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MODULATION OF TLR4-INDUCED VARIATIONS IN MITOCHONDRIAL CPT1 AND CPT2 EXPRESSION BY IMMUNOMODULATORY METABOLITES AND ITS CORRELATION ON THE SELECTIVE FUNCTIONAL DIFFERENTIATION OF MACROPHAGES

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DEGREE FINAL PROJECT IN BIOTECHNOLOGY

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Yo, Aitana Herrera Azcona, con DNI 78758961S, soy conocedora de la guía de prevención del plagio en la URV "Prevención, detección y tratamiento del plagio en la docencia: guía para estudiantes" aprobada en julio de 2017 (http://www.urv.cat/ca/vidacampus/serveis/crai/que-us-oferim/formacio-competencies-nuclears/plagi/) y afirmo que en este TFG no constituyen ninguna de las conductas consideradas como plagio por la URV.

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CENTRE INFORMATION

Madrid Institute for Advanced Studies (iMdea) in Food has the main objective to do world-class research in the fields of food, nutrition, and health, as well as to discover nutritional methods with economic and social value. Its founding mission is to transfer health and nutrition research into benefits for the Madrid Region's and Spain's economies, as well as for the well-being of the country's citizens.

Different nutrients and food products have been found to play crucial roles in the body's molecular systems, making nutrition a powerful tool in the prevention and treatment of chronic disease. The Institute's research focuses on the human body's molecular mechanisms, the physiological impacts of chronic disease, and how these are linked to the nutrient’s intake.

The objective of the Molecular Immunonutrition group lead by José Moisés Laparra is to study immunonutritional-based precision intervention strategies to selectively modulate innate immune responses with the aim of preventing the risk of gut-liver axis related diseases. In the research program Precision Nutrition and Cancer, one of the objectives is to understand how tolerance processes and immune system regulate antitumoral responses to apply the knowledge to develop long-lasting immune responses.

ABSTRACT

Immunometabolism, which is related to metabolic changes in effector cells of the immune system, constitute a critical determinant in the activation of specific immune responses. Carnitine-palmitoyltransferases (CPTs) can regulate the metabolic fate of fatty acids and palmitate-induced immunometabolic effects by innate immune receptors. The objective of this study was to evaluate the potential of butyrate, itaconate and plant protease inhibitors (PPIs) to modulate TLR4-induced response (bacterial lipopolysaccharide primed) on human macrophages. Thus, the effects on the enzyme activities in the mitochondrial and endo/lysosomal compartment were monitored, as well as the expression of mitochondrial CPT1/2 proteins and macrophage (HB8902® cells) phenotypic adaptations by flow cytometry. Butyrate and itaconate differentially affected, (improving and worsening, respectively) mitochondrial metabolism. Cell cultures subjected to the sequential exposure to both butyrate or PPIs and LPS, improved their mitochondrial activities in relation to LPS alone. Pre-treatment with PPIs and itaconate exerted positive effects normalizing endo/lysosomal activities compared to controls. All the assayed treatments promoted higher variations in CPT2 expression than in CPT1. LPS increased both CPT1 and CPT2 levels. Butyrate and itaconate when exposed together with LPS enhanced the distribution of macrophage phenotypes towards its proinflammatory profile (M1) and favoured downward trends in CPT2 expression. Either butyrate or itaconate increased the expression of CX3CR1 allowing their interaction with the tissue. In contrast, pre-treatment of cell cultures with PPIs induced a more normalized ratio between phenotypes distribution. These results open new perspectives on potential coadjuvant immunonutritional intervention strategies to classical pharmacological therapies.

Key words: macrophages, butyrate, itaconate, Toll-like receptor 4, carnitine palmitoyltransferases

INTRODUCTION

Palmitoylation is the covalent attachment of fatty acids to certain residues of peptides, typically to membrane proteins, and its precise function depends on the target protein being palmitoylated. Palmitoylation mediates the affinity of peptides for lipid rafts and facilitates their assembling. Protein clustering can impair innate immune function promoting the loss of 'Toll-like' receptors (TLR) signalling and, thereby the activity of the macrophage population that acts as a gatekeeper of immune response(s) against diseases. Immunometabolism, which is related to metabolic changes in the effector cells of the immune system constitute critical hallmarks in the activation of specific immune responses that take place in response to specific signalling pathways. A key result of these changes involves the influence on palmitoylation of different metabolites such as lipids, amino acids, polyamines, and products of the gut microbiota. A rich and diverse set of palmitoylated proteins has been discovered in macrophages, which alter the phenotype and modulate immunity and inflammation. The innate immune TLR4 receptor is a transmembrane protein whose molecular signalling is associated with palmitoylation processes through the activity of fatty acid synthase (FASN), and which is essential for the activation of the monocyte population. The hyperactivation of macrophages influence the severity and progression of various pathologies, such as non-alcoholic hepatic steatosis, cancer and respiratory disease caused by SARS-Cov2 virus.

1. Global burden of immunonutritional diseases

At the beginning of the 21st century, the world population is facing major and devastating pandemics; obesity and type 2 diabetes, with a great influence on liver dysfunction. This effect is mainly caused as a consequence of excessive lipid accumulation and characterized by hyperlipidemia, hyperglycemia, and cardiovascular complications (Benedict & Zhang, 2017).

Non-alcoholic fatty liver disease (NAFLD) has become the most common liver pathology worldwide affecting an estimated 15-30% of most populations due to a dramatic increase of risk factors such as obesity, sedentary lifestyle, and altered food supply and food preferences (Mitra et al., 2020). While non-inflammatory NAFLD incurs a high risk for the development of type 2 diabetes mellitus (T2DM) and of other major features of the metabolic syndrome (MS) (mainly cardiovascular complications like atherosclerosis and myocardial ischemia/infarction), the disease also affects morbidity and mortality (Paschos & Paletas, 2009). Thus 10-20% of subjects with NAFLD will suffer the severe variant of non-alcoholic steatohepatitis (NASH) (López-Velázquez et al., 2014), where fatty liver has progressed to massive hepatocyte apoptosis and hepatic inflammation, which sometimes ends in the development of hepatocellular carcinoma (HCC) (Benedict & Zhang, 2017; Kanda et al., 2018; Overi et al., 2020; Paschos & Paletas, 2009). Apoptosis of hepatic cells refers to mitochondrial dysfunction and lipoapoptosis

due to an excess of free fatty acids and uncontrolled oxidative processes (Kanda et al., 2018). These effects are highly dependent on macrophage function, which play central roles in coordinating immune responses that are associated to an increased expression of the fatty acid receptor (Hao et al., 2018; Viola et al., 2019), recently associated to metastasis and tumour growth and penetrance (Fernández et al., 2020; Hao et al., 2018).

In this context, while the endogenous factors are difficult to modulate, the environmental ones are predominant and addressable in a preventive or therapeutic strategy. Previous research has mainly focused on the influence of total calorie intake and consumption in the development of obesity, fatty liver, NASH and/or the MS. However, recent data suggest that the composition of the food, irrespective of its caloric level, as well as food influence on the host's intestinal immune system, may be even a more important factor preventing the most severe consequences from NAFLD or NASH (Eslamparast et al., 2017; Jasirwan et al., 2019; Perdomo et al., 2019).

2. Palmitoylation and its role in immunity

Palmitic acid (C16:0) (PA) is a long-chain saturated fatty acid, and a component of various lipids playing important roles in the cell membrane organization, signal transduction, and energy storage (Tzeng et al., 2019). Moreover, the palmitoyl chain can be attached to proteins (i.e., S-palmitoylation), where this modification can affect protein subcellular distribution and function (Spinelli et al., 2018). S-palmitoylation is a posttranslational modification of proteins consisting in a potentially reversible covalent attachment of palmitoyl chain to a cysteine residue(s) of proteins (Jin et al., 2021; Lin, 2021; Merrick et al., 2011). The palmitoylation is a two-step mechanism: i) First, palmitoyl-coA is bound to the Palmitoyl Acyl Transferase (PAT) enzyme and hydrolysed, forming a chemical intermediate, ii) Second, the acyl group is transposed to the target protein (Spinelli et al., 2018). As it is a reversible reaction, Acyl Protein Thioesterases (APT) can catalyze the thioester hydrolysis of palmitoylated cysteines in a process named depalmitoylation (Spinelli et al., 2018). The palmitoylome refers to all the palmitoylable proteins codified by the genome, which represents around a 10%. Interestingly, some human diseases including immunological, viral infections and cancer and neurodegenerative disorders are related to changes in the palmitoylome (Cho & Park, 2016; Spinelli et al., 2018; Yao et al., 2019).

S-palmitoylation contributes to the compartmentalization of proteins to distinct membrane domains (i.e., rafts) and has been found to be required for the correct targeting of some signalling proteins (Horejsi & Hrdinka, 2014). Rafts are nanodomains of the plasma membrane and some intracellular membranes, mainly of the Golgi apparatus, that are rich in sphingolipids, glycerophospholipids with saturated fatty acid chains, and cholesterol (Horejsi & Hrdinka, 2014). Notably, membrane rafts are sites of signal transduction by distinct receptors of immune cells involved in immune reactions. The latter include either those that are adaptative (i.e., T cell receptor (TCR), Fcε receptor

I, and Fcγ receptor II) and those related to innate immune responses (i.e., TLR4 that interacts with the Gram(-) bacterial lipopolysaccharide (LPS) and induce proinflammatory signalling and cytokines) (Horejsi & Hrdinka, 2014; Płóciennikowska et al., 2015).

Palmitoylation is a type of post-translational modification (PTM), also named protein acylation, and other fatty acids than PA are used in processes named as N-acylation (myristate) or O-acylation (Sobocinska et al., 2018; Spinelli et al., 2018). Less frequently, palmitate can also be attached to i) the amine group of various amino acids (glycine, cysteine, and lysine) giving N-palmitoylation or to ii) the hydroxyl group of serine or threonine in a process called O-palmitoylation (Guan & Fierke, 2011; Sobocinska et al., 2018). For example, secreted Wnt and ghrelin proteins are O-acylated with atypical fatty acid residues such as palmitoleate (C16:1) and octaonoate (C8:0) (Sobocinska et al., 2018; Zheng et al., 2016). Additionally, histone H4 could be O-palmitoylated at its serine residue in the nucleus (Sobocinska et al., 2018), which has special interest in the context of innate immune responses. Histone H4 O-palmitoylation regulates transcriptional activity (Zou et al., 2011) modulating downstream pro-inflammatory signals triggered by innate immune receptors (Sobocinska et al., 2018). ε-N-acylation is a type of acylation process that consist of attaching a fatty acid residue to the side chain of lysine by amide linkage (Kowalczyk et al., 2017; Lim et al., 2000). Examples of ε-N-myristoylated molecules are cytokines such as interleukin 1α (IL-1α) (Chiu et al., 2021) and tumour necrosis factor α (TNFα) (Udenwobele et al., 2017), the pro-inflammatory cytokines crucial in combating bacterial infections that are produced closely associated to TLRs signalling. This modification is also found in the so-called RTX toxins released by some pathogenic Gram-negative bacteria (Lim et al., 2000).

The innate immune system is strongly associated with obesity, insulin resistance, metabolic syndrome, and T2DM (Chan et al., 2017). Recent studies suggest that an important part of this interplay is due to NLR activation, in particular nucleotide oligomerization domain-like receptor 1 (NOD1) (Chan et al., 2017). This receptor resides as an inactive monomer in the cytoplasm and participates on the recognition of pathogen-associated molecular patterns (PAMPs) (Lin, 2021). NOD-like family is an innate receptor system that recognize several metabolites such as palmitate and it has been correlated to inflammatory dysregulations and metabolic disorders (Tzeng et al., 2019). Once NOD1/2 are activated, they recruit RIPK2 (receptor-interacting serine/threonineprotein kinase 2), that activates another kinase TAK1 (TGF-beta-activated kinase 1), that causes the activation of NF-kB (nuclear factor-kappa light-chain enhancer of activated B cells) and MAPKs (mitogen-activated protein kinase) (Lin, 2021). Recent analyses show that NOD1/2 palmitoylation lead to their membrane recruitment and NF-kB activation (Lin, 2021). So, membrane targeting caused by palmitoylation influences NOD1/2 signalling. Mutations in NOD1/2 palmitoylable cysteines cause a deficiency in NOD1/2 palmitoylation and interrupts NOD1/2 signalling and recruitment (Lin, 2021). This defective S-

palmitoylation is associated with inflammatory bowel disease and other inflammatory conditions (Y. Lu et al., 2019).

'Toll-like' receptors are essential players on the adequate development and trigger of innate immune signals that stem either at intestinal level or tissue injury to control tissue homeostasis and defence (Lin, 2021; Takeda & Akira, 2004). TLRs itself are not modulated by palmitoylation for membrane targeting. However, the are several TLR substrates and products that result palmitoylated to regulate TLRs activity. For example, correct MyD88 signalling through IRAK4 for innate immune responses are regulated by S-palmitoylation (Das et al., 2021a; Y. C. Kim et al., 2019). Furthermore, reducing the presence of endogenous and exogenous palmitic acid decreases MyD88 palmitoylation and TLRs signalling (Lin, 2021). In addition, fatty acid translocase CD36 that is closely associated to and modulates TLR4 signalling, is also regulated by a palmitoylation-depalmitoylation cycle (Das et al., 2021b; Lin, 2021). It has been reported by direct *in vivo* confirmation that it could occur a transcellular LPS, prototypical TLR4 agonist, uptake from the intestine into the portal vein involving CD36 and lipid rafts, with minor uptake via the canonical chylomicron pathway (Akiba et al., 2020).

Palmitoylation modulates metabolic pathways such as lipid metabolism and glucose homeostasis. Palmitoylation facilitates communication between the nucleus and mitochondria, which means that nutritional facts can enable mitochondrial genes transcription to modulate cellular metabolism (Jin et al., 2021). It has been showed that fatty acids utilization as well as nutrient-derived molecules such as insulin, modify protein palmitoylation (Spinelli et al., 2018). In this context, it takes importance the role that intestinal TLR4 plays as key determinant of insulin resistance (P. Lu et al., 2018). This receptor has been identified as a major factor preventing the metabolic syndrome.

2.1 Palmitoylation and cancer

Palmitoylation, especially *S*-palmitoylation, has an important role controlling diverse cellular mechanisms and homeostasis processes in human physiology (M. M. Zhang & Hang, 2017). Inaccurate regulation of this post-translational modification results in an anomalous activation of various signalling cascades involved in human disorders (Spinelli et al., 2018). Palmitoylation has an influence in at least four classic pathways present in cancer: i) cell proliferation, ii) resistance to cell death, iii) induction of angiogenesis and iv) activation of cell invasion and metastasis (Jin et al., 2021).

The most reported palmitoylated proteins are the RAS family, a group of guanosine triphosphate hydrolases (GTPases) responsible for cell proliferation and signalling (Ko & Dixon, 2018). Up to 30% of cancer events with uncontrolled cell growth and propagation, are characterized by an anomalous activation of the RAS oncogenes (Spinelli et al., 2018). For example, it has been shown that during leukemogenesis, HRAS and NRAS need to be palmitoylated to correct delivery to the plasma membrane through secretory system (Ko

& Dixon, 2018). Therefore, inhibition of NRAS oncogene palmitoylation avoid its localization and the hyper-activation of its signalling effectors. Inhibition of palmitoyl acyltransferases (PATs) could control the RAS family activity and could be useful to prevent cancer progression (Spinelli et al., 2018). Oppositely, another anti tumourigenesis method is based on the prevention of depalmitoylation by inhibiting acyl palmitoyltransferases (APT) enzymes, that can suppress tumour establishment (Ko & Dixon, 2018). Therefore, palmitoylation has both roles in organism, beneficial and harmful.

Recent studies have shown that PA enhances the cancer properties of the human hepatocarcinoma HepG2 cell line (Chong et al., 2020). This study showed that: i) PA increase intracellular hydrogen peroxide levels in HepG2, ii) an induction of the expression of proinflammatory cytokines such as TNF-α and IL6, iii) an activation of steatosis-associated hepatic fibrogenesis, iv) an increase of Cancer Stem Cells (CSC) and its sphere formation and v) an activation of the expression levels of pluripotent genes. Palmitoylation plays a key role inducing CSC activation in hepatocarcinoma development from patients with NAFLD. Thus, suppressing palmitoylation may be a significant approach for them to prevent liver cancer (Chong et al., 2020). It could be very interesting to promote a balance between palmitoylation and depalmitoylation to control cancer progression according to the type of tumourigenesis. Overall, targeting palmitoylation during cancer development could represent an important point of control since it plays dual roles, worsening or improving, tumour suppression and promotion.

2.2 Palmitoylation in viral infections

Viruses manipulate cells such as monocytes and macrophages as a tool to disseminate, replicate and long-term persistence in tissues (Nikitina et al., 2018). Monocytes usually respond to the infection expressing proinflammatory signals and antiviral molecules, whereas macrophages migrate to damaged tissue where they can be infected (Nikitina et al., 2018). Both of them could interact with other cell populations through direct cell-to-cell contacts and are involved in the immune response, that could be exploited by virus for dissemination and establishment (Nikitina et al., 2018).

Palmitoylation of viral proteins can notably affect virus assembly and infection, because could increase peptide hydrophobicity and affect its activity (McBride & Machamer, 2010; Sobocinska et al., 2018). Coronaviruses are enveloped positive strand RNA viruses, such as actual SARS-CoV virus, that cause a severe respiratory disease and death in 10% of infected patients (McBride & Machamer, 2010). It has been shown that cytoplasmic tail of S-protein is palmitoylated, but not much is known about the capacity of this alteration (McBride & Machamer, 2010).

It is well known that bacterial dysbiosis can shift immune system towards a cascade of inflammatory responses leading to multi organ damage. The gut microbiota has been

shown to be interconnected to the lung by a phenomenon called 'gut lung axis', which is bidirectional (Rajput et al., 2021). An immune-regulation of gut microbiome has been recovered by maintaining the homeostasis via various mechanisms in SARS-CoV-2 infection (Rajput et al., 2021). More research is needed but the production of short chain fatty acids (SCFAs) by the gut microbiota and its consequent immune stimulation seems to have a preventive effect on the COVID-19 (Rajput et al., 2021).

3. Functional differentiation of macrophages

Macrophages exhibit a broad phenotypic plasticity in response to changes in the tissue and tumour microenvironment (Mosser et al., 2021). The different bult of signals that stem from the disruption of tissue homeostasis and injury lead the infiltration of blood monocytes into the tissue where they differentiate into macrophage (Genin et al., 2015).

The concept of a classic (M1) and alternative (M2) functional differentiation of macrophages has become unrealistic due to phenotype development is a complex process that involves a combination of different stimuli. The current classification of macrophage immune activation is supported by: i) *in vitro* effects of immune-related ligands on the phenotype of macrophages and ii) *in vivo* evidence for distinct subsets of macrophages in disease (Martinez & Gordon, 2014).

- **M1 phenotype development**

The M1 phenotype is induced by three main stimuli: interferon gamma (IFN-γ), pathogen lipopolysaccharides (LPS) and granulocyte macrophage colony-stimulating factor (M-CSF) (Galván-Peña & O'Neill, 2014; Genin et al., 2015). These molecules induce proinflammatory macrophages and lead them to synthesize cytokines like IFN-β, TNF-α, IL-1β, IL-6 and IL-12, produce reactive oxygen (ROS) and nitrogen species (iNOS) (Genin et al., 2015; Martinez & Gordon, 2014).

The M1 activation could also take place through pathogen recognition receptors that interact with bacterial regions. A major mediator promoting proinflammatory macrophages is LPS. This is the prototypic agonist of the innate immune TLR4; triggering responses engaging adaptor molecules such as MyD88 and MaL/Tirap (Ciesielska et al., 2021; McKernan et al., 2020b). Thus, leading to the production of proinflammatory cytokines, chemokines and antigen presentation molecules (Martinez & Gordon, 2014). Mutations in TLR4 family cause susceptibility to infection with some bacteria and to develop bacteraemia (Martinez & Gordon, 2014). However, other studies show that LPS not only induces M1 phenotype but could also lead to the development of M2 biomarkers in certain macrophage cultures (Chanput et al., 2013; Seim et al., 2019; Steimle et al., 2019) depending on the exposure concentration.

- **M2 phenotype development**

It is well-known that M2 macrophages have opposite activity and effects to those developing a M1 phenotype, exerting anti-inflammatory effect. However, polarization is a more complex process that could result in different sub-phenotypes or categories of M2 macrophages, based upon various stimulating molecules and transcriptional changes (Martinez & Gordon, 2014). Moreover, their final status is not strictly anti-inflammatory, as it was initially thought. M2 phenotypes could be M2a (alternative activated), M2b (Type II M2 macrophages), M2c (deactivated) (Martinez & Gordon, 2014) and M2d (Le xun Wang et al., 2019).

The M2 stimulatory factors are cytokines (IL-4, IL-10, and IL—13), glucocorticoids and immune complex (IC) (Le xun Wang et al., 2019). M2a macrophages, known as wound-healing macrophages, are induced by IL-4 and IL-13. Consequently, M2a cells express mannose (CD206) receptors, attract IL-1 receptors (IL-1R), secrete pro-fibrotic factors (TGF-β), insulin growth factor (IGF) and fibronectin for tissue repairing (Le xun Wang et al., 2019). Moreover, IL-4 binding leads to a STAT6 activation and translocation, induces macrophage fusion, and decreases phagocytosis (Le xun Wang et al., 2019).

M2b macrophages are regulatory cells induced upon a combination between immune complexes (IC) and TLR agonists (Le xun Wang et al., 2019). Type II macrophages (M2b) express pro-inflammatory cytokines but also anti-inflammatory IL-10 and lower levels of IL-12, which is the functional converse of M1 cells (Le xun Wang et al., 2019). M2b cells regulate the immune and inflammatory responses, attenuating or aggravating the reaction, depending on the disease (Le xun Wang et al., 2019). Furthermore, M2 macrophages have different phenotypes and functions, and their specific markers have not been are not well stablished yet.

M2c (deactivated) macrophages are induced by IL-10 and have an anti-inflammatory role (Le xun Wang et al., 2019). The binding between IL-10 and its receptor causes an activation of the transcription factor STAT3, an inhibition of the pro-inflammatory cytokine expression and a release of IL-10 and TFG-β. IL-10 is secreted by Th2 cells, leukocytes and macrophages and inhibits lymphocytes. Another M2c role is to phagocyte apoptotic cells (Le xun Wang et al., 2019).

M2d cells are also named as tumour-associated macrophages (TAMs) and its phenotype is generated by a costimulation between TLR ligands and A2 adenosine receptor (A2R) agonists or by IL-6 (Le xun Wang et al., 2019). They produce high levels of IL-10, TGF-β and low IL-12, TNF-α and IL-1β which causes a pro-inflammatory reaction contributing to angiogenesis and cancer metastasis (Le xun Wang et al., 2019).

Collectively, rather than divide macrophage populations in M1 and M2 phenotype, both conditions do not necessarily exclude each other and often coexist (Martinez & Gordon,

2014). The consequent phenotype is a mixed macrophage population that depends on the balance of activating and inhibiting signals and tissue environment.

3.1 Metabolic reprograming: palmitoylation and macrophage differentiation

Metabolism is a key aspect of macrophage polarization. M1 macrophages are mainly present in hypoxic conditions and their energy is produced by glycolysis, induced by Hypoxia-Inducible Factor 1-alpha (HIF1α), which involves an increase of glucose uptake and the conversion of pyruvate to lactate (Galván-Peña & O'Neill, 2014). They switch their core metabolism from oxidative phosphorylation to glycolysis to control the production of ATP (Carroll et al., 2018). The intermediates of the Krebs cycle and pro-inflammatory cytokines production are also increased. During the activation the pentose phosphate pathway is induced and generates NADPH, ROS, and nitric oxide (Galván-Peña & O'Neill, 2014) that results in pro-inflammatory macrophages. Other immune cells like effector T cells and activated dendritic cells increase their glycolysis rate as macrophages to fulfil their energetic requirements (van den Bossche & van der Windt, 2018). Therefore, inhibition of glycolysis could represent a target for preventing pro-inflammatory responses. M2 macrophages are stimulated by parasitic products (IL4 and IL13) and result in anti-parasitic and tissue-repair cells (Galván-Peña & O'Neill, 2014). Their energy is originated from fatty acid oxidation, mitochondrial respiration (induced by STAT6 activation) and oxidative phosphorylation of glucose. In M2 macrophages, the β-oxidation of fatty acids in the mitochondria has an anti-inflammatory effect (Galván-Peña & O'Neill, 2014) while FA synthesis is associated with a pro-inflammatory outcome.

On one hand, lipid metabolism has an essential role in pro-inflammatory status. Fatty acid synthase enzyme has multiple catalytic domains and use acetyl-CoA and malonyl-CoA to synthesise palmitate (Carroll et al., 2018). Carroll and collaborators postulate that FASN has multiple functions, and its inhibition prevents a pro-inflammatory response in macrophages. On the other hand, cholesterol metabolism is a relevant pathway, high levels of its accumulation lead to macrophages to become foam cells and is related to metabolic disorders such as atherosclerosis. Interestingly, both pathways are interconnected, FASN is mainly regulated by sterol regulatory element-binding proteins (SREBPs), which are activated by low levels of cholesterol. Moreover, FASN products such as aceto-acyl-CoA promotes cholesterol production (Carroll et al., 2018).

FASN is essential for inflammatory signalling in response to inflammatory stimuli such as LPS and by TLR4 agonists (Carroll et al., 2018). Cholesterol is also a key component of the signalling platforms known as lipid rafts, which are essential for TLR4 signalling, because they facilitate TLR4 translocation (Carroll et al., 2018). Cholesterol has additional key functions including maintaining of cell membranes and fulfil its demand during macrophage activation. FASN deficiency in macrophages reduce inflammation and protect against metabolic illnesses, so it could be a target in disease prevention. Endogenous

fatty acids synthesized by FASN can be converted to palmitoyl-CoA and promote protein palmitoylation. In neutrophiles, a common adapter molecule in the TLR family (MYD88) is shown to be palmitoylated, what influences inflammatory signalling and regulates immune system (Y. C. Kim et al., 2019). De novo fatty acid synthesis and CD36-mediated exogenous fatty acid uptake contributed to MYD88 palmitoylation (Y. C. Kim et al., 2019). Consequently, endogenous fatty acids modulate innate immune functions and could be suggestes as a therapeutic option.

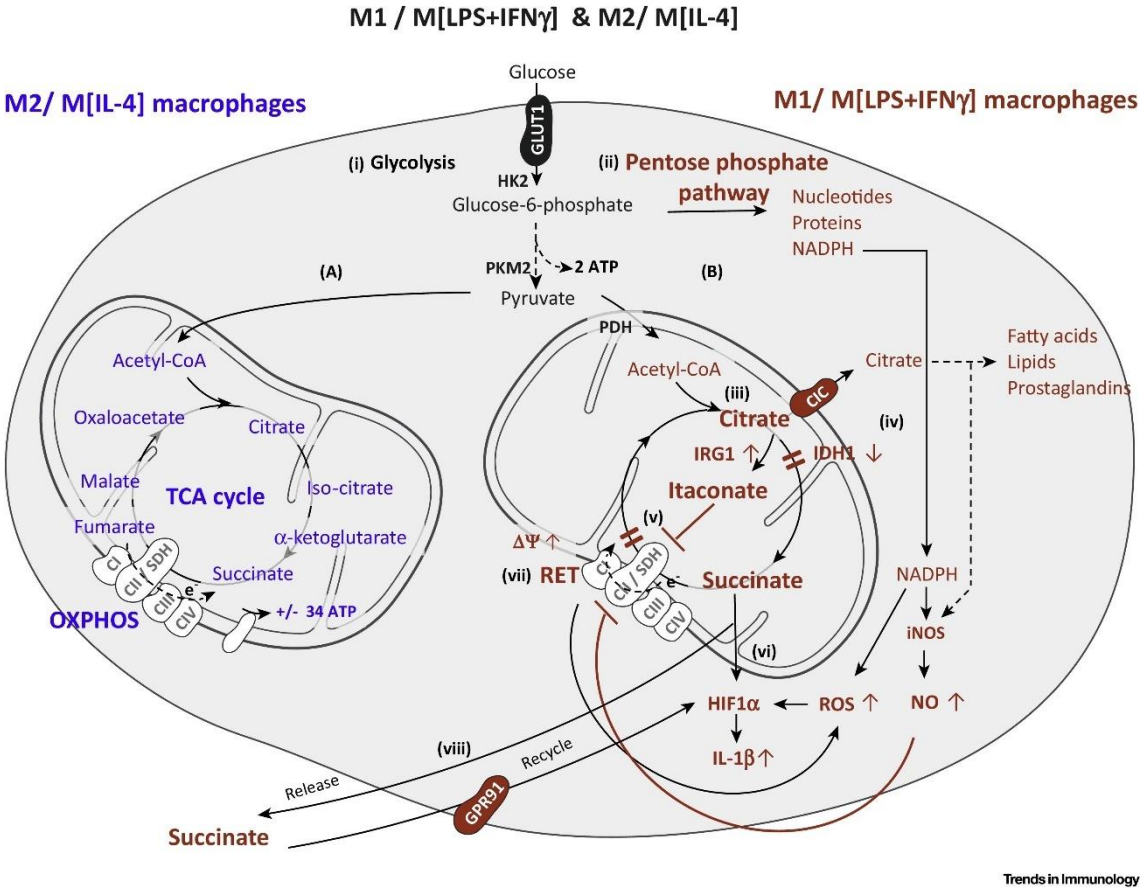


Illustration 1. *(A) M2 macrophages activated by IL-4, (i) glucose replenishes an intact tricarboxylic acid (TCA) cycle resulting in sustained ATP production via oxidative phosphorylation (OXPHOS). (B) M1 inflammatory macrophages are characterized by: (i) high glycolytic rates; (ii) an increased pentose phosphate pathway, reactive oxygen species (ROS) and nitric oxide (NO) production; and (iii) a disturbed TCA cycle.* (van den Bossche et al., 2017)

3.2 Role of macrophages in the tumour micro-environment

Tumour microenvironment is the environment around a tumour, including the cancer cells and surrounding blood vessels, immune cells, fibroblasts and signalling molecules. Macrophages are one of the most abundant immune cells and their presence in solid tumours reduce carcinoma survival (Nielsen & Schmid, 2017). In this context, tumour-associated macrophages (TAM) represent crucial regulators of the immune system and cancer, in some situations they limit tumour progression and in others has a negatively effect on cancer therapy (Ostuni et al., 2015).

The Warburg effect describes the charasteristic cancer cell metabolism, which is marked by increased glucose uptake and use (Jang et al., 2013). The chronic activation of aerobic glycolysis in cancer cells has been related to the activation of oncogenes or the loss of tumour suppressor proteins, implying that cancer progression is fundamentally accelerated (Jang et al., 2013). In this regard, the inhibition of glycolytic capability may help malignant cells to become less cancerous. Inflamed sites are known to have low oxygen levels, so nearly all cancer cells proliferate under hypoxia conditions (Galván-Peña & O'Neill, 2014). Hypoxia-inducible factor (HIF-1) can be expressed by different classical M1 activators (i.e., LPS) through NF-kB and cause the metabolic shift to M1 phenotype (Li et al., 2018). HIF-1 upregulates glucose transporters and modulates glycolytic enzymes, for example activation of pyruvate dehydrogenase kinase isozyme 1 (PDK1) or lactate dehydrogenase A (LDHA) (H. Zhang, 2015). Additionally, HIF-1 inhibits fatty acids catabolism repressing medium- and long-chain acyl-CoA dehydrogenases (MCAD and LCAD), that ends in an enhanced lipid accumulation, which is related to cancer cell proliferation (H. Zhang, 2015).

TAMs are typically associated with an M2-like polarization and are retained inside hypoxic areas to increment tumour angiogenesis (Nielsen & Schmid, 2017). In cancer and infectious diseases, some macrophages develop a M2b phenotype, which is known to promote tumourigenesis and parasitosis by blunting the immune and inflammatory response (Le xun Wang et al., 2019).

4. Role of the monocyte/macrophage population in non-communicable chronic diseases

Imbalances in the crosstalk between the immune and the metabolic systems lead to develop immunonutritional disorders such as obesity, malnutrition, T2DM and other features of the MS (Zmora et al., 2017). Immune-metabolic homeostasis involves interactions between immune and non-immune cells (Zmora et al., 2017). Here, monocyte/macrophage population has an essential role in maintaining proper metabolic regulation and homeostasis of iron, calcium, amino acids (Mosser et al., 2021) and especially lipids. Macrophages are present in metabolically active tissues as a resident population, as it occurs in white adipose tissue (WAT), and the liver (Bhargava & Lee, 2012).

Macrophages are recruited by the signalling of pro-inflammatory cytokines and chemokines to manage tissue disorders, generally induced by overload lipid influx, which aggravate the metabolic disorders (Raulien et al., 2017). Secondly, macrophages respond to different pathogenic or endogenic stimuli and develop their phenotype (Bhargava & Lee, 2012). Usually, M1 macrophages induce and M2 prevent metabolic diseases. Lastly, the pro-inflammatory response is resolved by IL-10 (induced by M1 and M2 cytokines), which deactivate macrophages. An absence of arresting signals is related to obesity, T2DM, and metabolic disorders because of a persistent pro-inflammatory stimulation and

increased lipid influx (Bhargava & Lee, 2012). This process is named as "sterile inflammation", where inflammation occurs in the absence of invading pathogens and when it is dysregulated, causes a defective wound healing that often results in chronic inflammatory disease with persistent tissue damage (Raulien et al., 2017).

The metabolic syndrome is a chronic inflammation caused by a feedforward cycle of inflammatory responses and metabolic stress (Bhargava & Lee, 2012). Macrophage M1 infiltration promotes inflammation in adipose tissue, liver, muscle, and pancreas in patients with obesity and T2DM. Nevertheless, lymphocytes also infiltrate before myeloid cells and control the activation state of adipose tissue macrophages. Regulatory T lymphocytes express anti-inflammatory cytokines which inhibit macrophage migration and induce a M2 phenotype (Esser et al., 2014), repressing adipose tissue inflammation. Overall, innate and adaptative cells cooperate to balance the homeostasis and inflammation in the control of obesity and T2DM.

Atherosclerosis is a common disorder derived from metabolic syndrome. Monocytes and macrophages are recruited into the subendothelial space due to high LDL and cholesterol intake, that accumulate and become oxidized (oxLDL) and promote a releasing of cytokines (Bhargava & Lee, 2012). Moreover, at early stages macrophages mediate the uptake of oxLDL by the scavenger receptor CD36, that leads to intracellular lipid accumulation and evoke an inflammatory response. Then, macrophages at advanced stages become foam cells and become apoptotic contributing to the formation of the atherosclerotic plaque necrotic core (Chen et al., 2019). In atherosclerotic patients, NLRP3 inflammasome is activated as well, that secretes IL-1β and IL-18 and also enhanced a pro-inflammatory state (Bhargava & Lee, 2012).

Non-alcoholic fatty liver disease (NAFLD) involves several lesions from harmless steatosis to steatohepatitis (NASH), which can lead to cirrhosis and hepatocarcinoma (Esser et al., 2014). These disorders could develop insulin resistance and obesity, and both have an accumulation of fat molecules and consequently a recruitment and activation of inflammatory cells. M1 development increase glucose influx for glycolysis, which is controlled by hypoxia-inducible factor 1α (HIF), a deletion of this factor develops a non-inflammatory state (Bhargava & Lee, 2012). Systemic hypoxia causes insulin resistance and NAFLD since HIF-1α activation inhibits physiological apoptotic cell death and promotes macrophage necrosis (Bhargava & Lee, 2012). Here, TLR4 has been identified as a key player determining the severity and pathological consequences of the disease.

5. Modulation of metabolic processes

5.1 Carnitine palmitoyltransferases in macrophage's phenotype

Elevated fatty acid levels in macrophages are known to play a role in fatty acid-induced inflammation, that can be neutralised by increasing triacylglycerol synthesis, reducing in consequence the lipid transport and activation of Fatty Acid Oxidation (FAO) (Gonzalez-

Hurtado et al., 2017). Carnitine palmitoyltransferases I and II (CPT1/CPT2) are mitochondrial enzymes that coordinate the transport of long fatty acids (LC-FAs) across the mitochondrial membrane binding them to carnitine molecules. CPT1 generates acylcarnitines, that cross the inner mitochondrial membrane to the matrix (Calle et al., 2019). Once inside, CPT2 of the peripheral inner membrane generates acyl-CoA from acylcarnitines to start Long Chain Fatty Acid β-Oxidation (LC-FAO) (Gonzalez-Hurtado et al., 2017). CPT1 enzyme is inhibited by malonyl-CoA.

The expression of CPT1a in macrophages has an influence on inflammation control and enhancing phagocytosis. It has been proved that constitutive active mutant of CPT1a enhance FAO in macrophages and reduced pro-inflammatory cytokines (Calle et al., 2019). However, CPT1 inhibition cause lipid droplets multiplication, up-regulation of iNOS expression and phagocytosis alteration, leading to an inflammatory state (Calle et al., 2019). The main controller of lipid metabolism is the peroxisome proliferator activated receptor gamma (PPARγ) transcription factor, which is activated upon IL-4 stimulation and upregulated lipid catabolism (Oyarce et al., 2021).

CPT2 enzyme is required to correct fatty acid β-oxidation and its inhibition led to a FAO-deficient macrophages that display M2 polarization markers after IL-4 stimulation, what elucidate that FAO is not indispensable for polarization (Nomura et al., 2019). Although FAO has no influence on polarization, it does have an important role in preventing the progression of atherosclerosis. Since it was shown that in CPT1 and CPT2 KO mice, it was enhanced the expression of CD36, the uptake of oxLDL and the transformation of M0Mϕ into foam cells (Nomura et al., 2019). In certain studies, it was shown that CPT2 is active with medium (C8–C12) and long-chain (C14–C18) acyl-CoA esters, whereas no activity was found with short- and very long-chain acyl-CoAs or with branched-chain amino acid oxidation (Violante et al., 2010). It was also reported that accumulation of acyl-CoA metabolites is involved in the intramitochondrial synthesis of acylcarnitines by CPT2, and abnormal acylcarnitines profiles are characteristic of most mFAO disorders (Violante et al., 2010).

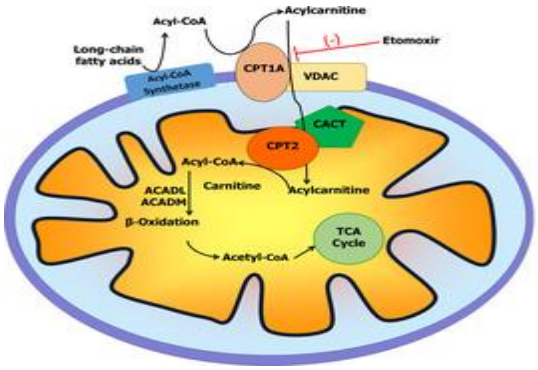


Illustration 2. *Carnitine-palmitoyltransferases I and II activities in the outer and inner mitochondrial membrane.* (Jernberg et al., 2017)

It is well known that *in vivo* macrophages display a mixed phenotype rather than M1 or M2 conventional phenotypes, and stimulatory molecules could be high density lipoproteins (HDL), free fatty acids or the abovementioned pro-inflammatory molecules.

There seems to be a correlation between the increase of CPT1a expression and the switch of M1 phenotype into M2 (Calle et al., 2019), or at least their markers. Nevertheless, FAO is not indispensable for macrophage polarization, high-fat diet-induced inflammation, oxidative stress, or insulin resistance (Divakaruni et al., 2018; Gilutz et al., 2012).

The repression of CPT1 and CPT2, as well as data from preclinical models lacking these enzymatic activities (CPT1/2 KO mice), are associated with greater severity and progression of tumourigenesis processes (Gao et al., 2009; Nomura et al., 2016; Xiong et al., 2020). These alterations seem to have an underlying mechanism affecting both cell proliferation, survival, and functionality where inadequate protein palmitoylation appears as a common link (Gratac, 2010).

5.2 Metabolic reprogramming of macrophage

5.2.1 TLR4 agonists

Lipopolysaccharides are known as TLR4 prototypical agonists and after its stimulation, TLR4 triggers two signalling pathways: MyD88-dependent and TRIF-dependent (MyD88-independent). Both routes translocate TLR4 from the plasma membrane to endosomes (Takeda & Akira, 2004), but the first one is faster than the second. In response to LPS or IFN-γ macrophages adopt a proinflammatory phenotype and suffer dynamic a metabolic reprograming that involves a two-stage remodelling of the Tricarboxylic Acid (TCA) cycle (Seim et al., 2019). In the early stage increases IL-6 and TNFα production and accumulation of intermediates citrate, itaconate and succinate. In the late stage decreases IL6, TNFα and metabolites, and TCA cycle get disrupted because of the inhibition of pyruvate dehydrogenase (PDH) and oxoglutarate dehydrogenase (Seim et al., 2019).

For a long time, plant seeds have been recognized as a good source of serine-type protease inhibitors, named as STPIs or PPIs, which have potential applications against cardiovascular, inflammatory, and neurological disorders (J. M. Laparra & Haros, 2019). Heat stable PPIs, have been identified from cereals, beans, and soya with a potential to stimulate innate immune responses, control tumour microenvironment and prevent HCC progression *in vitro* and *in vivo* (J. Laparra et al., 2019; J. M. Laparra & Haros, 2019; Llopis et al., 2020). PPIs from *Chenopodium quinoa* are constituted by glucosides and are typically found in the salt soluble fraction of seeds as part of protein complexes where monomeric units display an immunonutritonal and therapeutic capability (Srdić et al., 2020). Because of their disulfide-linked structure, these proteins are partially resistant to gastrointestinal enzymes (Srdić et al., 2020).

According to previous studies, TLR4 signalling is required for the progression of chemically induced hepatocellular carcinoma (Dapito et al., 2012). The development of a hepatic anti-tumour M1-macrophage phenotype ($F4/80^{+}$) can have significant implications preventing tumour progression in mice (J. M. Laparra & Haros, 2019).

Specifically, the immunonutritional fraction of *C. quinoa* increase the proportion and infiltration of M1 ($F4/80^+$) macrophages in injured livers *in vivo* and *in vitro* (J. M. Laparra & Haros, 2019; Llopis et al., 2020). Overall, PPIs from *C. quinoa* do not impair the production of inflammatory mediators (M-CSF and TNFα), maintaining the polarization of the antitumoural M1 macrophage phenotype (J. M. Laparra & Haros, 2019).

Moreover, after in vivo administration of PPIs from *C. quinoa* enhance gut microbial production of SCFAs such as butyrate and propionate (Llopis et al., 2020). These microbiota-derived metabolites have been shown to enhance intestinal hypoxia by activating a signalling axis via HIF1α, which improves epithelial barrier function and influences tissue function (Llopis et al., 2020).

Altogether, in hepatic pro-inflammatory environments, PPIs administration led to ameliorate cytokines and chemokines levels and show immunometabolic changes that could be correlated with TLR4 signalling stimulation (Llopis et al., 2020). These functional features of PPIs from C. quinoa enhancing immunometabolic adaptation could be useful to develop durable and long-lasting immune responses that can be used to control HCC aggressiveness (J. Laparra et al., 2019; J. M. Laparra & Haros, 2019).

5.2.2 Trained immunity strategies

For a long time, immunological memory was thought to be a unique feature of the adaptive immune response. This dogma has been challenged by a growing amount of literature demonstrating that innate immune cells (and even tissue-resident stem cells) can demonstrate adaptive characteristics (Netea et al., 2020). In this context, the metabolic adaptation of macrophages can help us develop intervention strategies in the field of 'innate immune memory'. The term 'trained immunity' refers to the long-term functional reprogramming of innate immune cells induced by exogenous or endogenous inputs (i.e., LPS or metabolites), which results in a different response to a second challenge when the cells return to their non-activated condition (Netea et al., 2020). The functional reprogramming of myeloid cells provides trained immunity during bacterial and fungal infections and could develop the alteration of several chromatin marks. Recent studies are examining stimulatory agents, such as activators of the NLRP3 or 'Toll-like' receptors to control cancer progression by activating the innate immunity system (Netea et al., 2020).

Cellular metabolism mediates the trained immunity by dependent epigenetic reprogramming of innate immune cells such as macrophages, so metabolites as itaconate or mevalonate could modulate the activity of chromatin-modifying enzymes (Netea et al., 2020).

5.3 Immunomodulatory metabolites: Butyrate and Itaconate

The gut microbiota is connected with the intestinal immune system and plays an essential role in the maintenance of homeostasis. Imbalances in gut microbiota composition could lead to proinflammatory responses and associates with diseases such as dysbiosis or inflammatory bowel disease in the host (Schulthess et al., 2019). To establish a stable microbiota, it is required diets rich in fibers which are the substrates of gut commensals, that process and fermentate them to yield short-chain fatty acids (SCFAs) which are volatile fat acids able to cross the blood-brain barrier via monocarboxylate transporters (Belizário et al., 2018). SFCAs have the potential to modulate the mucosal immune system and regulate the immune cells associated, such as T cells and macrophages. Acetate, butyrate, and propionate are some SFCAs produced by intestinal bacteria that promote intestinal epithelial barrier function (Schulthess et al., 2019).

5.3.1. Butyrate

Butyrate has been shown to cause metabolic and immunologic effects to macrophages that is in some aspect opposite to the well-known proinflammatory LPS stimulation in M1 macrophages (Schulthess et al., 2019). Macrophages differentiated in presence of butyrate display enhanced cell-intrinsic antimicrobial activity due to a shift in their metabolism, for example a reduced glycolysis. Butyrate increases the expression levels of adenosine monophosphate (AMP), which induces AMP kinase (AMPK), thus inhibiting the target of rapamycin in mammalian cells (mTOR). Inhibition of mTOR, the main regulator of autophagy, decreases glycolytic enzymes and increases the cleavage of a protein microtubule-associated (LC3) (Schulthess et al., 2019).Moreover, the presence of butyrate increases the level of ribulose 5-phosphate and reduces intracellular glucose, that potentiate the flux toward the pentose phosphate pathway that could contribute to generate NADPH and ROS species (Schulthess et al., 2019). One crucial enzyme of that pathway is glucose 6-phosphate dehydrogenase (G6PH), that stimulates proinflammatory responses and the expression of pro-oxidative genes due to MAPK and NF-kB pathways activation (Ham et al., 2013)

Butyrate controls the differentiation from monocyte to macrophage through histone deacetylase 3 (HDAC3) inhibition. HDACs remove acetyl groups on lysine residues from histones and non-histones peptides and regulate gene expression by modulating chromatin structure (Schulthess et al., 2019). The HDAC3 inhibition facilitates anti-bacterial functions of macrophages, drove their differentiation, altered their metabolism, and enhanced gene expression of bacterial killing genes. Interestingly, it has been shown that *in vivo* administration of butyrate restrains the proinflammatory cytokine production by LPS stimulation and reduces bacterial dissemination (Schulthess et al., 2019). In other research studies butyrate facilitates M2 macrophage polarization and function and have

anti-inflammatory effects on LPS-mediated M1 macrophages (Ji et al., 2016). Butyrate increases the migration of M2 macrophages and reduce the intestinal inflammation of mice with colitis disease. The reason of the enhance of M2 polarization could be the inhibited activity of HDAC, that promotes histone 3 lysine 9 (H3K9) acetylation, that lead the activation of the STAT6 signalling pathway which is required for IL-4 induced M2 polarization (Ji et al., 2016).

5.3.2 Itaconate

It could be from endogenous and exogenous. It is synthesized from the tricarboxylic acids cycle and its production is increased upon LPS treatment seemingly with the purpose of restraining macrophage immune responses to TLRs stimulation (Hooftman et al., 2020a). Itaconate is a regulatory molecule that could be degraded into pyruvate and acetyl-CoA and is present in multiple pathways (Qin et al., 2019). Interestingly, itaconate could alkylate cysteine residues on multiple proteins such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or NLRP3 and it is related to its anti-inflammatory properties (Hooftman et al., 2020a). Itaconate is reported to have an anti-inflammatory effect in inflammatory macrophages because it modifies specific cysteine of NLRP3 and inhibits inflammasome activation (Strelko et al., 2011b). NLRP3 is regulated metabolically and mitochondrial localization of NLRP3 precedes inflammasome activation. There are several stimuli implicated in NLRP3 activation such as reactive oxygen species (ROS), lysosomal disruption, potassium and chloride efflux and mitochondrial dysfunction (Hooftman et al., 2020a). Itaconate has been shown to be an important negative *in vitro* and *in vivo* regulator of NLRP3, which could have utility as a therapy in NLRP3-mediated diseases including autoinflammatory diseases such as Alzheimer (Hooftman et al., 2020a).

Itaconate has immunoregulatory effects through its ability to inhibit succinate dehydrogenase (SDH), that mediates the oxidation of succinate and its important in the TCA cycle, so itaconate-dependent inhibition leads to a succinate accumulation (Lampropoulou et al., 2017). Succinate is important in other immune pathways and controls IL1β expression, HIF1α activity and ROS production ((Lampropoulou et al., 2017);(Harding H & Ruslan, 2016). Lampropolou also suggests that itaconate decreases mitochondrial ROS production in LPS-treated macrophages. Itaconate attenuates the inflammatory response in stimulated macrophages by impairing the TCA cycle and causing a dependence on aerobic glycolysis by reducing the oxygen consumption (Harding H & Ruslan, 2016). In fact, itaconate appears to be the reason of the switch into aerobic glycolysis and the TCA cycle fragmentation that occur in M1 macrophages, which are a break at isocitrate dehydrogenase coupled to itaconate synthesis and decreased fumarate produced from succinate (Lampropoulou et al., 2017).

HYPOTHESIS

Immunomodulatory metabolites butyrate and itaconate may mediate immunometabolic effects influencing TLR4-induced signalling (LPS primed) and macrophage phenotype.

OBJECTIVES

The objective of this study is to evaluate the immunomodulatory potential of butyrate and itaconate as well as PPIs to modulate TLR4-induced immunometabolic effects derived from LPS stimulation on human macrophages.

To this end, specific partial objectives were established:

- To monitor the metabolic adaptations on enzyme activities in the mitochondrial and lysosomal compartments in human macrophage-like cells.
- To evaluate the modulation of mitochondrial carnitine palmitoyltransferases in human macrophage-like cells.
- To associate these changes to the selective functional differentiation of human macrophage-like cells.

MATERIALS AND METHODS

1. Cell culture

Human macrophage-like cells (WBC2649C, ATCC® HB8902™) were obtained from the American Type Culture Collection (ATCC) (Mannasas, VA, USA) and grown according to the standard conditions recommended. These cells are derived by the fusion of normal human peripheral blood leukocytes with the mouse macrophage cell line, RAW 264.

The basal medium for this cell line is ATCC formulated Eagle's Minimum Essential Medium (EMEM) (gibco, TermoFisher, catalogue nº 31095-029). To obtain the complete growth medium, Fetal Bovine Serum (FBS) (Cytiva, cat nº SV30160.03) was added to the base medium to achieve final concentration of 10% (v/v). Cells were seeded and maintained in 75 cm^2 cell culture flasks with vented filter cap from Eppendorf™ (catalog nº 0030711025) in 10mL EMEM medium and incubated at 37ºC and 5% CO_2. Flasks were non-treated and sterile, pyrogen-, DNase-, RNase-, human and bacterial DNA-free and non-cytotoxic. The medium renewals were prepared removing the old medium and adding fresh one every 2 or 3 days. Subcultures of different flasks were made displacing the half of the old medium, scraping the cells, adding fresh medium, dislodging the cells, and dispensing into the wells.

2. Experimental treatments

For the experiments, cells were collected in fresh EMEM and, after counting in a haemocytometer chamber (Neubauer), seeded at 30,000 cells/well in 12-well plates.

- **LPS addition**

Lipopolysaccharide used for the experiments was obtained from *Escherichia coli* O111:B4 (Merck, MA, USA) (Ref nº 437627). LPS was diluted in deionised (18.2 MΩ cm^2) water to prepare the stock solution, from it was added to the wells to achieve a final concentration of 100 ng/ml in the cell culture.

- **Plant serine-type protease inhibitors (PPIs)**

PPIs were obtained from homogenised seeds of *C. quinoa* obtained from local supermarkets. First, to 0,25g of *C. quinoa* it was added 5mL of PBS (1x) and then the tubes were incubated with shaking for one hour (Laparra and Haros, 2019). Afterwards, all tubes were put onto Amicon© (30kDa) filtration tubes and centrifuged (6,000rpm, 10min, 4ºC) to obtain clear filtrates. The fractions <30kD were polled before quantifying total protein content by using a Nanodrop. For the experiments, PPIs were added to the cell cultures at a final concentration of 50 µg/ml in serum free EMEM.

- **Butyrate solution**

From Sigma-Aldrich® sodium butyrate ($C_4H_7NaO_2$) (catalog nº B5887) a stock solution (0,05g/mL, w/v) was prepared in deionised water. For the experiments it was added from the stock solution to the cell cultures at a final concentration of 1.2 mM, 3.8 mM, and 11.3 mM in the well.

- **Itaconate solution**

From Alfa Aesar® itaconic acid ($C_5H_6O_4$) (catalog nº A15566) a stock solution (0,05g/mL, w/v) was prepared in deionised water. For the experiments it was added from the stock solution to the cell cultures at a final concentration of 0.9 mM, 2.6 mM, and 7.7 mM in the well.

3. Study of the metabolic adaptations in human macrophage-like cells

3.1 'MTT' test: Mitochondrial dehydrogenase activity

MTT monitors the mitochondrial enzyme activities. It is a colorimetric test in which metabolically active cells reduce yellow tetrazolium salt (MTT) to purple formazan crystals. These crystals precipitate and get dissolved, forming a solution to which their absorbance can be measured in a spectrophotometer Biochrom Asys UVM 340Z. There is a linear relationship between the darkness of the solution and the number of viable and metabolically active cells. This absorbance depends on the activity of dehydrogenase enzymes.

The MERK® MTT reagent (catalog nº CT01-5) (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) was prepared at a final concentration of 5mg/ml. This solution was diluted 1:10 (v/v) in a phosphate buffered saline (PBS) before being added

to cell cultures. Day 1 of the test: a 96-well plate was prepared with 10,000 cells/well and incubated for additional 4 hours. Day two: the treatments were applied: first, metabolites and PPIs were incubated for 1h; then cell cultures were challenged to LPS for additional 1h. Subsequently, the medium was spent out and 300 µl of MTT reagent was added to each well and allowed to incubate for one hour. Finally, the MTT reagent was removed and 150 µL of the solubilization solution was added to each well. The solvent consists of a base of acidic isopropanol with HCl 10% (v/v) and 10% (v/v) triton X100. The final step was to measure the absorbance of the microplate, background absorbance at 690nm and subtract from 570 nm measurement (570 nm – 690 nm).

3.2 'Neutral Red' test: Lysosomal activity

The neutral red uptake assay is a test use to estimate the activity of the endo/lysosomal compartment. Viable cells could take up dye neutral red by active transport and incorporate it into lysosomes, non-viable cells could not do this inclusion. After the incorporation of the dye, cells are washed with an acidified ethanol solution, and the dye is liberated from the cells. There is a linear relationship between the neutral red extractable and the lysosomal activity of viable cells in the microplate, which indicates the degree of cytotoxicity caused by the treatments.

The Sigma-Aldrich® Neutral Red Solution 0.33% (v/v) (catalogue nº N2889) (3-amino-7-dimethylamino-2-methyl-phenazine hydrochloride) (3.3g/L) was diluted 1:10 (v/v) in PBS (NR) before being added to cell cultures. Day 1 of the test: a 96-well plate was prepared with 10,000 cells/well and incubated for additional 4 hours. Day two: the treatments were applied: first, metabolites and PPIs were incubated for 1h; then cell cultures were challenged to LPS for additional 1h. Subsequently, the medium was spent out and 300 µl of NR reagent was added to each well and allowed to incubate for one hour. Finally, the NR reagent was removed and 150 µL of the solubilization solution was added to each well. The solubilization Solution consisted of 1% (v/v) acetic acid in 50% (v/v) ethanol. Then, absorbance was measured at 540 nm using a Biochrom Asys UVM 340Z spectrophotometer with background substraction at 690 nm.

4. Study of the selective functional differentiation of human macrophage-like cells

4.1 Flow Cytometry (FCM)

Flow cytometry (FCM) allows the simultaneous measurement of multiple fluorescences and light scatter induced by illumination of single cells while they are flowing through a sensing area. Cells were analysed and classified using fluorescent markers such as conjugated antibodies (O'Connor et al., 2001).

For the immunophenotyping analyses it was used a BD FACSCelesta™ flow cytometer. This instrument can detect and analyse up to 14 parameters in a single sample, using the

optical and electronics system, lasers, filters, detectors, optical paths, and signal processing technologies.

Subcultures were prepared for the experiment in a 12-well plaque with 30,000 macrophages each one and it was left to incubate at 37ºC for 24h. The following day the treatments were applied, first the metabolites (Butyrate, Itaconate and PPIs) (+1h of incubation) and then LPS (+1h of incubation). Eight wells were separated to be a control culture in which no treatments were added. After treatment, the medium was removed, 300 µL of PBS were added to each well and cells are lifted with the scraper. The next step was to protect macrophage surface markers using 50 µL of formaldehyde for each well. Then, cells from each well were transferred into a glass tube.

The fluorescence was measured using four conjugated antibodies: BD Horizont™ V450 Mouse-Anti-human CD44 (cat nº 561292), BD OptiBuild™ BV786 Rat Anti-Human CX3CR1 (cat nº 7444489), BD Pharmingen™ FITC Mouse Anti-Human CD68 (cat nº 562117) and BD Pharmingen™ PE Mouse Anti-Human CD206 (cat nº 555954). Each of the monoclonal antibodies bounds to a specific cell marker (CD44, CX3CR1, CD68 and CD206) and, by having a fluorochrome attached, allows macrophages to be identified.

The final stage began by adding one type of the antibodies (1 µl) to one of the control tubes, (every tube had one antibody) and incubating them for twenty minutes. The fluorescence of the tubes was analysed in the cytometer to establish the gating strategy for measuring the samples. Finally, the final step consisted of the joint addition of the four antibodies to each of the samples and measuring them in the cytometer.

4.2 Monitoring palmitoil-transferases expression by immunocytochemistry

Immunocytochemistry is a technique to confirm the expression and presence of a protein in a cell culture by using of a specific primary antibody that binds to it. The primary antibody interacts with the protein and by using a secondary antibody conjugated with a fluorophore, it can be visualized the presence/absence of the protein in the cells. For this technique it has been used a BD FACSCelesta™ flow cytometer.

Subcultures were prepared for the experiment in a 12-well plaque with 50,000 macrophages each one and it was left to incubate at 37ºC for 24h. The following day the treatments were applied, first the metabolites (Butyrate, Itaconate and PPIs) (+1h of incubation) and then LPS (+1h of incubation). Then, the medium was removed.

The first step was to fix the cells with a 2% (v/v) paraformaldehyde solution, adding 300µL to each well and allowing them to incubate for 3 minutes. After the cells were detached, they were centrifuged at 1,400 rpm for 5 minutes at room temperature. The second step was to permeabilize the cells with 300 µL of 20% (v/v) ethanol in PBS (20EtOH:80PBS), followed by another centrifugation. Supernatant was removed and then

200 µL of PBS was added to each Eppendorf. Later, 100 microliters from each tube were split into two different Eppendorf (one for each primary antibody).

The next step was to add the primary antibodies to carnitine palmitoyltransferases 1 and 2 (CPT1/CPT2). The ones used were abcam Rabbit mAB to CPT1A (0,604mg/mL) (cat nº ab220789) and Invitrogen CPT2 Policlonal Antibody (1mg/mL) (cat nº PA-81290). For each tube 1µL of the antibody was added to obtain a 1/100 dilution. Then, incubated at 4ºC for 30 minutes. The final step was to add the secondary antibody IRDye® 680RD Goat anti-Rabbit (0,5mg) (cat nº 926-68071). Finally, using the Ac and PBS a 1/500 dilution (v/v) was made and from this dilution 1µL was added to each tube. Then, left the tubes incubating during 24h at 4ºC.

To bring out the inhibitory experiment of CPT1/2, cultures were pre-treated with Perhexiline Maleate ($C_{19}H_{35}N•C_4H_4O_4$) STEMCELL Techology™ (catalogue nº 100-0267) during 20 minutes after the metabolite's exposure.

5. Statistical analysis

Statistical analysis was performed using Statgraphics© Plus program (version 5.1, Rockville, MD, USA). For normally distributed data, ANOVA with the post hoc Tukey test was applied. Statistical significance was established at $p<0.05$ for all comparisons.

RESULTS AND DISCUSSION

1. Metabolic response(s) to TLR4 agonists.

Taking advantage of the previously reported differences of the capacities of LPS and PPIs to interact with TLR4, it was compared their influence on the metabolic adaptation in macrophage's mitochondrial and endo/lysosomal compartments (**Figure 1**). The results show a time-dependent effect, exclusive for the mitochondrial compartment (Fig. 1A), which displays a similar trend for both LPS and PPIs. The longer time of exposure, the lowest MTT conversion values. However, both samples increased the metabolic activity of the endo/lysosomal compartment. Here, exposure time has no influence in the neutral red dye uptake (Fig. 1B). This may interpret the results as an immunometabolic suppressive effect on mitochondrial activities, while the endo/lysosomal compartment remains functioning.

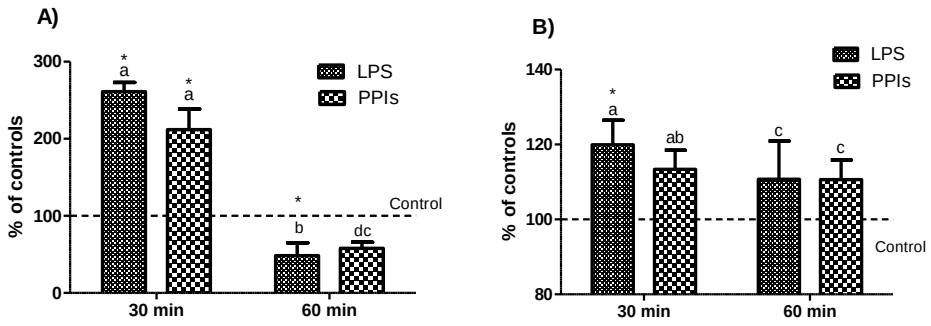


***Figure 1**. Mitochondrial activity levels of HB8902© in response to LPS and an extract (<30kDa) obtained from C. quinoa (PPIs) (n = 6). (A) MTT conversion due to dehydrogenases activity (B) Neutral red uptake due to endo/lysosomal activities. Results are expressed as mean ± SEM. a-d Different superscript letters indicate statistical differences (p < 0.05). Dotted line represents the metabolic activity measured in in untreated control cultures. * Asterisks show statistical different to the control level.*

Innate immune TLR4 is integrated into cellular networks that work together regulating cell metabolism and signalling pathways. TLR4 activation sequentially triggers two signalling cascades (Takeda & Akira, 2004), i) one involving TIRAP and MyD88 adaptor proteins and induced in the plasma membrane, whereas ii) the second engaging adaptor proteins TRAM and TRIF begins in early endosomes after endocytosis of the receptor (Ciesielska et al., 2021). Early evidence showed that LPS-induced HIF1α shuts down mitochondrial respiration (Palsson-Mcdermott et al., 2015). In addition, the tricarboxylic acid cycle gets disrupted because of the inhibition of the pyruvate dehydrogenase and oxoglutarate dehydrogenase complexes, leading to TCA intermediaries' accumulation (Seim et al., 2019) such as succinate and malate (Galván-Peña & O'Neill, 2014). Otherwise, proteome assays of human macrophage-like cells reported the negative effect of PPIs from *C. quinoa* down-regulating the expression of HIF-1α (Srdić et al., 2020). Thus, our results allow us to hypothesize that reduction in MTT conversion values could be mediated by the inhibitory effect on pyruvate kinase that results from both LPS and PPIs stimulation (H. Zhang, 2015);(Srdić et al., 2020). This effect will sequentially impact the progression and metabolite flow within tricarboxylic acids cycle. TLR4/MyD88-dependent signalling contributes to aggravate proinflammatory processes, whereas the TLR4/TRIF-dependent pathway helps to better control the metabolic function and inflammation (Srdić et al., 2020). The endocytosis of TLR4 finishes the MyD88-dependent signalling, while the following endosome maturation and lysosomal degradation of TLR4 determine the duration and magnitude of the TRIF-dependent pathway. In this sense, PPIs from *C. quinoa* modulate the pattern in expression levels of different Rab proteins (a type of Ras proteins responsible for the building and transport of vesicles) contributing to a delayed progression for the early endosome (Srdić et al., 2020), which could play a key role controlling the breadth of TLR4 signalling.

2. Metabolite-induced changes in the mitochondrial enzyme activities

The immunonutritional capability of PPIs to influence the harmful LPS-mediated effects on mitochondrial function (Ciesielska et al., 2021) was evaluated to achieve a better understanding of their potential (**Figure 2**). In contrast to the behaviour observed for the independent exposure, the trend of the joint exposure in mitochondrial conversion of MTT results unaltered in relation to control cultures (Fig. 2A). LPS is considered the prototypical TLR4 agonist. At molecular level, TLR4 activation by LPS triggers downstream signalling to engage the MyD88 adaptor molecule towards severe proinflammatory response(s) (Lijian Wang et al., 2009). Alternatively, proteome studies have shown TRIF-mediated signalling as the most likely molecular pathway associated to PPIs-mediated TLR4 activation (Srdić et al., 2020). These differences, together with the suggested delayed progression of early endosomes could be responsible for the observed results.

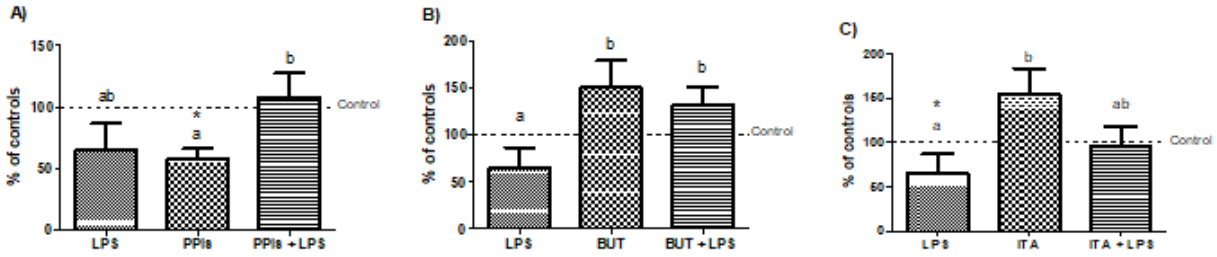


***Figure 2**. Changes in mitochondrial activity levels of HB8902©. (A) Cells incubated with an extract (<30kDa) obtained from C. quinoa (PPIs) and/or LPS (n=6). (B) Cells incubated with Butyrate 3.8 mM (n=13) and/or LPS (n=6). (C) Cells incubated with Itaconate (0.9 mM) (n=13) and/or LPS (n=6). Results are expressed as mean ± SEM. a-b Different superscript letters indicate statistical differences ($p < 0.05$). * Asterisks indicate statistical differences to the control level.*

The influence of both metabolites on the mitochondrial conversion of MTT was evaluated (Fig. 2B-C). Exposure of human macrophage-like cells to either independent butyrate (Fig. 2B) or itaconate (Fig. 2C) significantly ($p<0.05$) increased MTT conversion values in comparison to the LPS exposure. Only cell cultures subjected to the joint exposure of butyrate and LPS exhibited an upward trend in MTT conversion level, compared to cultures treated with LPS alone. Butyrate has been shown to reduce the glycolytic capacity and reserve in macrophages via histone deacetylase (HDA)-mediated gene downregulation affecting also mTOR kinase activity (Schulthess et al., 2019). From these literature data, it was expected the shutting down of mitochondrial enzyme activities, in contrast, we found out the MTT conversion values increased (Fig. 2B). However, these substantial changes in glycolysis reported in the bibliography are related to a 150 mM of butyrate exposure (Schulthess et al., 2019) and it is known that glycolysis affect mitochondrial activity, but it is carry out in cytoplasm.

In the present study, to identify a potential inhibitory effect of butyrate and itaconate on mitochondrial enzyme activities, different concentrations of these metabolites (butyrate or itaconate) were analysed (**Figure 3**). The mitochondrial activity induced by

butyrate is directly proportional to the concentration (Fig. 3A), whereas the activity caused by itaconate is inversely proportional (Fig. 3B).

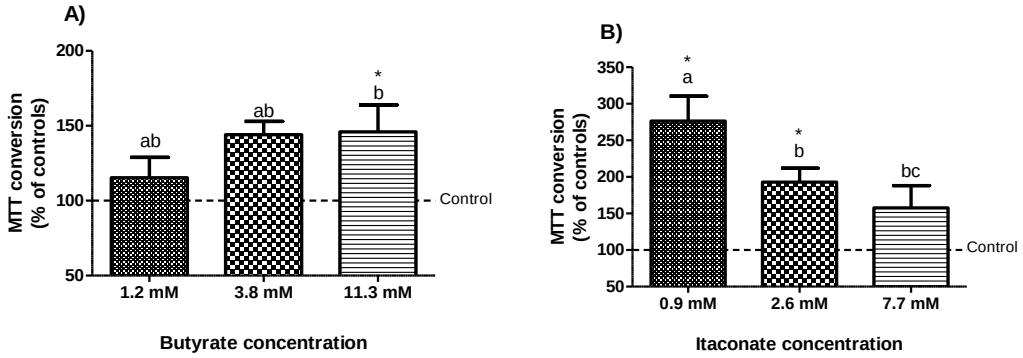


Figure 3. *Mitochondrial activity of HB8902 cells after exposure with butyrate (A) and itaconate (B). Three different concentrations of the metabolites were performed to dilucidated the optimal concentration that modify mitochondrial activity. Results are expressed as mean ± SEM.* $^{a-b}$ *Different superscript letters indicate statistical differences ($p < 0.05$). * Asterisks indicate statistical differences to the control level.*

In human macrophages-like exposed to butyrate, using concentrations between 3.8 mM and 11.3 mM (Fig. 3A) caused little variations of MTT conversion, opposite as it was observed between 1.2 mM and 3.8mM concentrations. In previous studies, the possible toxic effects of different concentrations of butyrate were monitored, seeing that at 4mM butyrate induced apoptosis in murine peritoneal macrophages (Ramos et al., 2002). According to the latter study and taking into the account the results of (Fig. 3A), it was decided to use 3.8mM of butyrate to do the treatments. Additionally, it was confirmed that with lower concentrations of butyrate, mitochondrial changes were also observed, as it was reported in other literature data (Ji et al., 2016), where they used 1.2 mM of butyrate and noticed significant metabolic changes. Considering the aforementioned, the increased mitochondrial activity of this study (Fig. 2A) does not correlate with the reduced glycolytic capacity reported certain studies (Schulthess et al., 2019). It may be due to the differences between butyrate concentrations: 3.8 mM used in Fig. 2A, and 150 mM used in (Schulthess et al., 2019) study or because glycolysis takes place outside the mitochondria.

Three possible concentrations of itaconate for the treatments (Fig. 3B) were also analysed. It was observed that concentration values were inversely proportional to the MTT conversion, suggesting that increasing itaconate concentrations caused decreasing MTT values. According to literature, macrophages have been shown to have intracellular concentrations of itaconate ranging from 40 µM to 8 mM (Harding H & Ruslan, 2016; Michelucci et al., 2013; Strelko et al., 2011a), whereas growth-inhibiting concentrations of itaconate range from 5 mM to 100 mM (McFadden & Purohit, 1977; Michelucci et al., 2013; Naujoks et al., 2016). Taking into consideration endogenous concentration of itaconate and results of Fig. 3B, it was decided to use 0.9 mM for the treatments.

In itaconate-treated macrophages, the mitochondrial activity was enhanced as it is observed an increase in the MTT conversion values (Fig. 2C). Nevertheless, these results are not in accordance with literature. Several studies affirm that mitochondrial respiration should decrease after itaconate treatment through SDH inhibition, a key enzyme of the complex II of the electron transport chain and the TCA cycle (Harding H & Ruslan, 2016; Lampropoulou et al., 2017). Moreover, further research has shown that itaconate decreases oxygen consumption and increases dependence on aerobic glycolysis (Harding H & Ruslan, 2016). Itaconate covalently modifies the cysteines of key glycolytic enzymes and proteins (Keap1 and glutathione) and impairs glycolytic flux mainly through inhibition of fructose-bisphosphate aldolase A (Qin et al., 2019). The latter study could suggest that the increase in mitochondrial activity of the Figure 2C might be due to a compensation of glycolysis inhibition is taking place by increasing the TCA cycle or by non-mitochondrial respiration.

3. Metabolite-induced changes in the endo/lysosomal capacities

The distribution of TLR4 to the endo/lysosomal compartment determines both its downstream signalling and the receptor retrieval towards the plasmatic membrane or proteasome degradation. Thus, the changes in neutral red uptake as marker of the endo/lysosomal compartment were analysed (**Figure 4**). No statistical differences were achieved, only a trend to increase endo/lysosomal activity after the independent addition of the metabolites, PPIs or LPS. However, the joint treatment of PPIs or itaconate with LPS normalizes the values to controls' (Fig. 4A-C).

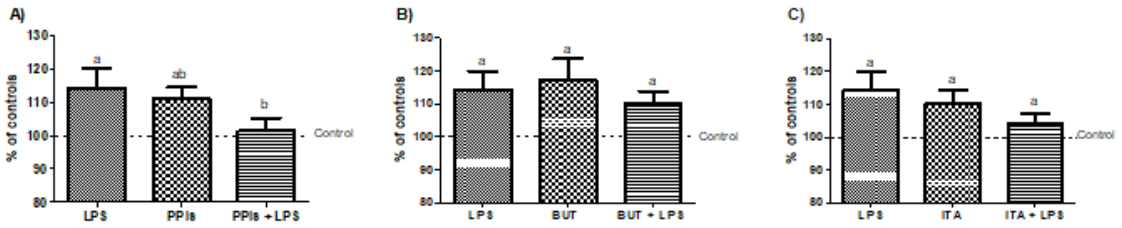


Figure 4. *Changes in endo/lysosomal activity levels in HB8902© cells. (A) Cells incubated with an extract (<30kDa) obtained from C. quinoa (PPIs) and/or LPS (n=6). (B) Cells incubated with Butyrate 3.8 mM (n=13) and/or LPS (n=6). (C) Cells incubated with Itaconate (0.9 mM) (n=13) and/or LPS (n=6). Results are expressed as mean ± SEM. a-b Different superscript letters indicate statistical differences (p < 0.05). * Asterisks indicate statistical differences to the control level.*

PPIs from *C. quinoa* modify the pattern in expression levels of different Rab proteins supporting an impact in late endosome membrane trafficking (Srdić et al., 2020). Rab proteins are GTPases responsible for compartmentalization of the endocytic pathway into early, recycling, late, and lysosomal routes; and coordination of individual transport steps from vesicle budding to fusion of them (Wandinger-Ness & Zerial, 2014). The results (Fig. 4A) do not explain a completely disrupted endo/lysosomal pathway of PPIs because PPIs red uptake levels are upper than control ones.

Overall, it seems that butyrate treatment led to an increasing trend of endo/lysosomal activity (Fig. 4B), while a combination exposure of the metabolite with LPS seems to decrease that tendency. Butyrate treatment causes an increment of adenosine monophosphate (AMP), an inducer of AMP kinase that inhibits mTOR, the expert regulator of autophagy (F. Wang et al., 2020b). Regardless, no conclusion could be drawn because there are not significant differences.

Macrophages enable specifically transport itaconate into intracellular vacuoles, a common residence of intracellular bacteria (Harding H & Ruslan, 2016). Additionally, some studies report that the majority of itaconate is secreted to the supernatant to generate local extracellular concentration against bacteria (Harding H & Ruslan, 2016; Strelko et al., 2011a), suggesting that with itaconate exposure the endo/lysosomal compartment remains active. In (Fig. 4C) results may confirm literature data because of an upward trend after itaconate exposure in comparison to the control values. However, it could be hypothesized that the joint exposure of itaconate and LPS (Fig. 4C) ameliorate the LPS-induced TLR4 endosomal internalization and signalling cascade.

4. Changes in CPT1/CPT2 protein expression

Fatty acid oxidation through mitochondrial carnitine palmitoyltransferase-I (CPT1) and -II (CPT2) plays key roles determining macrophage polarization and phagocytic functions (**Figure 5**). At first glance, all the assayed treatments promoted variations in CPT1 protein expression to a lesser extent than in CPT2. Only cell cultures exposed to LPS exhibited a significant ($p<0.05$) increase in CPT1 expression, while variations in this enzyme for all other groups of treatment (butyrate, itaconate or PPIs) did not differ ($p>0.05$) to those values of controls (Fig. 5A-C). When considering the joint addition to influence TLR4-mediated effects on CPT1 expression, only butyrate enabled a significative ($p<0.05$) CPT1 and downregulation in relation to the LPS-induced effects (Fig. 5B). It was also quantified a significant ($p<0.05$) increase in CPT2 protein expression in those cultures exposed to LPS alone, as well as those cultures incubated with butyrate alone (Fig. 5B). All the joint additions favoured downward trends in CPT2 expression; however, none of those reached statistical significance ($p>0.05$).

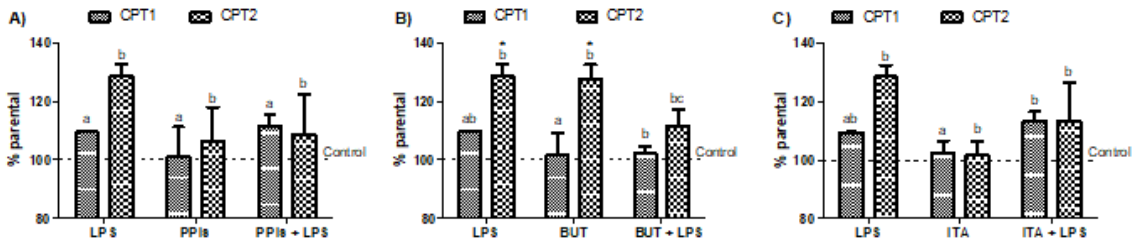


***Figure 5**. Carnitine palmitoyltransferases I and II (CPT1 and CPT2) in HB8902© cells. (A) Cells incubated with Butyrate 3,8mM and/or LPS. (B) Cells incubated with Itaconate (0,9mM) and/or LPS). (C) Cells incubated with an extract (<30kDa) obtained from C. quinoa (PPIs) and/or LPS. Results (n=3) are expressed as mean ± SEM. a-b Different superscript letters indicate statistical differences ($p < 0.05$). *Asterisks show statistical differences to the control level.*

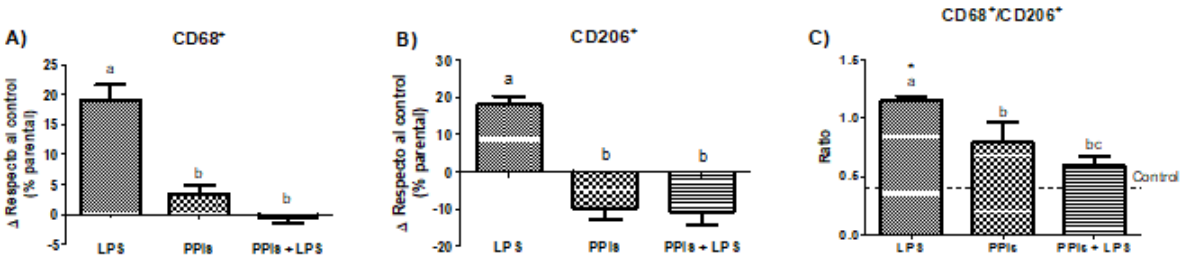
LPS decreases fatty acid oxidation pathway-dependent oxidative phosphorylation and induces pyruvate metabolism stimulation without affecting other pathways (Gonzalez-Hurtado et al., 2017; Nomura et al., 2016). The abovementioned difference between LPS- and PPIs-induced TLR4 signalling due to the MyD88 and TRIF adaptor molecules, respectively, favours the downward trend in CPT2 expression when both agonists are exposed together (Fig. 5A).

Our results suggest that butyrate induces regulation of fatty acid metabolic processes associated to an increased trend in the β-oxidation pathway (Fig. 5B), what could explain the raised mitochondrial activity of the Figure 2B results. At same time, it has been described the butyrate-induced overexpression of the HIF1α (J. Zhang et al., 2020) that upregulates TLR4 (S. Y. Kim et al., 2010) and fatty acid translocase (CD36). CD36 differently regulates macrophage responses to LPS (Biedroń et al., 2016). This interaction seems to take place when considering the joint addition of butyrate and LPS, and could explain, at least in part, the observed results.

As for itaconate, this is one of the most highly induced endogenous metabolites in LPS-activated macrophages (Diskin & Pålsson-McDermott, 2018). This metabolite specifically blocks NLRP3 inflammasome activation by reducing the NLRP3-NEK7 (member of the family of mammalian NIMA-related kinases) interaction, likely due to modification of a specific cysteine C548 on NLRP3 (Hooftman et al., 2020b). Hence, it can be hypothesized that LPS-activated macrophages requiring fatty-acid synthesis and FASN activity for adequate activation of NLRP3 could result interfered. This effect could explain, at least in part, the downward trend of mean values for CPT2 in cultures exposed to the subsequent addition of LPS and itaconate (Fig. 5C).

5. Changes in macrophages phenotype

M1 and M2 are the two ends and diametrically polarized $CD68^+$ (inflammatory/antitumoral), and $CD206^+$ (anti-inflammatory/protumoral), respectively, macrophage phenotypes (**Figure 6**).



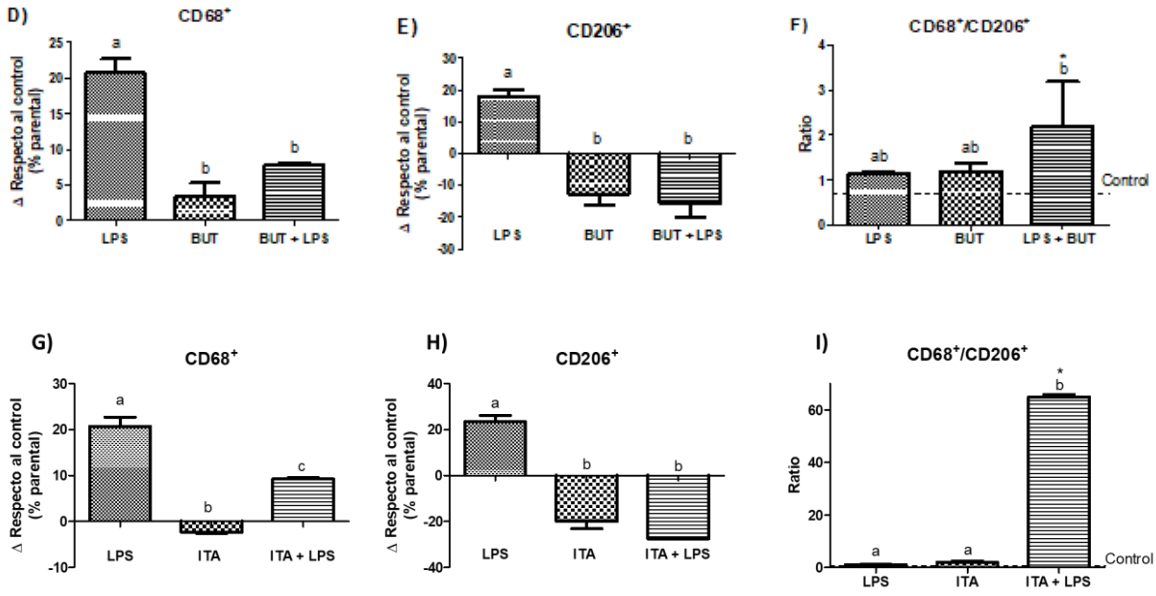


Figure 6. *Phenotypical changes in HB8902© cells. Two different phenotypic markers were looked at by flow cytometry (CD68+ typically M1 and CD206+ typically M2). All samples have n=3. (A), (B) and (C) Cells incubated with an extract (<30kDa) obtained from C. quinoa (PPIs) and/or LPS. (D), (E) and (F) Cells incubated with Butyrate 3.8 mM and/or LPS. (G), (H) and (I) Cells incubated with Itaconate (0.9 mM) and/or LPS. (A) (D) and (G) represent the positive increase of CD68+ macrophages; (B), (E) and (H) represent the positive increase of CD206+ macrophages; and (C), (F) and (I) represent the CD68+/CD206+ ratio. Results are expressed as mean ± SEM. a-b Different superscript letters indicate statistical differences ($p < 0.05$). * Asterisks indicate statistical differences to the control level.*

Macrophage phenotype developed after LPS treatment shows both $CD68^+$ and $CD206^+$ markers (Fig. 6A-B), with more presence of $CD68^+$. Literature studies have diverse conclusions about macrophage polarization under LPS stimuli. Most of the studies affirm that after LPS stimulation of TLR4, MyD88 pathway is triggered, macrophages suffer a metabolic reprograming and adopt a proinflammatory phenotype, as shown in Figure 6A (Chanput et al., 2013; Ciesielska et al., 2021; Genin et al., 2015; Martinez & Gordon, 2014). However, in other literature data it was showed that LPS could also enhance the expression of $CD206^+$ (M2) marker but to a lesser extent (Martinez & Gordon, 2014; Steimle et al., 2019). Additionally, other studies show a correlation between the increase of CPT1a expression and the switch of M1 phenotype into M2 (Calle et al., 2019), that would also justify the $CD206^+$ enhanced phenotype by LPS (Fig. 6B-E-H).

After the LPS treatment, the expression of the key metabolic regulator Pyruvate Kinase M2 (PKM2) in macrophages could be induced. In certain research it has been displayed that the activation of PKM2 inhibits LPS-induced HIF1α and IL1β, that attenuates LPS induced proinflammatory M1 macrophage phenotype while promoting typical M2 markers (Palsson-Mcdermott et al., 2015) at the same time that promoting those of M1, as it is observed in (Fig. 6A-B). Both PKM2 and HIF1α inhibit LPS-induced glycolytic reprogramming and succinate production (Palsson-Mcdermott et al., 2015). Succinate triggers the activation of HIF1α, that hinders mitochondrial respiration; and IL1β, which enhances proinflammatory response (Galván-Peña & O'Neill, 2014; Seim et al., 2019;

Takeda & Akira, 2004). So, if succinate is inhibited, the proinflammatory state (Fig. 6A-B-C) and mitochondrial respiration (Fig. 2A) are not totally reached.

In PPIs treatment, macrophages show a positive increase in $CD68^+/CD206^+$ ratio (Fig. 6C), related to a M1 phenotype development. PPIs acts as a TLR4 agonist and triggers TRIF-mediated signalling, generating a proinflammatory response (Srdić *et al.*, 2020). However, when stimulation of PPIs is done in conjunction with LPS, both TRIF-dependent and Myd88-dependent signalling pathways are enhanced. Due to the fact that stimulation with PPIs is done at a higher concentration (50µg / mL vs. 100ng / mL of LPS), the TRIF pathway is over-activated, so the expression of CXCL10 is promoted, which feeds back TLR4 and causes the Myd88 signalling inhibition. It could be the reason of the decreasing the $CD68^+/CD206^+$ ratio when the exposure of LPS and PPIs is joint (Fig. 6A-C).

Butyrate treatments induce an increased $CD68^+/CD206^+$ ratio (Fig. 6F), where a greater development of the M1 phenotype is interpreted. It could be justified because the HIF1α increasing expression stimulates TLR4 receptor and inhibit fatty acid oxidation, both situations lead to a M1 phenotype development. It can be also noticed that butyrate could ameliorate the metabolic reprogramming of LPS towards a M1 phenotype, due to the joint exposure decreases the CD68+ markers (Fig. 6D). However, the treatment with butyrate and LPS still cause an increment of $CD68^+/CD206^+$ ratio (Fig. 6D) comparing to the ratio of LPS alone, suggesting that butyrate, rather than modulating the effect of LPS, has a synergistic effect. However, in the previous section, it was mentioned that butyrate enhanced the expression of CPT2 (Fig. 5B), which is related to an increased fatty acid oxidation, associated with M2 phenotype. These controversial results could be explained because butyrate promotes HIF1α expression, that could inhibit, at least in part, the fatty acid oxidation, and the result is an increase in M1 markers. This effect added to the proinflammatory effect of LPS may explain the synergistic effect of LPS plus butyrate (Fig. 6F).

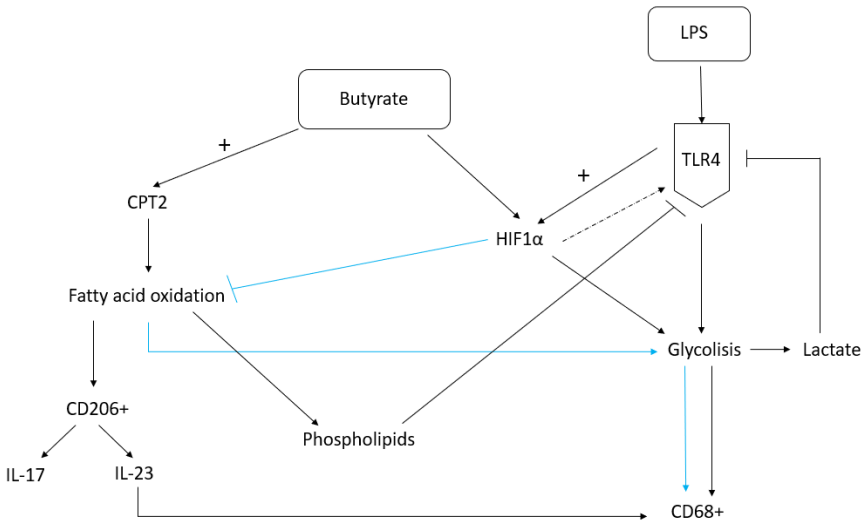
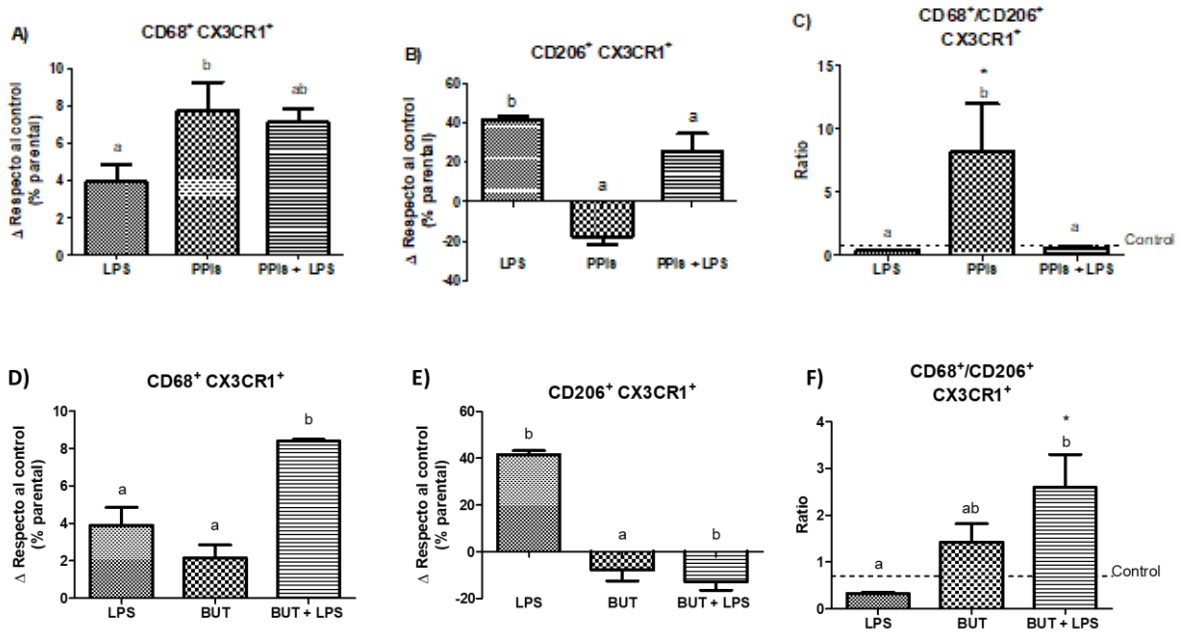


***Illustration 3**. Schematic representation of the possible effect caused by the subsequent treatment of LPS followed by butyrate in macrophages.*

Macrophages stimulated with itaconate metabolite show a slight increase of $CD68^+$ markers (Fig. 6G), while the $CD68^+/CD206^+$ ratio is slightly positive (Fig. 6I). Taking into consideration that CPT1 and CPT2 (Fig. 5C) were not significantly modified by itaconate exposure alone, phenotype development is not interconnected to fatty acid oxidation. In several researches, itaconate has been displayed to have an anti-inflammatory phenotype (Hooftman et al., 2020a; Lampropoulou et al., 2017; Qin et al., 2019), what could explain, at least in part, the almost zero ratio that appears in the Figure 6I. However, itaconate has demonstrate to have dual effects as an immunosuppressant and anti-bacterial metabolite (Harding H & Ruslan, 2016; Lampropoulou et al., 2017). Thereby, results of this study (Fig. 6B-H-I) could be endorsed by the anti-microbial activity of itaconate reported in (Harding H & Ruslan, 2016; Lampropoulou et al., 2017) studies, but the $CD68^+/CD206^+$ ratio suggest that the phenotype is between M1 and M2 (atypical M1). On the other hand, the increase $CD68^+/CD206^+$ ratio due to joint exposure of itaconate and LPS suggest that together have proinflammatory properties due to HIF1α potentiated expression from both sides, LPS-TLR4-HIF pathway and itaconate-SHD inhibition, and HIF1α stimulation.

- **Changes in the capacity of macrophages to interact with the tissue**

During the phenotype development of macrophages in metabolic and inflammation disorders, their infiltration is crucial. In **Figure 7** it has been measured $CD68^+/CD206^+$ markers and ratio in combination with the CX3CR1 marker of tissue interaction and infiltration. The expression of the fractalkine receptor CX3CR1 has been identified as a key regulator of macrophage function at sites of inflammation (Burgess et al., 2019).



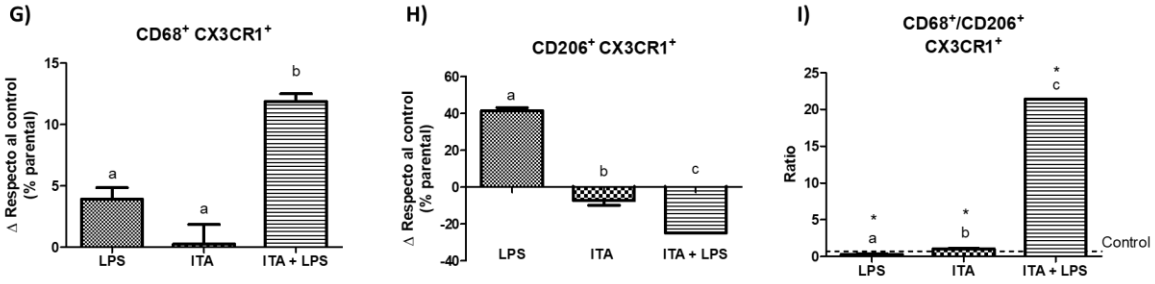


***Figure 7**. Phenotypical changes in HB8902© cells. Two different phenotypic markers were looked at by flow cytometry (CD68+ typically M1 and CD206+ typically M2). The other markers are CX3CR1, related to a control of macrophage infiltration and CD44, that is related to an inhibition of infiltration. All samples have n=3. (A), (B) and (C) Cells incubated with an extract (<30kDa) obtained from C. quinoa (PPIs) and/or LPS. (D), (E) and (F) Cells incubated with Butyrate 3.8 mM and/or LPS. (G), (H) and (I) Cells incubated with Itaconate (0.9 mM) and/or LPS. (A) (D) and (G) represent the increment of CD68+ macrophages; (B), (E) and (H) represent the increment of CD206+ macrophages; and (C), (F) and (I) represent the CD68+/CD206+ ratio. Results are expressed as mean ± SEM. a-b Different superscript letters indicate statistical differences (p < 0.05). * Asterisks indicate statistical differences to the control level*

LPS exposure in macrophages promotes the expression of the CX3CR1 marker in both $CD68^+$ and $CD206^+$ (Fig. 7A-B) cells, in the latter with more presence, so the $CD68^+/CD206^+$ ratio is almost zero. The stimulation of TLR4 with LPS is linked to a greater infiltration of M1 macrophages into the tissue (Surmi & Hasty, 2008), but our results (Fig. 6A-B-C) report also a M2 development after LPS- stimulation. Macrophages with M2 phenotype also infiltrate into the tissue, usually to resolve the inflammation, it has been published that M2 macrophages present more CX3CR1 markers than M1 ones (Burgess et al., 2019; Gupta et al., 2012; Surmi & Hasty, 2008).

After stimulation of macrophages with PPIs, of those presenting the infiltration marker ($CX3CR1^+$), the majority show a pro-inflammatory phenotype ($CD68^+$) (Fig. 7A), which causes a significant ($p<0.05$) increase in the $CD68^+/CD206^+$ ratio (Fig. 7C). PPIs stimulation of TLR4, is related to the infiltration of activated pro-inflammatory macrophages into the tissue and their interaction (McKernan et al., 2020a; Surmi & Hasty, 2008). The joint exposure of LPS and PPIs promote that $CD68^+$ macrophages express CX3CR1 but to a lesser extent than $CD206^+$ ones (Fig. 7A-B-C), which might be interpreted as the stimulation of LPS that is causing the M2 phenotype is stronger than that enhanced by PPIs.

Butyrate treatment promotes the development of M1 phenotype macrophages with potential to interact to the tissue and infiltrate (Fig. 7D). This is explained because, as seen previously (Fig. 6B-C-D), butyrate increases the expression of HIF1α while blocking fatty acid oxidation. Both events are signals that promote macrophage infiltration into the tissue (Henze & Mazzone, 2016; Imtiyaz & Simon, 2010; Sutti et al., 2015). It is observed that butyrate has a significant ($p<0.05$) greater capacity to counteract the effect towards the M2 phenotype of LPS during joint exposure (Fig. 7F), hypothetically because both could stimulate TLR4 direct- and indirectly at the same time as inducing HIF1α. Otherwise,

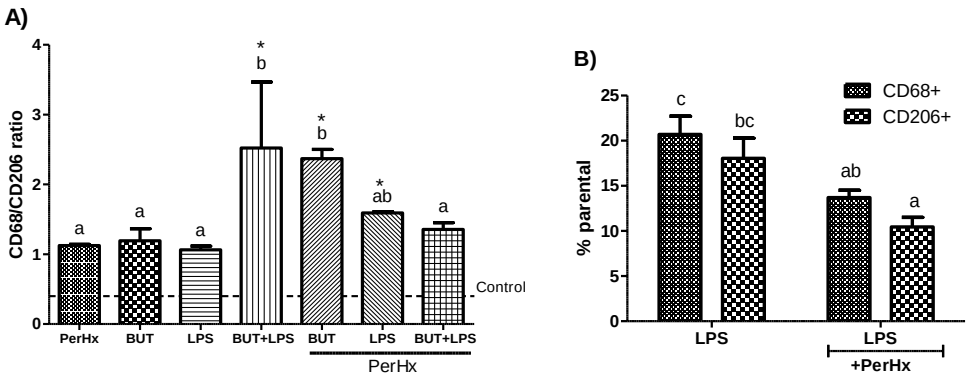
as mentioned above, itaconate by itself is not capable of sufficiently or correctly stimulating macrophages to develop a clear phenotype (Fig. 6G-H), so the presence of the marker is not observed in the results CX3CR1 in them (Fig. 7G-H). However, during itaconate and LPS treatment, it is observed a significant ($p<0,05$) increase of the $CX3CR1^+$ $CD68^+/CD206^+$ ratio (Fig. 7H). It is known that itaconate inhibits inflammasome development, causing an altered fatty β-oxidation, which is also related to macrophage infiltration (Surmi & Hasty, 2008) and could explain our results.

- **Changes in the migratory capacity of macrophages**

Infiltration is a complex process where not only CX3CR1 participates, but also other molecules such as CD44, a cell-surface glycoprotein involved in cell–cell interactions, cell adhesion and migration. CD44 is necessary for macrophage migration inhibitory factor (MIF) activity. MIF antagonizes the action of glucocorticoids, upregulates TLR4 expression and suppresses apoptosis, supressing macrophage infiltration (Shi et al., 2006). It has been analysed the expression of both markers related to migration, CD44 and CX3CR1, in $CD68^+$ and $CD206^+$ macrophages (**Annex 1**-**Figure 8**). No differences were observed between the results (Fig. 7 and Fig. 8), which lead us to suggest that this marker does not result affected by immunomodulatory signals.

6. Influence of CPT1/2 determining macrophage phenotype.

Perhexiline (maleate salt) (PerHx) has been used to block fatty acid oxidation inhibiting CPT1, and to a lesser extent CPT2. This causes a shift in substrate utilisation from long chain fatty acids to carbohydrates (enhanced glycolysis), resulting in glucose and lactate production and increased ATP synthesis for the same O_2 consumption (Gilutz et al., 2012). It has been analysed the influence of butyrate, LPS, and a combination of both treatments on perhexiline-treated macrophage cultures (**Figure 9**). Butyrate was chosen since, according to our results, it appears to better control TLR4-induced effects on CPT1/2 expression.



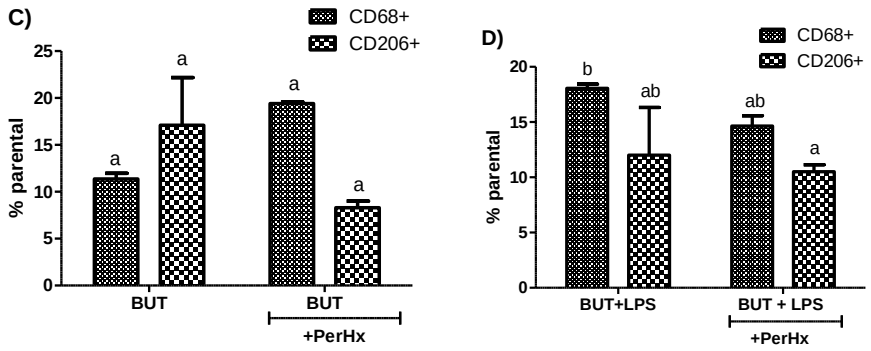


Figure 9. *(A) Phenotypical changes in HB8902© cells expressed by CD68+/CD206+ ratio after perhexiline, LPS or/and butyrate treatments alone and in combination. Two different phenotypic markers were looked at by flow cytometry (CD68+ typically M1 and CD206+ typically M2). (B) % Parental of CD68+ and CD206+ populations after perhexiline or not, LPS (B), butyrate (C) and joint (D) treatments. All samples have between n=3 and n=4. Results are expressed as mean ± SEM. a-b Different superscript letters indicate statistical differences (p<0.05). *Asterisks indicate statistical differences to the control level. In (A) * indicates stastistical differences to CD68/CD206+ ratio control.*

As a consequence of the metabolic redirection caused by PerHx, cultures mostly generate an imbalance towards the $CD68^+$ phenotype (Fig. 9A). Cultures pre-treated with PerHx do not alter the effect of butyrate and LPS on the phenotype developed by cell cultures (Figure 9). Notably, the positive synergy between butyrate and LPS, increasing the functional differentiation of macrophages towards their $CD68^+$ phenotype, presents a downward trend: it decreases approximately 50% of the mean values after exposure with perhexiline (Fig. 9A). These changes are motivated by the negative variation of the $CD68^+$ population (21.5% lower), offset by the positive, to a lesser extent, of $CD206^+$ (12.5% higher) (Fig. 9D). After treatment with perhexiline, the activity of CPT1/2 is also conditioned, this downward trend could be because the inhibition of CPT1 modifies the signalling of phospholipids through lipid rafts, which hinder the signalling towards $CD68^+$ by TLR4. This selective orientation of the phenotype is concordant with the metabolic drift towards greater glycolytic activity.

The differences on relative variations of the $CD68^+$ and $CD206^+$ populations (Figure 9B-C-D) of the cultures exposed to butyrate or/and LPS and those cultures treated with PerHx support its inhibitory activity on CPT1/2. However, this inhibition does not seem to be absolute because it is also present a certain proportion of $CD206^+$ macrophages (Fig. 9B-C-D). Alternative activated macrophages (M2) play essential roles on the resolution of proinflammatory processes, preventing the so called chronic 'sterile inflammation'. These macrophage population displays an increase in transcriptional programs associated with mitochondrial biogenesis and oxidative mitochondrial metabolism (fatty acid oxidation)(Divakaruni et al., 2018). During etomoxir treatment, another CPT1/2 inhibitor, it was observed that FAO is not essential to M2 phenotype development (Divakaruni et al., 2018), because after the treatment, CD206+ markers were still observed. The results of Figure 9 could allow us to suggest that FAO is still functioning after CPT1/2 inhibition by perhexiline. Perhexiline maleate was also recently found to inhibit the activity of

mTORC1 (Gilutz et al., 2012). In certain studies butyrate has shown to act similarly as perhexiline inhibiting mTOR pathway in cancer cells (Pant et al., 2017) but in other studies contrarily (F. Wang et al., 2020a), activating it. Collectively, taking into consideration the high susceptibility to the metabolic control on macrophage's selective differentiation and function, from a functional perspective it might interpret the results as CPT1/2 activities playing important roles to preserve adequate immunometabolic signals within the phenotypic balance of macrophages.

CONCLUSIONS

Both metabolites, itaconate and butyrate, exhibited immunomodulatory potential to HB8902 human-like macrophages, probably trough the indirectly TLR4 stimulation. Nevertheless, butyrate showed a more marked effect modulating macrophage metabolism.

During the single exposure of metabolites, only butyrate increased the expression of CPT2, and none of them had an influence through the expression of CPT1. Conversely, itaconate, butyrate and PPIs were able to normalize the CPT2 expression values caused by LPS stimulation, showing a downward tendency. However, CPT1 expression was not modulated by the treatment of both metabolites and PPIs individually. Interestingly, the joint exposure of LPS followed by itaconate or PPIs, exhibited the same trend increasing the CPT1 expression.

Butyrate exposure generated a positive differentiation of macrophages towards a pro-inflammatory phenotype, as concluded from the upward trend in the $CD68^{+}/CD206^{+}$ ratio. In addition, it was also observed that the subsequent exposure of LPS and butyrate had a synergistic effect, enhancing the development of the $CD68^{+}$ phenotype in macrophage culture.

FUTURE PERSPECTIVES

Controlling fatty acid metabolism through TLR4 signalling may be a target for regulating the activity of the monocyte macrophage population in pro-inflammatory environments. In general, these results open new perspectives on possible biotechnological strategies for immunonutritional intervention, which could complement classical pharmacological therapies.

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SELF-ASSESSMENT

I remember the moment when I decided that I wanted to study Biotechnology and how emotion, nerves and uncertainty invaded me. And now, I find myself writing my final degree project four years later, with a backpack of experiences and people on my back. When the moment to decide where to do my internship came, I was sure that I wanted to participate in a research group in which they were investigating on nutritional strategies to prevent diseases. And thanks to my stay in the molecular immunonutrition group, I have discovered my passion. In the future I would like to dedicate myself to research the great potential that food intake has on immunity.

These months of the internship, in a different city from the one I grew up in and studied in, have been enriching. I have learned to have critical thinking, to be decisive and to have willpower. I have had to learn to be efficient in the laboratory and accomplish the objectives that I had to reach. In addition, doing this work in English has been a great challenge for me and that in the past I would never have imagined that I would achieve it. I'm proud of myself I did it and this experience will probably bring me benefits in the future. It has not been an easy road, there have been moments when it seemed that carrying out the project was impossible and where everything went uphill. But I really think that it was worth it. I have ended up wanting to continue contributing knowledge to the scientific world and to nourish myself with other researchers. I hope I will have the opportunity to continue learning and training for much longer.

I would like to thank the people who have surrounded me these months and throughout my university years, who have supported me. And of course, to my parents, who have watched over me from the distance. And finally, I would like to dedicate this project to my two sisters and my brother, to encourage and motive them, because even with the wind against you, you can go far.

ANNEX

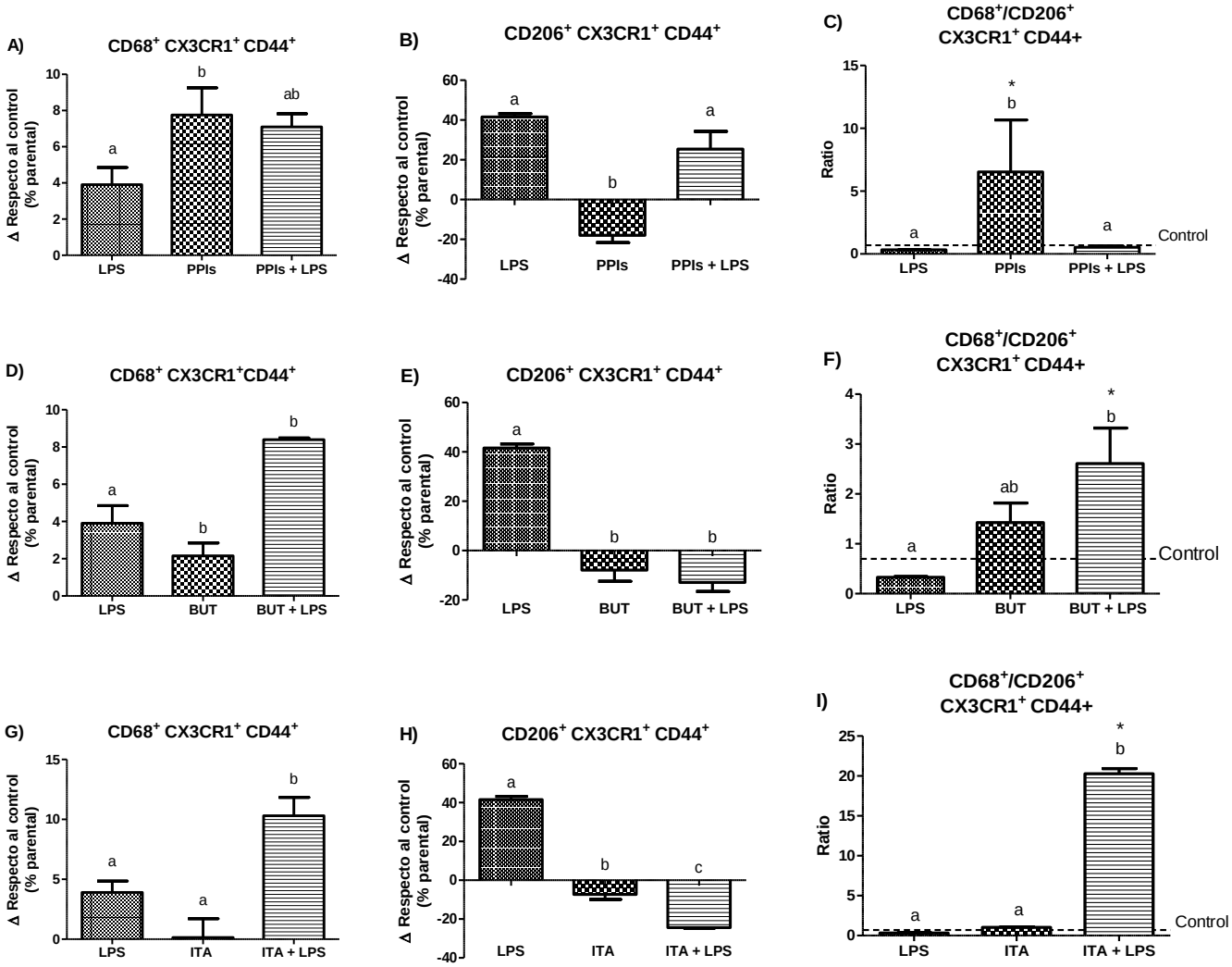

***Figure 8**. Phenotypical changes in HB8902© cells. Two different phenotypic markers were looked at by flow cytometry (CD68+ typically M1 and CD206+ typically M2). The other markers are CX3CR1, related to a control of macrophage infiltration and CD44, that is related to a inhibition of infiltration. All samples have n=3. (A), (B) and (C) Cells incubated with an extract (<30kDa) obtained from C. quinoa (PPIs) and/or LPS. (D), (E) and (F) Cells incubated with Butyrate 3.8 mM and/or LPS. (G), (H) and (I) Cells incubated with Itaconate (0.9 mM) and/or LPS. (A) (D) and (G) represent the increment of CD68+ macrophages; (B), (E) and (H) represent the increment of CD206+ macrophages; and (C), (F) and (I) represent the CD68+/CD206+ ratio. Results are expressed as mean ± SEM. [a-b] Different superscript letters indicate statistical differences (p < 0.05). * Asterisks indicate statistical differences to the control level.*