumption rates (OCR) in intact cells were measured by Hansatech Oxigraph in a thermostatically controlled chamber (T=37°C) equipped with a stirring device and a gas-tight plug which has a narrow port enabling addition of solutions by a microsyringe.

Cells were seeded into Petri dishes (100mm) or Flasks (T25) and when they reached 70% confluence, they were incubated under different conditions. After treatment, cultured cells were detached from the plate by trypsinization, counted by Burker's chamber method and centrifuged for two times at 1200 RPM for 5min, first in their growth medium and then washed in PBS (Phosphate buffered saline). The cellular pellet was suspended in specific respiration buffers and put into the reaction chamber of the oxigraph, after 10-15min of baseline stabilization. After achievement of the stationary resting oxygen consumption rate (OCR_{RR}), 1.0μg/mL of oligomycin was added, followed by the addition of 10μM of the uncopuler FCCP. The measured OCRs were corrected for the 2μM rotenone-insensitive respiration, to be sure that the oxygen consumption was attributable only to the mitochondrial respiration. Regarding MoDCs assays, following cross-checks we established to perform all the respirometric measurements at 37°C since changing the temperature of the oxygraph chamber from 37 to 39 °C did not result in appreciable changes of the OCRs.

Respiration Buffer for RAW 264.7: 50mM KH_2PO_4, 10mM Hepes, 1mM EDTA, 10mM glucose, pH 7.4

Respiration buffer for MoDCs: AIM-V.

Polarographic measurements in permeabilized cells: mitochondrial respiration measurements using exogenous substrates can help to define a general mitochondrial bioenergetic profile and its dysregulation, because changes in respiration may correlate to altered mitochondrial substrate

oxidation. Therefore, the effect of specific substrates of the respiratory chain on mitochondrial respiration of MoDCs was analyzed by measuring the rate of oxygen consumption by Hansatech Oxigraph at 37 °C, with 20×10^6 cells/ml suspended in 0.25mM sucrose, 10mM KH_2PO_4, 27mM KCl, 40mM Hepes, 1mM $MgCl_2$, 0.5mM EGTA, 0.1% BSA, pH 7.1 supplemented with 5μg digitonin/10^6 cells. Addition of digitonin, which permeabilizes specifically plasma membrane, with negligible effect on intercellular membrane compartments, enabled access of the utilized substrates to mitochondria.

According to a well-established protocol, a step-by-step analysis of various segments of the respiratory chain was assessed by giving substrates for feeding electrons into the subsequent respiratory chain complexes. After cell-permeabilization, in the presence of 5μM FCCP to achieve the maximal respiration capacity, respiratory substrates and inhibitors were added as follows: a)10mM Pyruvate + 2mM Malate (with no inhibitors) for CxI+CxIII+CxIV; b)10mM Succinate in the presence of 2 μM Rotenone for CxII+CxIII+CxIV; c)160μM decil-Ubiquinol in the presence of 20mM Malonate for CxIII+CxIV;

d)10mM Ascorbate + 0.2mM N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) in the presence of 2μM Antimycin A + 2μM Myxothiazol for CxIV. The measured OCRs were corrected for the 10mM Sodium Azide-insensitive respiration and normalized to the cell number.

2.5 Activities of Complex I and Complex IV

For RAW 264.7 cells, the specific activities of Complex I (NADH: ubiquinone oxidoreductase) and Complex IV (cytochrome *c* oxidase) were assayed spectrophotometrically. Cultured cells were detached from the plate by trypsinization, counted by Burker's chamber method and centrifuged for two times at 1200 RPM for 5 minutes, first in their growth medium and then washed in PBS (Phosphate buffered saline). The cellular pellet was then suspended in the specific Resuspension Buffer, frozen–thawed and ultra-sound-treated.

Complex I was assayed following the NADH oxidation by measuring the initial 10μM rotenone-sensitive rate of 50μM NADH oxidation (ε340 nm=6.22 mM^{-1} cm^{-1}) in the presence of 200μM decyl-Ubiquinone (dUQ) as electron acceptor and 3mM KCN.

Complex IV was assayed by following the initial 2mM KCN-sensitive rate of 20μM ferro-cytochrome *c* oxidation (ε_{550nm} = 19.1 mM^{-1} cm^{-1}) in 10mM Tris HCl, 1 mg/ml bovine serum albumin, pH 8.0, under aerobic conditions in the presence of 1μM Antymicin A, 10μM Rotenone.

Resuspension Buffer: Tris HCl 20mM, EGTA 2mM, KCl 40mM, Saccharose 0,32M.

2.6 Determination of Citrate Synthase activity

Citrate synthase activity was measured spectrophotometrically. Cultured cells were detached from the plate by trypsinization, counted by Burker's chamber method and centrifuged for two times at 1200 RPM for 5 minutes, first in their growth medium and then washed in PBS (Phosphate buffered saline). The cellular pellet was then suspended in the Resuspension Buffer (Tris HCl 20mM, EGTA 2mM, KCl 40mM, Saccharose 0,32M), frozen–thawed and ultra-sound-treated. After that, the suspension was added to a reaction mixture consisting of 100mM Tris HCl buffer (pH 8), 0.3mM AcetylCoenzyme A, 0.1mM DTNB, and 0.2% (v/v) Triton X-100. The reaction was started by the addition of 0.5mM oxaloacetate by following the reduction of DTNB at 412nm (ε=13.6mM^{-1}cm^{-1}).

2.7 Metabolic flux analysis

Oxygen consumption rate (OCR) and extra-cellular acidification rate (ECAR) were measured in adherent RAW 264.7 cells with a XF96 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA, USA). Control and compound-treated macrophages were seeded each in 4 wells (experimental replicates) of a XF 96-well cell culture microplate (Seahorse Bioscience) at a density of 15–20 × 10^3 cells/well in 200µL of DMEM and incubated for 18h at 37 °C in 5% CO_2 atmosphere without or with 10ng/ml LPS ± 3mM pHPP. After replacing the growth medium with 180µL of bicarbonate-free DMEM pre-warmed at 37 °C, cells were preincubated for 30min before starting the assay procedure. After baseline measurements of OCR, each well was sequentially supplemented with 20µL of Oligomycin, 22µl of FCCP and 25µl of Rotenone to reach working concentrations of 1µM, 0.8µM and 1µM respectively. After baseline measurements of ECAR each well was sequentially supplemented with 10mM Glucose, 1µM Oligomycin, 50mM 2-deoxy-D-Glucose (10mM). The first injection is a saturating concentration of glucose to measure the rate of glycolysis under basal conditions. The second injection inhibits mitochondrial ATP production and shifts the energy production to glycolysis, with the subsequent increase in ECAR, revealing the cellular maximum glycolytic capacity. The final injection inhibits glycolysis through competitive binding to glucose hexokinase and the resulting decrease in ECAR confirms that the ECAR produced in the experiment is due to glycolysis. In this

way the Seahorse XF Glycolysis Stress Test used on the Seahorse XFp Extracellular Flux Analyzer provides a method to assess the key parameters of glycolytic flux: Glycolysis that is the ECAR rate reached after the addition of saturating amounts of glucose; the Glycolytic capacity that is the cellular glycolytic response to energetic demand from stress and the Glycolytic reserve, defined as the difference between Glycolytic Capacity and Glycolysis rate, indicating the capacity available to utilize glycolysis beyond the basal rate.

2.8 Live cell imaging of ROS and RNS

Murine Macrophages RAW 264.7 and MoDCs were cultured at low density on 6 well plates and incubated with the following probes: 10μM 2,7-dichlorofluorescin diacetate (which is converted to dichlorofluorescein –DCF- by intracellular esterases) for 30min and 5μM 4-amino-5-methylamino-20,70-difluorofluorescein (DAF-FM) diacetate for 45min, for detection of H_2O_2 and nitric oxide (NO) respectively. Stained cells were washed with PBS and captured at EVOS digital microscope in LED illumination for transmitted light and in blue illumination for green fluorescence. Images analysis was performed by the Software ImageJ, by calculating the fluorescence intensity as pixel density.

2.9 Live cell imaging of mitochondrial $Ca^{2+}$and mitochondrial membrane potential

MoDCs were cultured at low density on 6 well plates and incubated for 20 min with 2μM tetramethylrhodamine ethyl ester (TMRE) to monitor mitochondrial membrane potential (mtΔΨ) and 5μM RHOD-1AM, a cationic fluorescent Calcium indicator which undergoes potential-driven uptake into mitochondria. Stained cells were washed with PBS and captured at EVOS digital microscope in LED illumination for transmitted light and in green illumination for red fluorescence. Images analysis was performed by the Software ImageJ, by calculating the fluorescence intensity as pixel density.

2.10 TNFα measurement

MoDCs were cultured in 6-well plates and incubated 3h with 1mM L-NAME at 39°C and 37°C 5% CO_2, while parallel controls were treated with vehicle at 39°C and 37°C 5% CO_2. TNFα concentrations were measured after 3h in cell supernatants using a commercially available Quantikine ELISA Immunoassay Kit for Human TNF-alpha (R&D Systems, Minneapolis, MN).

2.11 NO· and IL-6 measurements

RAW 264.7 cells were plated in 6-well plates and used the following day. Cells were stimulated by adding 10 ng/ml *Escherichia coli* LPS (serotype O111:B4) in presence or absence of graded concentrations of the tested compounds (EP, pHPP). Nitrite/nitrate and IL-6 concentrations were measured after 18 h in cell supernatants using commercially available Griess reaction kit (Oxis International, Portland, OR) and Quantikine Immunoassay Kit for IL-6 (R&D Systems, Minneapolis, MN).

2.12 Western Blotting analysis

Aliquots containing 40μg of proteins from each cell lysate were subjected to SDS polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories; Hercules, CA, USA) using Trans Blot Turbo Transfer System. Membranes were probed with the following primary antibodies: HIF-1α (1:1000) and β-actin (1:5000). After incubation with corresponding suited horseradish peroxidase-conjugated secondary antibody (1:2500), signals were developed using the enhanced chemiluminescence kit (ClarityTM Western ECL Substrate, Bio-Rad) and the ChemiDoc Imaging System XRS + (BioRad) and analysed with the ImageJ Lab 4.1 software. The intensity of bands was normalized to the β-actin signal.

2.13 Real-Time Reverse Transcriptase-PCR analysis

RAW 264.7 cells were harvested at 24 h in 1 ml of TRI-Reagent as directed by the manufacturer (Molecular Research Center, Inc.). Bromochloropropane was used for the extraction. The final RNA pellet was dissolved in nuclease-free water and quantified using a GeneQuant pro UV spectrophotometer (GE Healthcare). Extracted RNA (1μg /reaction) was converted to single-stranded cDNA in a 20μl reaction using the Reverse Transcriptase System Kit (Promega) as directed by the manufacturer. The mixture was heated to 70 °C for 10min, maintained at 42 °C for 30min, and then heated to 95 °C for 5min using a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA). TaqMan Gene Expression Assays for mouse iNOS and 18S RNA (endogenous control) and real-time PCR reagents were from Applied Biosystems (Foster City, CA). Reaction mixtures for PCR were assembled as follows: 10μl TaqMan Universal PCR Master Mix, 1μl of each Gene Expression Assay mix, 1μl cDNA template and 7μl of water. The real time RT-PCR analysis was performed using ABI Prism 7900HT (University of Pittsburgh Genomics and Proteomics Core

Laboratories) by incubating at 50 °C for 2min, 95°C for 10min, 95 °C for 15s, and 60°C for 1min; the two final steps were repeated for 40 cycles. Each sample was assayed in duplicate and the values were averaged. A ΔΔCt relative quantification method was used to calculate mRNA levels for MCP1 in the samples. Results were first normalized relative to 18S mRNA expression levels and then to the control (calibrator).

2.14 Statistics

Data are presented as means +/- SEM, and analyzed using two-tailed student t-tests. A value of $P<0.05$ was considered statistically significant.

Results

In the first part of this study we used LPS-stimulated murine macrophage RAW 264.7 as an *in vitro* model of sepsis to investigate alterations of mitochondria-related functions and the impact on this of p-hydroxyphenylpyruvate (pHPP).

3.1 Effect of pHPP on the LPS-induced immuno-phenotype changes in RAW 264.7

First of all, in order to test the reliability of our model, we incubated macrophages o.n. with 10ng/ml LPS to test if they could be really activated by this toxin.

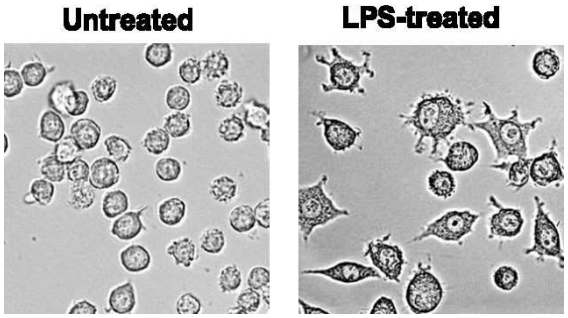

Fig.5. LPS-mediated activation of macrophages. Cultured cells were treated with 10 ng/ml LPS for 18 h and morphological changes were analyzed by light transmission microscopy.

As shown in Fig. 5, the overnight treatment of macrophages with LPS resulted in a dramatic change in the morphology of the cells that from a rounded shape acquired a dendritic-like appearance with formation of pseudopods. This morphological change is diagnostic of the transition of macrophages from a quiescent (M0) to an activated (M1) state and it is also accompanied by production of pro-inflammatory cytokines like IL-6 (Fig. 6). Co-incubation of LPS with pHPP prevented dose-dependently the release of IL-6, more efficiently than EP at equimolar concentrations. Indeed, Fig. 6 clearly illustrate that while 10 mM pHPP completely inhibited IL-6 production, a similar concentration of EP caused only a 40 % decrease of the cytokine release.

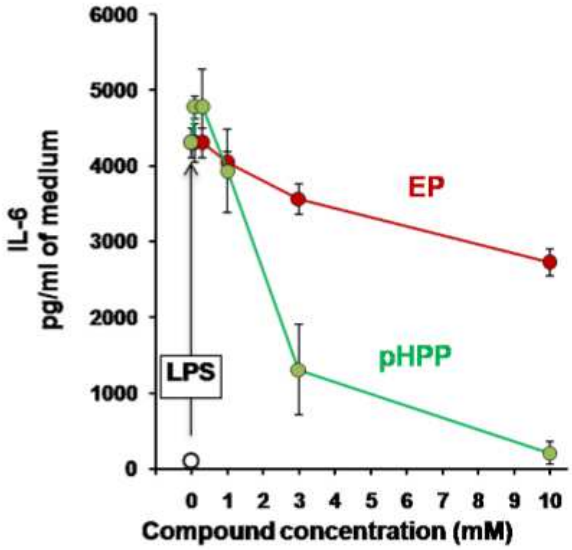

Fig. 6. Effect of graded concentrations of EP and pHPP on IL-6 release in LPS-treated RAW 264.7 macrophage cell line. Cultured cells were treated with LPS as in the absence and in the presence of the indicated concentrations of either EP or pHPP and assayed for the IL-6 released in the culture medium described in Materials and Methods.

However, in spite of this marked anti-inflammatory effect, pHPP-treatment had no impact on the LPS-induced morphological change, as shown in figure 7.

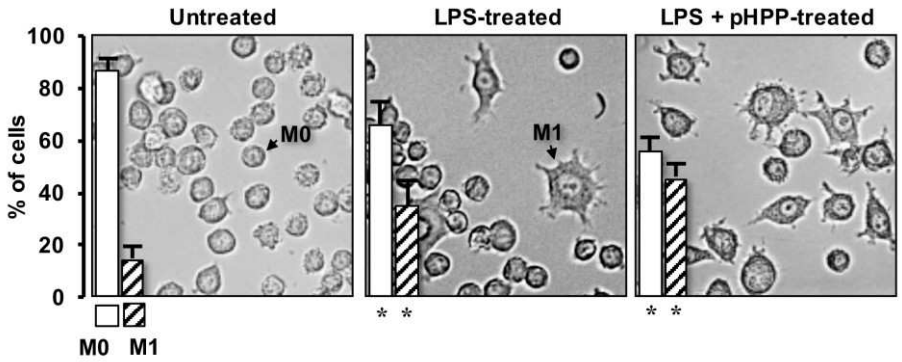

Fig 7. Effect of pHPP on the LPS-induced immuno-phenotype changes in RAW 264.7. Cultured cells were treated with 10ng/ml LPS and co-incubated with 3mM pHPP for 18 h and morphological changes analyzed by light transmission microscopy. The histograms on the left show the morphometric analysis carried out on images collected from 3 independent condition in each biological replicate.

3.2 pHPP as antioxidant in the macrophage immune response

Next we assessed the well-established effect of LPS on the expression of the *NOS2* gene (coding for iNOS) and nitric oxide (NO) production. As shown in figure 8, LPS-treatment resulted in a marked up-regulation of the iNOS expression and consequent production of NO, assessed as NO_2^-/NO_3^- formation released in the culture medium. pHPP prevented dose-dependently both these effects, while equimolar concentrations of EP resulted much less efficient than pHPP.

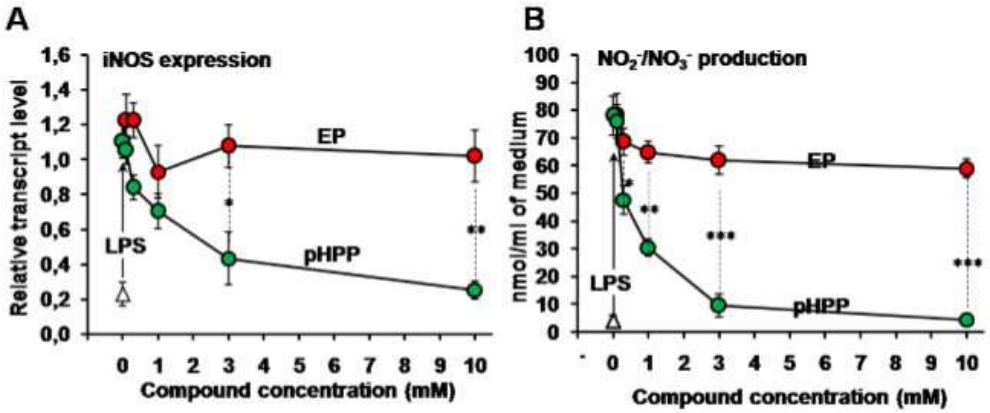

Fig. 8. Effect of graded concentrations of EP and pHPP on iNOS expression and nitrite/nitrate release in LPS-treated RAW 264.7 macrophage cell line. Cultured cells were treated with 10 ng/ml LPS for 18 h in the absence and in the presence of the indicated concentrations of either EP or pHPP and assayed for iNOS mRNA level in cell lisates (A) and for the nitric oxide (NO) derivatives nitrite/nitrate in the supernatant (B). * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

The antioxidant activity of pHPP under pro-oxidative stressing conditions was verified by imaging with the intracellular-trapped fluorescent probes DAF and DCF, using EVOS Digital Microscope, as indicated in the Methods Section. In our model, pHPP is able to prevent almost completely both the basal and the LPS-mediated over-production of NO (Fig. 9), detected by DAF, whose specificity was assessed by the NOS pan-inhibitor L-NAME.

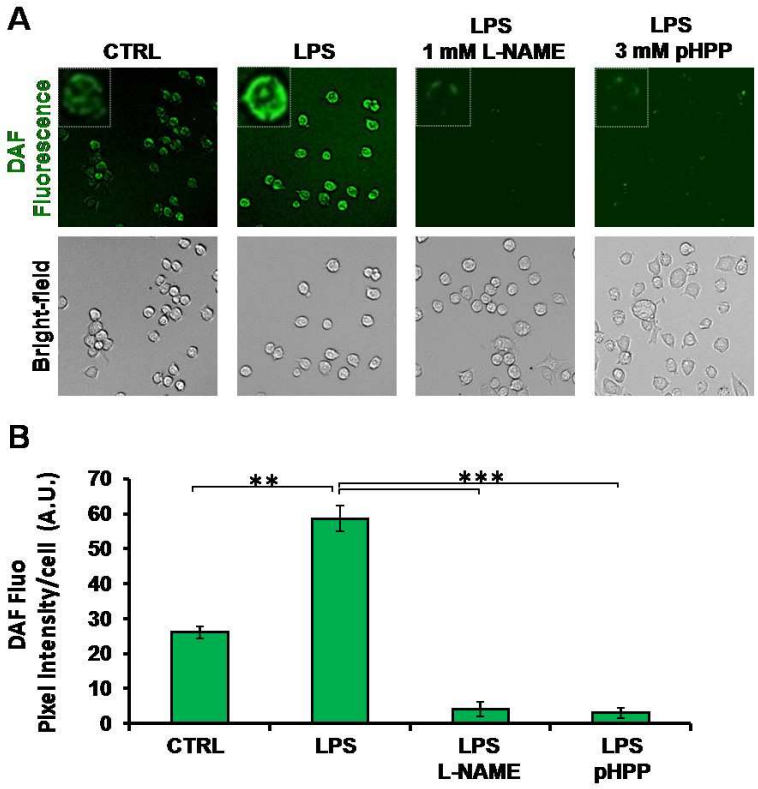

Fig 9. Fluorescence microscopy imaging of cellular production of NO in LPS-treated RAW 264.7 macrophage cell line and effect of pHPP. ** P<0.01, *** P<0.001.

Along with the generation of NO, LPS stimulation of macrophages can also induce a marked alteration of the cellular redox state due to the production of reactive oxygen species (ROS). Interestingly, also this LPS-induced overproducion of ROS was completely abolished by co-treatment with pHPP, as shown in figure 10.

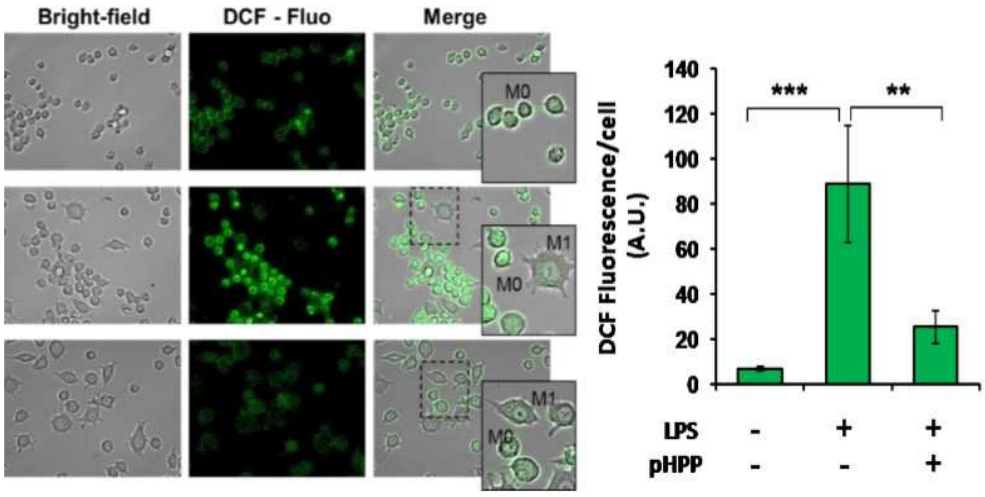

Fig 10. Fluorescence microscopy imaging of cellular production of peroxides in LPS-treated RAW 264.7 macrophage cell line and effect of pHPP. ** P<0.01, *** P<0.001.

3.3 pHPP_as bio-energizer in the macrophage immune response

As ROS and RNS, even if important for the bactericidal activity of macrophages, are at the same time responsible of oxidative damages and inhibition of the respiratory chain Complex I and IV, we measured oxygen consumption rates (OCR) in intact cells by Hansatech Oxigraph. The graph in figure 11 shows that LPS-mediated activation of macrophages caused a significant 50% decrease of the resting endogenous oxygen consumption (OCR_{RR}) compared with untreated or pHPP-treated cells. In the presence of the ATP-synthase inhibitor oligomycin the OCR was sharply reduced in all the conditions. This remaining respiratory activity is an indirect measurement of the passive proton leak and it is not coupled to ATP production. The difference between the OCR_{RR} and the OCR_L indicates the effective mitochondrial oxygen consumption that was used to drive ATP production (OCR_{ATP}). Therefore, considering the significant reduction of the ATP-linked respiration in LPS treated-cell, it can be deducted that LPS-treatment of RAW 264.7 led to an oxidative phosphorylation failure. Notably, a complete prevention of the LPS-mediated reduction of the endogenous respiratory activity and partial recovery of the ATP-linked respiration was observed following co-incubation with pHPP and even if not statistically significant, a trend to increase the OCR was observed treating unstimulated cells with pHPP. Also when macrophages were

co-incubated with DCA, a known activator of PDH, the LPS-mediated inhibition of cell respiration was completely prevented thus suggesting an impairment of pyruvate oxidation in LPS-treated cells.

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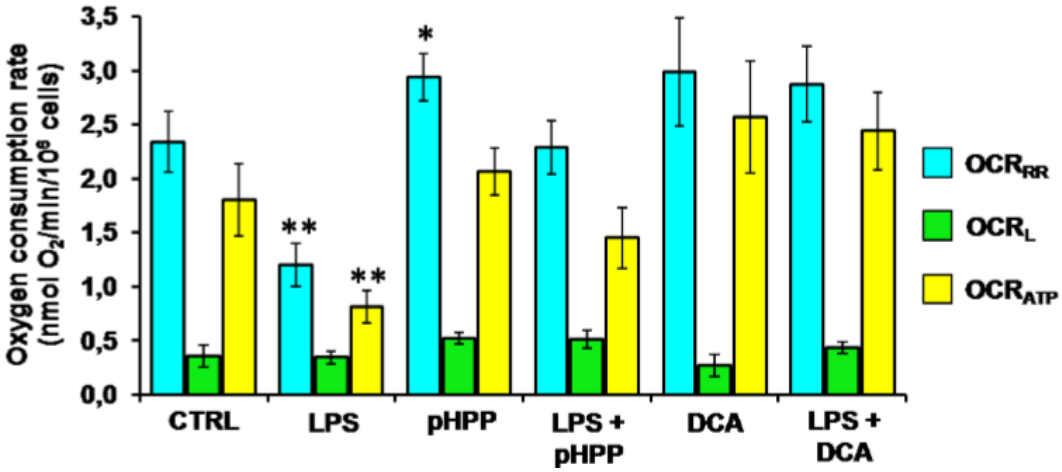


Fig 11. Effect of pHPP and DCA on mitochondrial respiration in LPS-stimulated RAW 264.7. ** P<0.01 vs the corresponding OCRs of all the other conditions. *P<0.05 vs the corresponding OCRs of CTRL.

To verify that the LPS-induced reduction of oxidative phosphorylation was linked to a shift towards a glycolytic metabolism, we measured the metabolic fluxes in cultured cells by the SeaHorse technology. As shown in Fig. 12A, the inhibitory effect of LPS on the OCR and the preserving effect of pHPP were substantially confirmed also in the uncoupled state. In addition, we assessed the extracellular acidification rates (ECAR) as an indirect measurement of the glycolytic flux, confirming that LPS treatment induced a significant increase of glycolytic flux, reserve and capacity and that this metabolic switch was fully prevented by co-incubation with pHPP (Fig. 12B). All together this results clearly show that LPS-treatment caused a depression of the mitochondrial oxidative phosphorylation, pushing the glycolytic flux and that pHPP was able to largely prevent this metabolic shift (Fig. 12C).

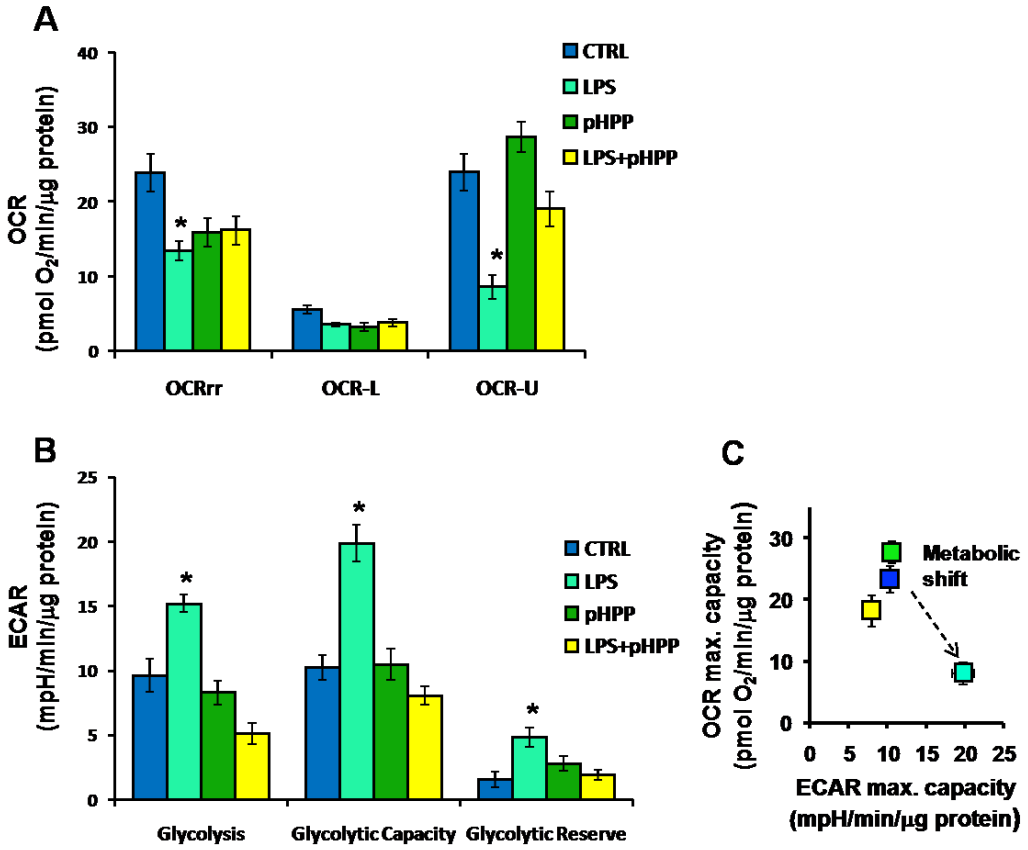


Fig.12 Metabolic flux analysis in LPS-treated RAW 264.7 macrophage cell line and effect of pHPP. Cells were seeded in a 96-wells cell culture microplate and incubated 18h without or with 10ng/ml LPS ± 3 mM .The histograms in (A) and (B) show the oxygen consumption rates (OCR) and the extra cellular acidification rates (ECAR) respectively assayed by the SeaHorse platform as described under Materials and Methods and normalized to the protein content of the cells removed from each well at the end of the assay. The OCR were corrected for the residual OCR measured after the addition of the CxI inhibitor rotenone (not shown). (C) Energy map obtained plotting the maximal ECAR and OCR capacity measured in (A) and (B). *P<0.05 vs the corresponding OCRs and ECARs of all the other conditions.

As regards the activity of the respiratory chain complexes controlling the respiratory flux [79], the specific enzymatic activities of Complex I and IV were inhibited by LPS to a similar extent of the respiratory chain and pHPP was able to completely prevent this enzymatic inhibition (Fig. 13).

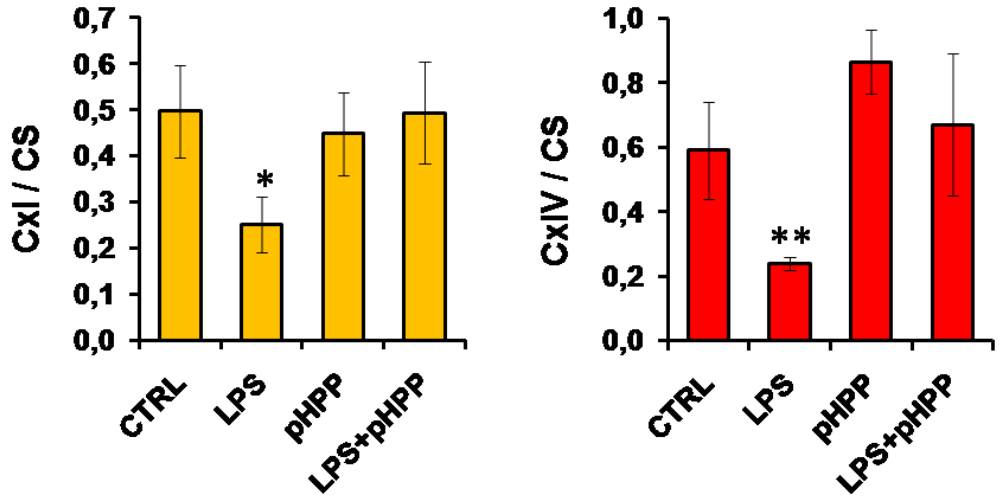


Fig. 13. Activity of mitochondrial respiratory chain complexes. RAW 264.7 cell were incubated with the indicated compounds. Then cells were lysated and the specific enzymatic activity of the NADH dehydrogenase (CI) and cytochrome c oxidase (CIV) assessed by spectrophotometric assays under condition of saturating substrates as detailed under Materials and Methods. The measured activities were normalized to the citrate synthase activity. *P<0.05 **P<0.01 vs the corresponding activities of all the other conditions.

Next, we verify if the pro-inflammatory background observed in septic patients could bring on inhibition of the mitochondrial respiratory activity as observed in LPS-stimulated RAW 264.7 and if this was sensitive to the bioenergyzing power of pHPP. For this aim, the culture medium of TLR4-expressing normal dermal human fibroblasts (NHDF) [80] was supplemented with sera from a cohort of septic patients obtained in collaboration with the Critical Care Unit of the Foggia School of Medicine, while sera from healthy donors were used as control. Their respiratory activity was assessed after 18h incubation and figure 14 shows that treatment of NHDF with septic serum caused a significant 50 % reduction of the mitochondrial respiratory efficiency as compared with NHDF treated with normal human sera, probably due to the presence of plasmatic LPS. Importantly, co-incubation of septic sera with pHPP can prevent, also under this condition, the mitochondrial OxPhos impairment. In this case, pHPP had a significant stimulatory effect of respiration also on NHDF incubated with normal non-septic sera.

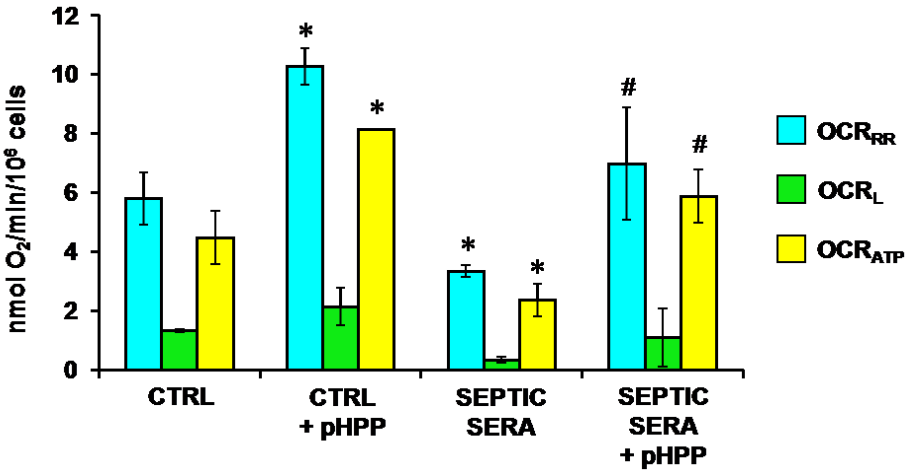


Fig. 14. Measurement of mitochondrial respiratory activity in septic sera-treated NHDF human fibroblasts and effect of pHPP. *P<0.05 vs CTRL # P<0.05 vs Septic Sera.

The hypoxia-inducible factor 1 is a transcription factor controlling the expression of genes involved in the glycolysis under hypoxic conditions. Although HIF-1α subunits are typically induced in hypoxia, they can also be expressed in macrophages and dendritic cells in normoxia upon inflammatory stimulation (see Introduction 1.5). Indeed, figure 15 shows that treatment of RAW 264.7 cells with either the hypoxia-mimetic $CoCl_2$ or LPS induced a significative normoxic stabilization of the transcription factor subunit alpha (HIF-1α) compared with its low basal expression in untreated cells due to proteasomal degradation. Co-treatment of LPS with pHPP resulted in no change in the basal level of the HIF-1 α subunit.

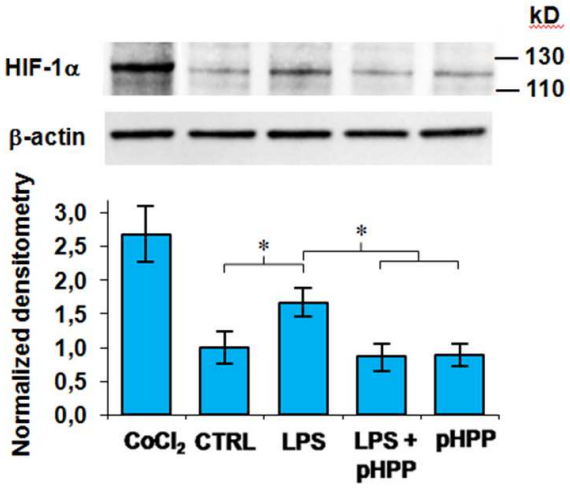


Fig 15. Expression of HIF-1α in LPS-treated RAW 264.7 cell line and effect of pHPP. *P < 0.05

3.4 Thermal regulation of MoDCs immune activation

In the second part of this research project, we focused on the direct effect of fever on innate immune cell metabolism, mimicking the fever-like thermal challenge by the incubation of Monocyte-Derived Dendritic Cells (MoDCs) for 3h in a humified 5% CO_2 incubator at 39°C. MoDCs were obtained after 6 days of differentiation with IL4 and GM-CSF from circulating human monocytes, isolated from blood of healthy volunteers collected in collaboration with the local blood bank (O.O.R.R. Foggia), as indicated under Materials and Methods.

First of all we verified the effect of a mild hyperthermia on MoDCs immune activation. The thermal shock did not cause significant changes both in the cell morphology and in the cell viability but it induced a three-fold increase of TNFα release by MoDCs exposed to 39°C as compared with cells left at 37°C that served as control (see Fig. 16), confirming the well known stimulatory effect of feverish temperatures on the innate immune response.

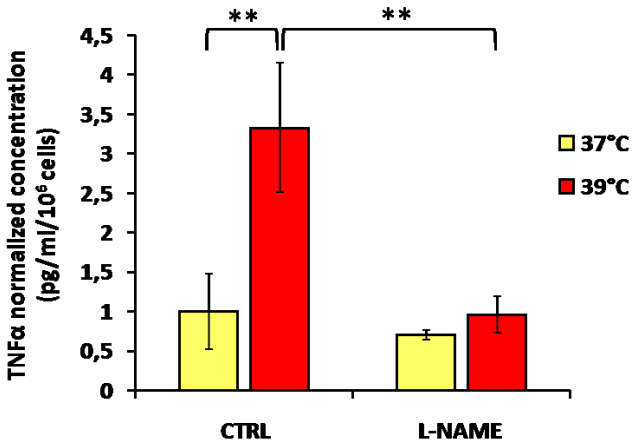


Fig.16 Effect of mild-hyperthermia on TNFα release in MoDCs. Cultured cells were incubated at 39°C for 3h in the absence and in the presence of 1mM of the NOS pan-inhibitor L-NAME and assayed for the TNFα released in the supernatant as described in Materials and Methods. ** $P<0.01$.

This effect was also demonstrated by a wide iNOS-dependent overproduction of NO by heated cells, whose production was detected by imaging using the fluorescent probe DAF (Fig.17). Fig. 17 displays a significant increase of the DAF-related fluorescent signal in the 39 °C-treated MoDCs as compared with normothermic MoDCs.

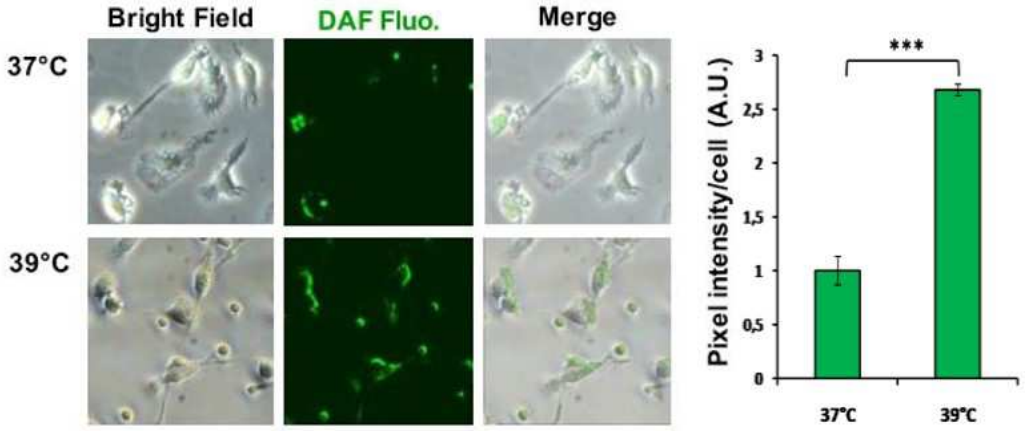


Fig.17 Fluorescence microscopy imaging of cellular production of NO in hyperthermia-treated MoDCs. *** P<0.001.

Of note, incubation of MoDCs with the NOS pan-inhibitor L-NAME during the thermal shock prevented the hyperthermia-induced release of TNFα (Fig.16).

3.5 Thermal regulation of mitochondrial oxidative phosphorylation in MoDCs

Next, we carried out respirometric analysis experiments aimed at characterizing the mitochondrial oxidative phosphorylation system in monocytes and MoDCs, to confirm the strict linkage between immune function and switches in the bioenergetic profile.

The results are summarized in figure 18 that shows that differentiation of monocytes into MoDCs was accompanied by a more than five-fold increase in the endogenous OCR_{RR}, the uncoupled OCR_U and the ATP-linked respiration. Moreover MoDCs respiratory activity is sensitive to hyperthermia showing a significant 50% decrease in both endogenous OCR_{RR} and ATP-linked respiration and a 25% inhibition of the uncoupled respiration OCR_U after 3h of incubation at 39°C, suggesting a hyperthermia-induced shift towards a glycolytic metabolism. As regards monocytes, they did not undergo this metabolic switch after hyperthrmic treatment and showed similar respiratory activities in both CTRL and heated cells.

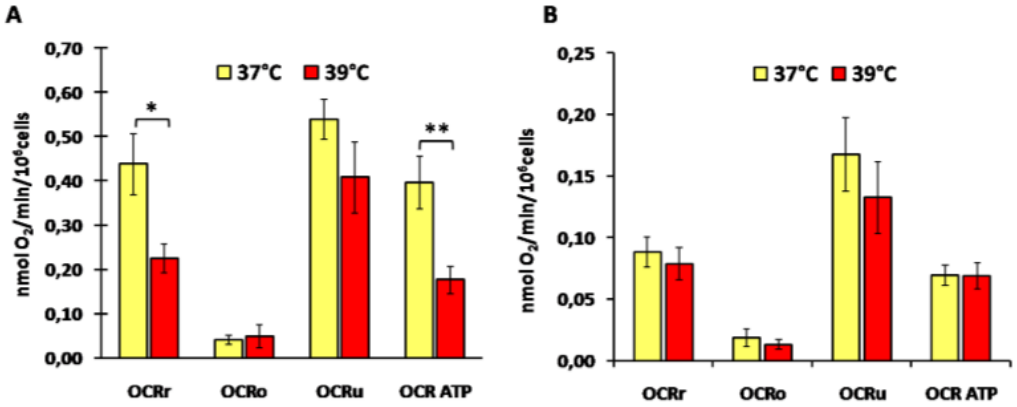


Fig.18 Effect of mild hyperthermia on mitochondrial respiration in MoDCs (A) and Monocytes (B). *P<0.05. ** P<0.01.

To confirm the bioenergetic alterations observed, we next carried out functional imaging of mitochondria in live cells by the fluorescent lipophylic probe TMRE, that accumulate in mitochondria driven by the mitochondrial transmembrane potential (mtΔΨ). Figure 19 shows that the probe TMRE was loaded to a lesser degree into the mitochondria of the 39 °C-treated MoDCs as compared with the 37 °C-treated cells, indicating a reduced steady-state of mtΔΨ in thermal-stressed cells.

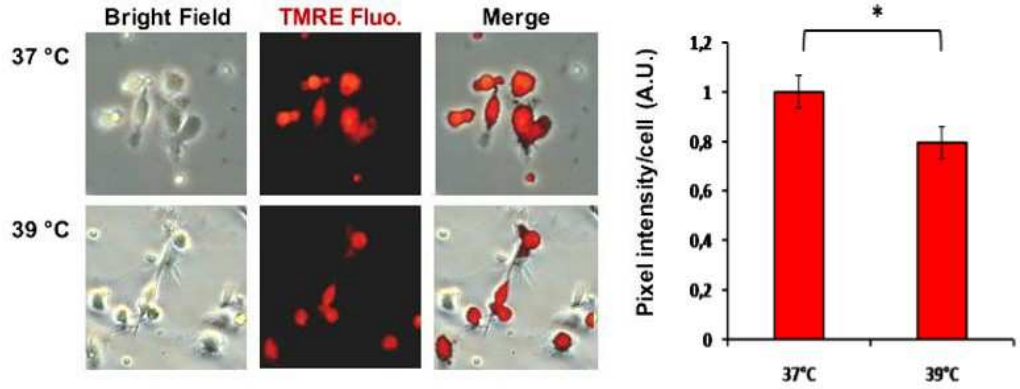


Fig.19. Fluorescence microscopy imaging of mitochondrial transmembrane potential (ΔΨ) in hyperthermia-treated MoDCs. *P<0.05.

To further dissect the observed impairment of the respiratory activity in thermally challenged MoDCs, we measured the maximal (uncoupled) respiratory fluxes by exogenous substrates in digitonin-permeabilized cells, supplying electrons to specific segments of the mitochondrial respiratory chain, in particular: piruvate+malate for CxI+CxIII+CxIV, succinate for CxII+

CIII+CIV, decil-Ubiquinol for CxIII+CxIV and ascorbate+TMPD for CxIV (Fig. 19). Respiratory fluxes were markedly decreased in 39°C-treated cells when either the pyruvate+malate or decyl ubiquinol ($dUQH_2$) were used as reducing substrates to respectively fuel CxI+CxIII+CxIV or CxIII+CxIV segment of the respiratory chain. Conversely, when succinate or ascorbate/TMPD are used to fuel CxII+CxIII+CxIV or CxIV segment respectively, no significant changes in the OCR between the 37°C- and 39°C-treated MoDCs were observed.

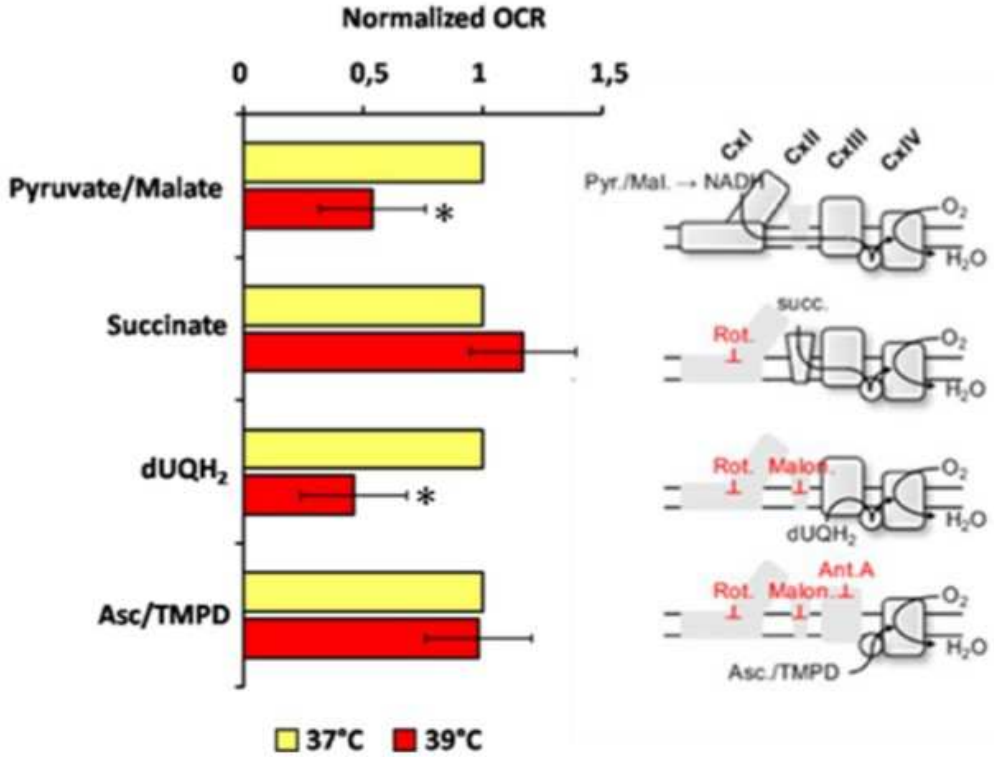


Fig.20 Uncoupled respiration rates in digitonin-permeabilized MoDCs. *P<0.05.

Interestingly, heated MoDCs also shown a moderate increase of the extramitochondrial oxygen consumption rate in the presence of Rotenone (a specific inhibitor of Complex I of the respiratory chain) and we demonstrated that it is partially attributable to a greater production of ROS as result of hyperthermia-induced activation of a proinflammatory phenotype in MoDCs.

The intracellular ROS generation was assessed by imaging, using the redox-sensitive fluorescent probe DCF and the results attained illustrate that the 39°C-treated MoDCs exhibited a significant 50% increase in the DCF-related fluorescence signal as compared with normothermic cells (Fig.21).

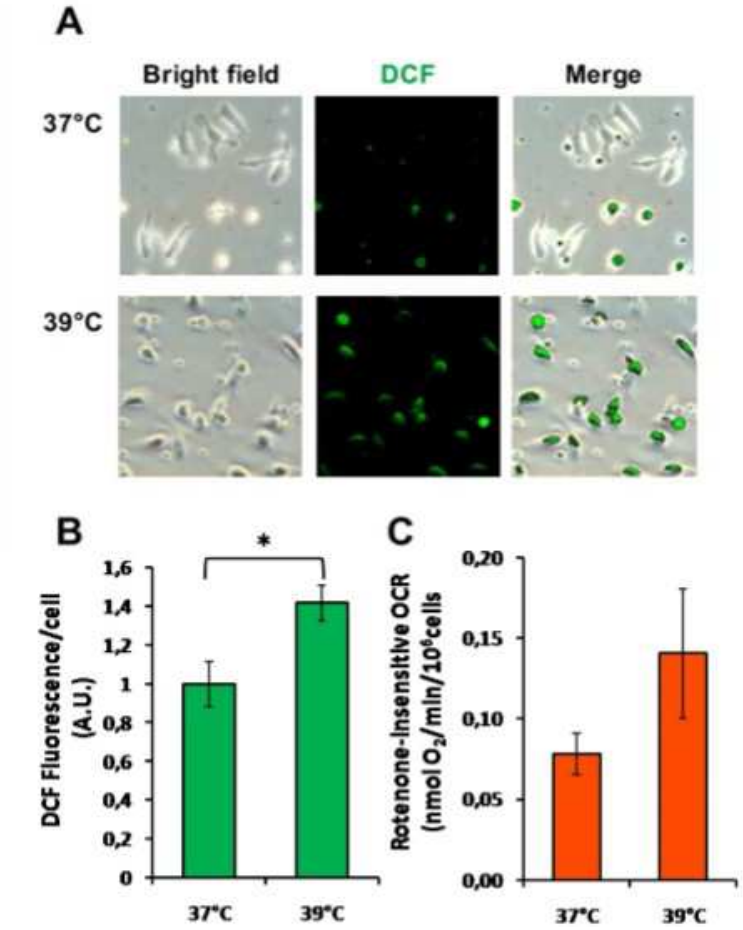


Fig.21. Fluorescence microscopy imaging of cellular production of peroxides in hyperthermia-treated MoDCs. *P<0.05.

As Gupta and Coll. have already demonstrated that febrile temperatures induce changes in the release of factors like heat stress HSP70 by macrophages and MoDCs, we asked if this was linked to the observed metabolic reprogramming in MoDCs. To this aim MoDCs were incubated at 37°C in the culture medium of cells previously incubated for 3h at 39°C and then analyzed at the Hansatech Oxygraph for the OCR. The oxymetric assay revealed that the treatment of MoDCs with the 39°C-conditioned medium was able to cause, even under normothermic conditions, the inhibition of the mitochondrial respiratory activity (Fig. 22).

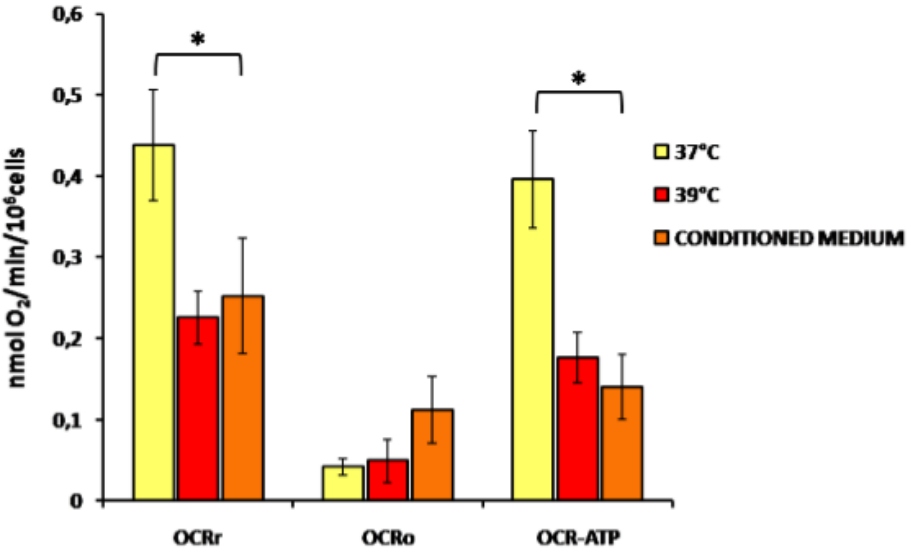


Fig.22. Effect of 39°C-conditioned medium on mitochondrial respiration in MoDCs. MoDCs were incubated at 37°C with the supernatant of cells previously incubated for 3 h at 39°C and polarographically tested for the OCR. *P<0.05.

Notably, the inhibition of the mitochondrial respiratory activity observed in the 39°C-treated cells is an irreversible process because when after 3 hours at 39°C, MoDCs were kept to 37°C for further 3 hours in a fresh medium, the mitochondrial OxPhos activity was not recovered (Fig. 23).

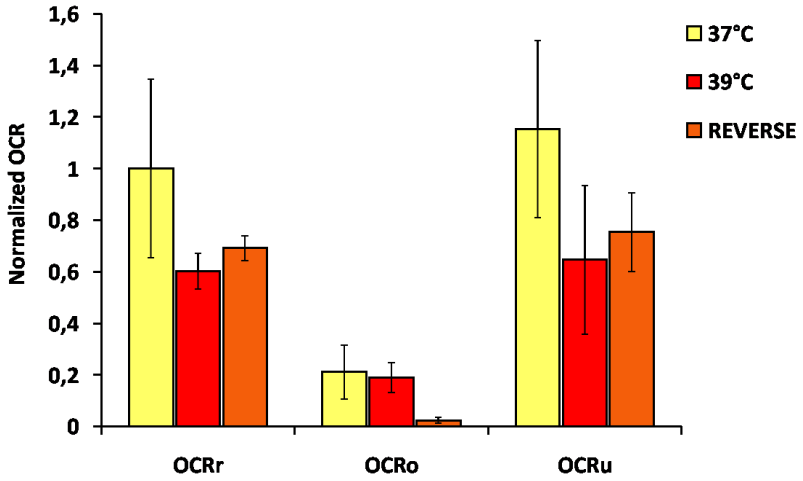


Fig.23 Reverse of the hyperthermia-induced inhibition of the mitochondrial respiratory activity. MoDCs were incubated for 3 hours at 39°C, then the culture medium was changed with a fresh medium and cells kept to 37°C for further 3 hours before being tested for the OCR.

3.6 Ca^{2+} signalling in thermal regulation of MoDCs immune response

Intracellular Ca^{2+} plays important roles as second messenger to regulate several signalling pathways and recent studies have underlined its involvement in many aspects of immune response regulation [81]. The role of mitochondria in intracellular Ca^{2+} signalling relies mainly in its capacity to take up Ca^{2+} from the cytosol and thus modulate the cytosolic [Ca^{2+}], acting as local Ca^{2+} buffers in specific cellular regions [82]. Imaging in live cells of mitochondrial calcium (mt-Ca^{2+}) was assessed by the specific mitotropic Ca^{2+}-sensitive fluorescent probe Rhod-1.

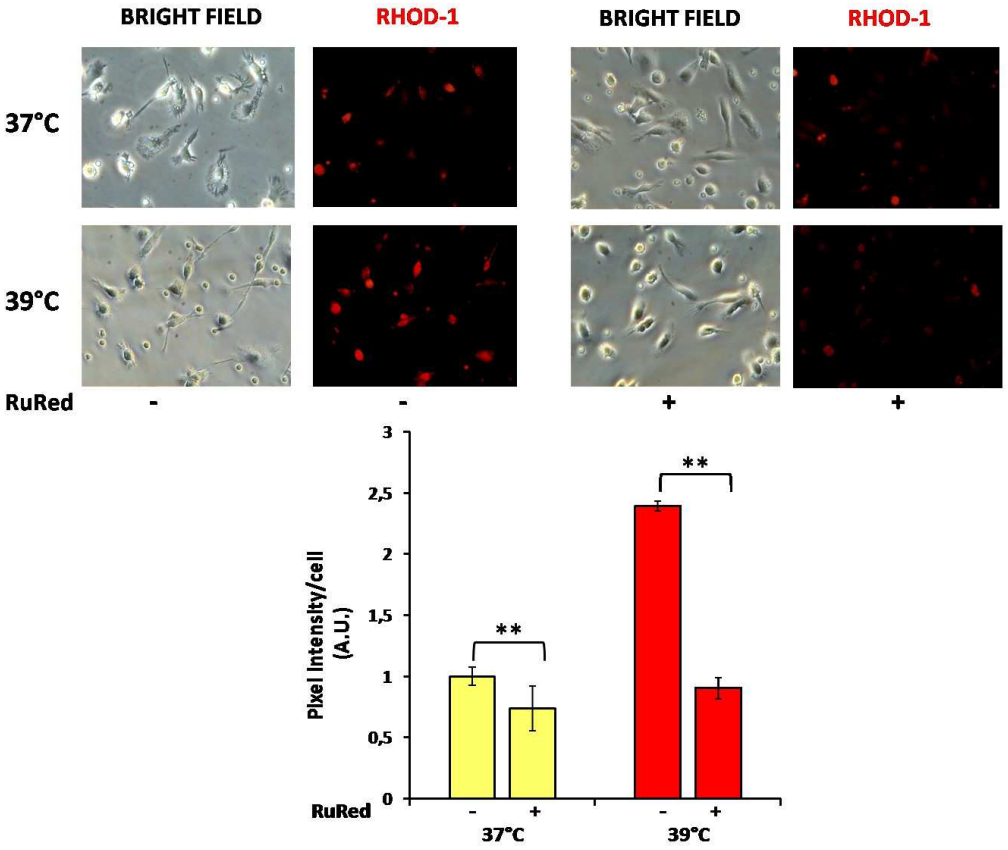


Fig.24. Fluorescence microscopy imaging of mitochondrial Ca^{2+}. Cultured cells were incubated at 39°C for 3h in the absence and in the presence of 5μM of the mitochondrial calcium uniporter inhibitor Ruthenium Red. Mitochondrial [Ca^{2+}] was assesd by the mitotropic Ca^{2+}-sensitive fluorescent probe Rhod-1. **P<0.01.

Fig.24 illustrates that a significant rise of the mt-Ca^{2+} takes place in the 39°C-treated MoDCs as compared with normothermic cells and that this uptake

was completely prevented in the 39°C-treated cells by incubation with the mitochondrial calcium uniporter inhibitor Ruthenium Red. Of note, incubation of MoDCs with Ruthenium Red during the 39°C treatment partially prevented the inhibition of the mitochondrial OxOhos efficiency elicited by the mild hyperthermia (Fig. 25).

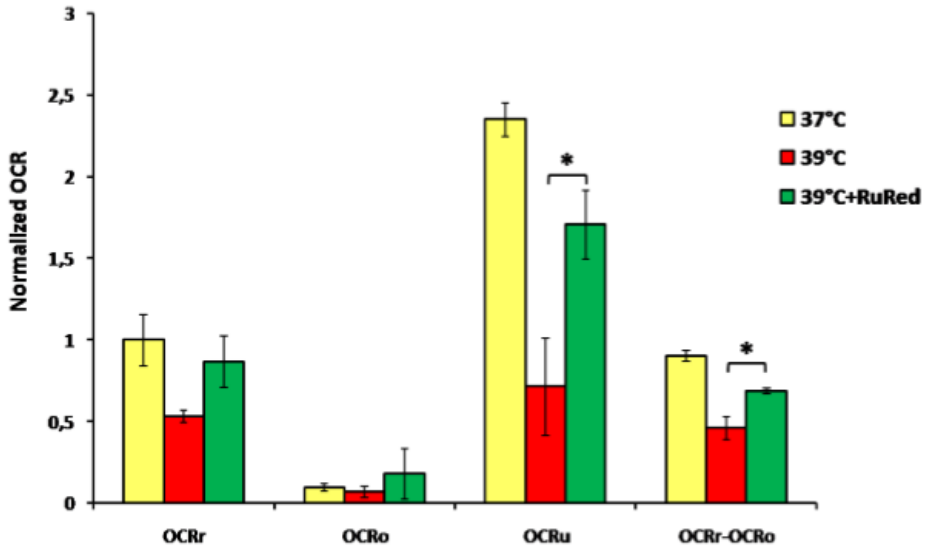


Fig.25. Effect of of the mitochondrial calcium uniporter inhibitor Ruthenium Red on mitochondrial respiration in hyperthermia-treated MoDCs. *P<0.05.

Discussion

Recent studies on the metabolism of immune cells have highlighted the tight link between metabolic state and phenotype of these cells. Their metabolism not only characterizes different immune cell phenotypes in response to alterations in cellular signalling, but it also alters signalling to drive immune-cell phenotype. This is linked to changes in the redox homeostasis and/or accumulation of metabolites, which serve as signal transducers activating/suppressing defined sets of transcription factors. On this basis, targeting mitochondrial metabolism is becoming an attractive therapeutic strategy for modulation of the immune response in inflammatory patological states. The innate immune system, including macrophages, neutrophils, Dendritic Cells and natural killer T cells, is a conserved system acting as a first-line of defense.

4.1 Para-Hydroxyphenylpyruvate inhibits the LPS-mediated metabolic priming of macrophages

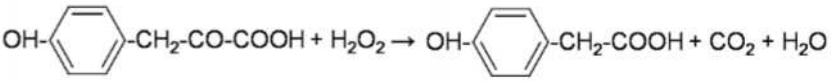
The innate immune system, including macrophages, neutrophils, Dendritic Cells and natural killer T cells, is a conserved system acting as a first-line of defense. Macrophages are monocytes-derived cells that play important roles in the innate immune system and include: resident macrophages that patrol their surroundings and maintain homeostasis, macrophages that attack invading pathogens and tumor cells, macrophages that orchestrate the process of wound healing and macrophages that resolve inflammation. Since one cell cannot perform all these divergent tasks simultaneously, it was suggested that macrophages can be differently activated, depending on the signals received from the microenvironment. Thus, macrophages exhibit an enormous plasticity [83] and dynamically shift from one form of activation to another according to the conditions [84]. Activation of Toll like receptors (TLR) by danger-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs), such as LPS, induces innate immune cells toward a transition from a quiescent M0 to an activated M1 state, characterized by changes in the metabolic state, production of pro-inflammatory cytokines and high phagocytic and bactericidal activity.

On the basis of the results presented, a working model linking PAMPs and DAMPs-mediated immune response to mitochondrial metabolism will be put forward along with suggestions for its therapeutic control.

First of all, the results of the present study show that the overnight treatment of macrophages with LPS induced a marked morphological change of RAW 264.7 cells, diagnostic of cell-activation (Fig. 5), confirmed by production of pro-inflammatory cytokines like IL-6 (Fig. 6). Indeed, in response to phagocytic particles and chemoattractant stimuli (like LPS), FcγRs, RTKs and GPCRs share common downstream effectors that could be regulated by kinases and phosphatases for coordinated responses, leading to the reorganization of actin cytoskeleton required for regulation of cell shape, adhesion, migration and phagocytosis. In this context, the Rho GTPases such as Rac and Cdc42 are involved in all these processes [85]. pHPP prevented dose-dependently and more efficiently than EP the release of IL-6, but it had no impact on the LPS-induced morphological changes, because these effects are liked to different signalling pathways (Fig. 7). The production of pro-inflammatory cytokines is indeed under control of the transcription factor NF-kB [86], one of the major down-stream target of the TLR4-related signalling pathway, while FcγRs, RTKs and GPCRs are involved in the actin cytoskeleton reorganization.

LPS-activated RAW 264.7 cells also exhibit a sharped rise in ROS and RNS production mostly due to inducible Nitric Oxide Synthase (iNOS) and NADPH oxidase 2 (NOX2) augmented activities. iNOS and NOX are key mediators of macrophage immune response that play central roles in the intracellular killing of pathogens. NOX2 is a major source of free radicals and its respiratory burst is well described in LPS activated neutrophils and macrophages [87]. It can be transcriptionally induced and post-transcriptionally activated by LPS because both ROS generation and the activation of the redox-sensitive transcription factor NF-kB are mediated by a direct interaction of TLR4 with with a member of the NADPH oxidase (NOX4) [88]. Downstream of NF-kB, an increase in the expression of proinflammatory genes including inducible nitric oxide synthase (iNOS) is also usually observed [89]. This was confirmed in RAW 264.7 macrophages, where LPS-treatment caused a marked up-regulation of the iNOS expression and consequent overproduction of NO, measured by its by-products NO_2^-/NO_3^- released in the culture medium and also detected by fluorescence microscopy. In our cellular model pHPP prevented dose-dependently both these effects more efficiently than EP (Fig. 8 and 9) and it also abolished the LPS-induced oxidative burst (Fig. 10). ROS production not only enhances phagocytic and bactericidal activity of M1 macrophages but also directly activate redox-sensitive transcription factors, like NF-kB [90], priming immune cells to pro-inflammatory commitment. In this scenario we propose

that the anti-inflammatory effects of pHPP can be mainly attributable to its direct antioxidant activities [91] which, depending on the cheto-enol tautomer involved, can scavenge either H_2O_2 and $ONOO^-$ or OH• and NO•. The pyruvoyl mojety present in pHPP can react with peroxides leading to decarboxylation according to the reaction:



while the enol tautomer can extend the delocalization of the π electrons of the aromatic phenol ring, acquiring the ability to scavenge one-electron unpaired species such as O2•-, OH•, NO•. Interestingly, macrophages constitutively express the inflammatory cytokine macrophage migration inhibitory factor (MIF), which in addition to sustain inflammatory responses through the suppression of anti-inflammatory effects of glucocorticoids [92], can catalyze the tautomerization of pHPP toward the enol tautomer. Althought the biological function of MIF catalytic activity is still unclear, it might have fortuitously provided additional antioxidant properties to the administered pHPP under the experimental conditions of this study.

On the basis of our observation, pHPP can be considered an effective scavenger of ROS and RNS that could be used as therapeutic agents for various pathological conditions mediated by redox dependent phenomena such as sepsis, to prevent oxidative damage and multiorgan energetic failure as well as dysregulated pro-inflammatory commitment of immune cells.

In addition to their role in the intracellular killing of pathogens, ROS and RNS are directly linked to the switch of M1 macrophages to "aerobic glycolysis". As mentioned before, during early inflammation, polarisation towards M1 macrophages is dependent on NOX2 activation which, via protein tyrosine phosphatase oxidation and AKT activation, increases trafficking of glucose transporters to the membrane and glucose uptake for glycolysis [89]. At the same time, the activation of redox-sensitive transcription factors, like NF-kB increases the expression iNOS leading to the formation of NO [89]. The reaction between superoxide $O_2^{\bullet-}$ and nitric oxide NO leads to the production of peroxynitrite, to support the killing of bacteria and further recruitment of other immune cells, but also responsible for nitrosylation and consequent inhibition of iron-sulphur (Fe-S) cluster proteins in the Complex I of the electron transport chain [93]. Moreover, NO, competing with O_2, on the cytochrome c oxidase (CxIV) has a direct inhibitory effect of the terminal electron acceptor of the mitochondrial respiratory chain, slowing-down of the electron transfer rate [94].

In this way, under pro-inflammatory conditions dysfunctional mitochondria become a major source of ROS [95] because of accumulation of redox intermediates up-stream of CxIV and enhanced electron leakage [96]. In parallel, mitochondrial efficiency is likely to be compromised via ROS/RNS-mediated inhibition of the respiratory chain and glycolysis is promoted. The development of mitochondrial dysfunction is among the main causes of multiple organ dysfunction syndrome (MODS) and it is mostly due to the metabolic reprogramming in infected tissues. In this scenario, preventing or reversing the OxPhos impairment may represent an effective therapeutic strategy in the treatment of sepsis.

Consistent with this model we found a significant 50% inhibition of the mitochondrial resting endogenous oxygen consumption and of the ATP-linked in intact RAW 264.7 challenged with LPS (Fig. 11). To notice the specific activities of CxI and CxIV of the respiratory chain were inhibited by LPS to a similar extent of the respiratory activity in solubilized cells under normoxic setting, suggesting permanent (nitro)oxidative modification of the respiratory complexes and excluding the competitive inhibition of NO and ROS (Fig. 13). To verify that the reduction of mitochondrial respiratory activity corresponded to a shift toward a glycolytic metabolism, metabolic flux analysis was assessed by SeaHorse technology, revealing that the LPS-induced decrease in mitochondrial OxPhos activity was accompanied with a consistent rise of the extracellular acidification rates (ECAR), indicative of a compensatory up-regulation of the aerobic glycolysis, resembling the Warburg effect observed in tumours (Fig.12).

A major transcription factor controlling the expression of the glucose transporters and glycolytic enzymes is the heterodimeric transcription factor HIF-1, whose β subunit is constitutively expressed, while its α subunit is constitutively transcribed but immediately degraded in normoxia following its hydroxylation by O_2-dependent prolyl hydroxylases (PHDs) [97]. Although HIF-1α subunits are typically induced in hypoxia, hypoxia-independent stabilization of HIF-1α has been described under pro-inflammatory conditions where high levels of succinate or increased ROS production, that oxidizes Fe^{+2}, may cause PHDs inactivation and HIF-1α stabilization [98]. Also ligation of TLRs, such as the binding of LPS to TLR4, stimulate HIF-1α expression through NF-kB pathway and hypoxia shows a synergistic effect with LPS [99]. NF-kB-mediated iNOS expression causes increase in concentrations of NO that following the subsequent increase in peroxynitrite can stabilize HIF-1α in normoxia by nitrosylation-mediated inhibition of PHDs activity, probably

targeting their Fe^{+2} catalytic site [100]. In this way, during inflammation, HIF-1α is stabilized by inactivating prolyl hydroxylase activity and transactivated by the NF-kB pathway [33]**,** resulting in the activation of the gene encoding the pyruvate dehydrogenase kinase (PDK) [34] and in the production of glycolytic enzymes, glucose transporters and pro-inflammatory cytokines [101]. HIF 1α pathway is essential for M1 macrophages survival and immune function, considering that they are mainly found in hypoxic environments and therefore have to rely on glycolysis for their ATP production. Consistent with this notion we found in LPS-stimulated RAW 264.7 a significant stabilization of HIF-1α as compared with the unstimulated resting macrophages (Fig. 15). To notice, pHPP inhibited all the above described alterations in LPS-stimulated RAW 264.7. Indeed a complete prevention of the LPS-mediated reduction of the mitochondrial respiratory activity was observed following co-incubation with pHPP, as well as the prevention of the inhibition the specific enzymatic activities of CxI and CxIV in solubilised cells. Likewise, the stabilization of HIF-1α was also inhibited. Thanks to antioxidant power of pHPP, ROS and RNS levels would decline, reducing respiratory chain complexes permanent and competitive inhibition as well as PHDs inactivation; HIF1α would then be less likely to be nitrosylate, and therefore would be more likely to be degraded and glycolytic enzyme and pro-inflammatory cytokines production would decline. In addition to being an antioxidant, pHPP is also considered a bioenergizer because it is an intermediate of the catabolic pathway whose end-products Fumarate and Acetoacetate can enter into mitochondria and support the Krebs Cycle either as intermediates or substrates, preventing cellular energetic failure induced by LPS stimulation. Notably, also when macrophages were co-incubated with DCA, a known activator of pyruvate dehydrogenase (PDH), the LPS-mediated inhibition of cell respiration was completely prevented thus suggesting an impairment of pyruvate oxidation in LPS-treated cells. In fact, DCA removes the block of the respiratory activity caused by LPS-signalling, leading the shift from glycolytic to aerobic metabolism by inhibiting the PDK, the selective inhibitor of pyruvate dehydrogenase (PDH). So, on the basis of these results, we hypothesized that the incresed respiratory activity in presence of pHPP was due to a risen availability of reducing equivalents from Krebs Cycle, otherwise blocked by LPS intracellular signalling mediated by HIF1α and PDK, that results in PDH inhibition. Interestingly the fact that primary human fibroblasts treated directly with septic sera also showed the depressed respiratory phenotype observed with the LPS-stimulated RAW 264.7 (Fig. 14) highlights the general relevance of this observation.

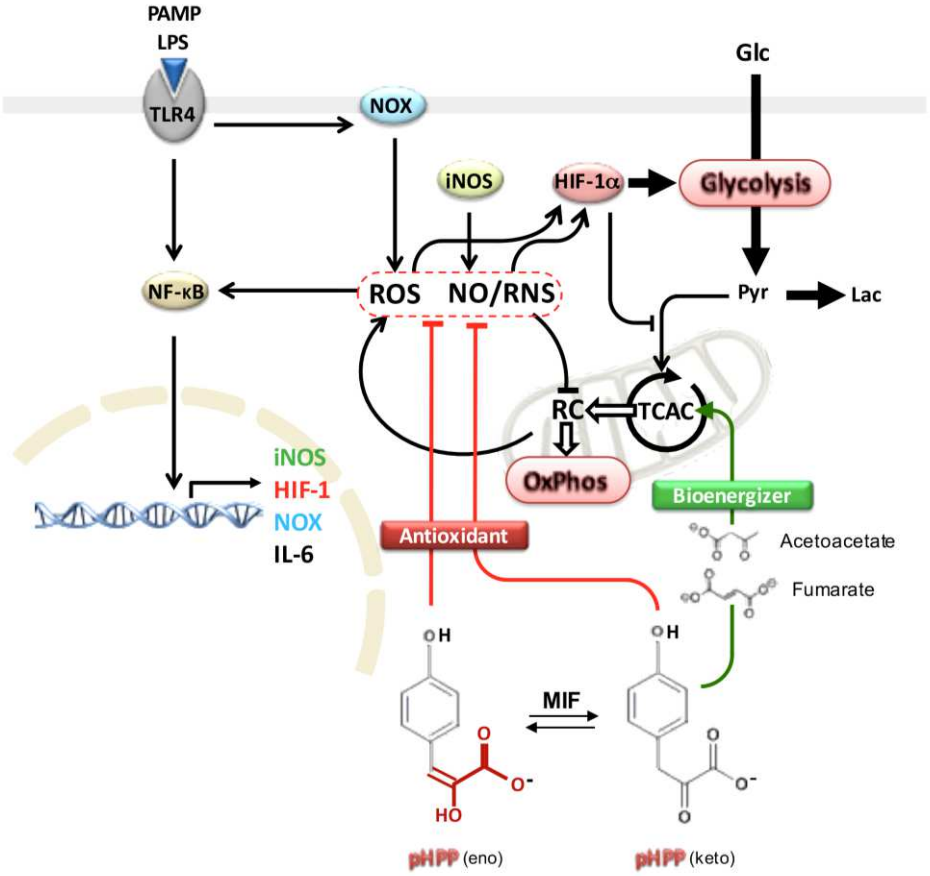


Fig.26 Schematic drawing of the anti-inflammatory effect of pHPP. The scheme highlights the effect of pHPP as resulting from its combined reactive species scavenger and respiratory substrate-supplier actions.

In conclusion we propose that the anti-inflammatory and metabolic effects of pHPP are mainly linked to its ROS and RNS scavenging activities. These would counteract a number of redox sensitive processes like activation of the transcription factors NF-kB, stabilization of HIF-1α and the respiratory chain activity. In addition, the production of terminal catabolites of the tyrosine degradation pathway would directly fuel the Krebs cycle thus favouring the OxPhos metabolism. All together these effects would hinder the pro-inflammatory metabolic priming of macrophage, as well as of other immune cell type, candidating pHPP as a promising drug in diseases hallmarked by disproportionate activation of the immune system, like sepsis.

4.2 Febrile Temperature and metabolic phenotype in Monocyte-Derived Dendritic Cells

The innate immune response can be also stimulated by noninfectious stimuli like elevated temperatures as confirmed in previous studies showing thermal activation of dendritic cells and other immunocytes like macrophages and neutrophils. Fever is a preserved symptom associated with improved survival and resolution of many infections [47]. However, as in the cases of PAMPs-induced immune response, in conditions of extreme inflammation, like sepsis, fever can lead to neurological injuries instead of being a beneficial symptom. The induction of fever involves specific interplay between the innate immune system and neuronal circuitry and in this context Dendritic cells represent the most potent and versatile antigen-presenting cells, finely orchestrating the cooperation between innate and adaptive immunity [19].

In the second part of study we have deepened the effect of fever-like temperatures on the cell physiology of Monocyte-Derived Dendritic Cells (MoDCs) mimicking feverish conditions by incubating cells for 3h in a humified 5% CO_2 incubator at 39°C. Notably, the experimental protocol adopted in this study foresaw a relatively smaller increase of the ambient temperature (i.e. a Δt=2°C over the normothermic condition) and a shorter exposure to it (i.e. 3 h) as compared with previous reports.

The results obtained show that MoDCs exhibited hyperthermia-induced commitment from a quiescent to an activated immune state as evidenced by production of TNF-α and NO (Fig. 16), confirming the stimulatory effect of elevated temperatures on the pro-inflammatory and phagocytic potential of Dendritic cells [51].

As activation of phagocytic cells is linked to changes in the redox homeostasis, we verified the effect of mild hyperthermia on the cellular redox state by imaging, demonstrating that 39°C-treated MoDCs are characterized by a significant increase in both ROS and NO production (Fig. 17-21), accompanied by a marked decrease in the mitochondria-related respiratory activity and consequently in the OxPhos efficiency, likely linked to a switch to a gycolytic metabolism (Fig. 18-19). Conversely, undifferentiated monocytes, did not undergo the metabolic shift towards a glycolitic metabolism after thermal shock. These cells indeed exhibited low respiratory activity in both untreated and heat-treated samples thus suggesting that monocytes likely have to rely on glycolysis also in physiological conditions, losing the sensitivity of the mitochondrial activity to thermal stress (Fig.18).

As better detailed in the previous paragraph, the mitochondrial respiratory chain complexes are both major producers and targets of reactive oxygen and nitrogen species, that are able to impair mitochondrial respiratory activity. As a consequence of a defective electron transfer throughout the respiratory chain, a significant overproduction of mitochondrial reactive oxygen species takes place according to the ROS-induced ROS release (RIRR) mechanism, whereby ROS generated by NOX would cause further production of ROS by the mitochondrial respiratory chain [102].

A step-by-step analysis of various segments of the respiratory chain of MoDCs, revealed specific alterations at the level of the NADH-ubiquinone oxidoreductase (CxI) and of the ubiquinol-cytochrome c oxidoreductase (CxIII) (Fig. 20). Intriguingly, both the respiratory complexes utilize the CoQ as substrate and depletion of CoQ has been reported in septic shock and associated with the inflammatory cascade [103]. Moreover, a growing body of evidence suggests that treatment of cells with TNF-α results in an electron transport inhibition at the level of CIII [104] [105] followed by an increased generation of ROS in mitochondria [106] and we found increased production of both TNF-α and ROS in hyperthermia treated MoDCs. The inhibitory effect of TNF-α could be attributable to the activation of sphingomyelinases that cleave membrane sphingomyelin resulting in the formation of ceramide [107]. which can move to mitochondria where directly interacts with CxIII, inhibiting cytochrome b reoxidation in the Q-cycle with subsequent generation of ROS. However, the inhibition of the mitochondrial respiratory activity observed in the 39°C-treated cells is an irreversible process, because re-conditioning of the 39°C-treated MoDCs to normothermia did not cause a significant recovery of the mitochondrial OxPhos activity, suggesting a permanent, covalent modification of the respiratory complexes (Fig.23).

The observed inhibition of the mitochondrial respiratory activity in 39°C-treated cells is probably linked to the release of factors recognized as DAMPs in the culture medium, since the treatment of MoDCs with the 39°C-conditioned medium under normothermic conditions resulted in the inhibition of the mitochondrial respiratory activity (Fig. 22). Some DAMPs can engage TLRs to induce and amplify the inflammatory response mediating NF-kB activation. [108]. The earliest event following a rise in the body temperature is the increased expression of a large family of heat shock proteins (HSPs), which acting as chaperonins protect the cell by stabilizing unfolded proteins. Under non-stressing conditions the heat shock transcription factor (HSF-1) is bound to HSPs which inhibit its activity. Under stressful conditions such as heat shock,

pH shift or hypoxia, HSPs are recruited to client proteins and let HSF-1 move into the nucleus where it activates transcription of HSP coding genes. Consistently, a gene expression analysis carried out on MoDCs exposed for 3 h at 39°C resulted in a significant 4 to 10 fold up-regulation in the expression of HSP70, HSP105, HSP40, HSP60 (A. Liso et al., unpublished data). Although HSPs are usually intracellular chaperon, heat stress can induce HSP70 release from cells into the extracellular environment where it can act as a DAMP to stimulate macrophages and DCs [109]. Extracellular HSP70 and other HSPs engage multiple surface receptors, including CD91, scavenger receptor A, CD40, TLR2 or TLR4, leading to the release of nitric oxide, TNF, IL-6, IL-1β and IL-12 [110]. Therefore, we suggest that in our experimental model the mild hyperthermia-induced activation of MoDCs is mediated by autocrine stimulation by HSPs via binding to TLRs. This would imbalance the cellular redox homeostasis activating redox sensitive transcription factors like NF-kB, that enhances the expression of pro-inflammatory genes including cytokines such as TNFα [89]. In this context, the attenuation of redox state alterations could reduce the pro-inflammatory activation of hyperthermia-treated MoDCs. Indeed, incubation of MoDCs with the NOS pan-inhibitor L-NAME during the thermal shock was able to prevented the hyperthermia-induced release of TNFα (Fig.16).

In addition we investigated the mitochondrial calcium level in thermally stressed MoDCs since intracellular Ca^{2+} plays important roles as second messenger in the immune response regulation [81] and mitochondria modulate the cytosolic [Ca^{2+}], acting as local Ca^{2+} buffers [82]. The result obtained clearly show that the 39°C-treated cell displayed a larger uptake of cytosolic of Ca^{2+}, due to increased release from intracellular stores or from transmembrane transient receptor potential (TRP) channels, a family of cationic thermo- and chemo-sensitive channel acting as molecular sensors of changes in body temperature. In particular the vanilloid transient receptor potential subfamily (TRPV) comprises a number different isoforms heat-sensitive to a wide range of temperature values. This property of TRP channels is relevant in the contexts of immune cells activation and maturation. Although initially discovered in the sensory neurons, subsequent reports have suggested the expression and functional role of thermosensitive TRPV channels in different immune cells, especially macrophages and dendritic cells, where they can induce immunogenicity after exposure to heat or capsaicin [111].

Coherently with this observations, we found a significant rise of the mt-Ca^{2+} in the 39°C-treated MoDCs that was completely prevented by incubation

with the mitochondrial calcium uniporter inhibitor Ruthenium Red (Fig. 24). So it is likely that hypethermia-induced activation of TRP channels drives increase in the intracellular level of Ca^{2+}, then modulated by mitochondria which buffer excessive rise in cytosolic Ca^{2+} by importing it *via* a ruthenium red-sensitive calcium uniporter. High levels of mitochondrial Ca^{2+} may decrease the $\Delta\mu H^{+}$ and consequently the efficiency of ATP synthesis and can detach cytochrome c from the inner membrane slowing down the flux of electrons between CIII and CIV of the mitochondrial respiratory chain [112]. In addition, Ca^{2+} activates the mitochondrial isoform of a nitric oxide synthase, thereby releasing NO• inhibiting cytochrome c oxidase [113]. The synergistic combination of these two events causes accumulation of reducing equivalents upstream of cytochrome c oxidase, resulting in electron leakage to O_2 with increased formation of $O_2^{\bullet-}$ especially by CI [114], therefore establishing a self-inhibition [115]. Indeed, the hyperthermia-induced inhibition of the mitochondrial OxPhos can be partially prevented blocking Ca^{2+} entry into the mitochondria by incubation of MoDCs with Ruthenium Red during the 39°C treatment (Fig. 25).

On the basis of the results presented, we can infer that in the innate immune system both microbial and physical stimuli share common signalling pathways, altering the metabolic state for cell commitment toward immune activation. Hyperthermia-induced priming of MoDCs through an autocrine mechanism mediated by HSPs and macrophage pathogenic stimulation by LPS activate TLRs, inducing marked changes in the redox homeostasis due to NOX and iNOS activation, leading to $O_2^{\bullet-}$ and $NO^{\bullet}$ generation. This unbalance of the redox state, in addition to enhance intracellular killing of pathogens, activates defined sets of transcription factors like NF-kB leading to enhanced expression of iNOS, pro-inflammatory cytokines and HIF 1, rewiring cell metabolism and priming cells for immune response. Moreover, $NO^{\bullet}$ is a competitive inhibitor of CIV of the respiratory chain, whose inhibition leads to accumulation of reducing equivalents into the up-stream complexes CxI and CxIII. This enhances the possibility of electron leaks with production of $O_2^{\bullet-}$ and other ROS that may account for the observed impairment of the respiratory chain activity and consequent switch to aerobic glycolysis driven by HIF-1α stabilization. The reaction between $NO^{\bullet}$ and $O_2^{\bullet-}$ leads to the formation of peroxynitrite ($ONOO^{-}$), causing nitrosylation and consequent inhibition of CxI. In this context, the enhanced mitochondrial ROS production is counteracted by antioxidant enzymes, such as the glutathione peroxidase which converts H_2O_2 in $2H_2O$ molecules by oxidizing gluthatione 2GSH in GS-SG, but accumulation of GS-SG leads to the reversible glutathionylation of sulphydryl groups of protein

cysteins, exerting an inhibitory effect on the respiratory chain complexes [116]. Intriguingly, the aforementioned differential gene expression analysis on the 39°C-treated MoDCs unveiled a significant ≈3 fold decrease of the manganese-superoxide dismutase (SOD2), which is the mitochondrial isoform of the ROS-scavenging enzyme. In general this metabolic reprogramming up-regulates the aerobic glycolysis and suppresses the mitochondrial pyruvate oxidation giving cells enhanced biosynthetic capacity and at the same time allowing cells to adapt and survive in metabolically restrictive conditions, such as hypoxia.

A further possible thermal sensor might be provided by the TRP channels whose activation increases the intracellular level of Ca^{2+}, modulating the immune response, while the observed increase of the mitochondrial Ca^{2+} in our cellular model might be an adaptive mechanism of mitochondria to buffer the thermally induced cytosolic Ca^{2+} increase.

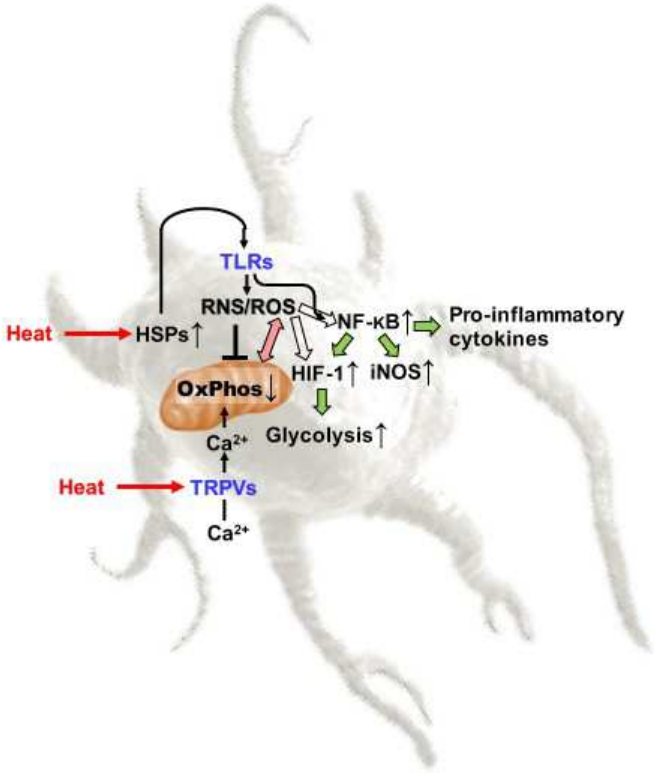


Fig. 27 Working model of the effect of mild hyperthermic conditioning of MoDCs on cell metabolism and pro-inflammatory activation.

In conclusion the results presented in this study support the emerging notion that immunological response involves specific metabolic reprogramming of immune-competent cells and point out that both physical and biological stimuli are able to rewire cellular metabolism in order to induce immune

activation. However, in cases of dysregulated immune response like sepsis, organ failures are associated with fundamental alterations of cellular metabolism and in this scenario modulation of the metabolic rewiring in specific check-points by antioxidants (L-NAME and pHPP), bioenergizers (pHPP) and channel inhibitors (Ruthenium Red) could represent an effective pharmacological strategy to hamper the pro-inflammatory metabolic priming of immune cells and cope with the tissue energy crisis, attenuating certain symptoms induced by endotoxemia as well as by febrile temperatures.

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