NUTRITIONAL IMMUNOTHERAPY: TARGETING IMMUNOMETABOLISM WITH BIOACTIVE FOOD COMPOUNDS

Nieves Beatriz Martínez Micaelo

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Nutritional Immunotherapy: targeting immunometabolism with bioactive food compounds

Doctoral Thesis

Directed by Dr. Maria Teresa Blay Olivé

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FAIG CONSTAR que aquest treball, titulat "Nutritional Immunotherapy: targeting immunometabolism with bioactive food compounds", que presenta Neus B. Martínez Micaelo per a la obtenció de títol de Doctor, ha estat realitzada sota la meva direcció al Departament de Bioquímica I Biotecnologia d'aquesta universitat I que acompleix els requeriments per poder optar a Menció Europea.

Tarragona, 15 de maig de 2014

La directora de la tesi doctoral

Dra. M. Teresa Blay Olivé

Als de casa...

"My brain is only a receiver, in the Universe there is a core from which we obtain knowledge, strength and inspiration. I have not penetrated into the secrets of this core, but I know that it exists."

Nikola Tesla

Nikola Tesla (1856-1943). Austro-Hungarian electrical and mechanical engineer, Tesla was one of the greatest and most enigmatic scientists of the 20th century. Tesla obtained over 700 patents in fields including electricity, robotics, radar and wireless transmission of energy. Tesla's discoveries laid the groundwork for many of the technological advances of the 20th century.

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Summary

Nutritional immunotherapy is based on promoting health through the dietary intake of natural bioactive compounds found in food, such as the procyanidins and docosahexaenoic n-3 polyunsaturated fatty acid (DHA), optimising the functionality of the host's own immune system and improving its role in the preservation of the body against destabilisers of homeostasis. The research that this thesis encompasses is focused on the role of the nutritional profile in the regulation of immunometabolism.

To accomplish this purpose, in the first part of the work presented in this thesis, we determined whether macrophages, the effector cells of innate immunity, could differentially sense dietary composition. We demonstrated that bioactive food compounds modulate, at the molecular level, the functionality of macrophages by hindering macrophage activation. Furthermore, this immunomodulatory effect is compound-dependent, relying on the intrinsic factors of each compound, such as chemical and nutritional properties, to determine its bioactivity.

The second part was aimed at determining the role of the dietary pattern within the complex crosstalk of immunity and metabolism using an *in vivo* model of diet-induced obesity. We determined that immunometabolic regulation depends on the nutritional profile. While a diet based on foods with a high energy content can weaken immunometabolism, the presence of bioactive foods such as procyanidins or DHA can strengthen the relationship.

Within the third part, we evaluated the role of gene-diet interactions in the phenotypic expression of obesity-related complex traits. Using a diet-induced obesity model, two distinct genetic backgrounds (phenotypically different inbred rat strains) were immunometabolically challenged. Our results revealed that interactions between genetic and dietary factors are fundamental for the susceptibility of a genotype to diet-induced obesity.

We established that the nutritional profile is a powerful tool to target the functionality of the immunometabolic axis. We further concluded that bioactive compounds present in food improve the efficiency of this axis, thereby promoting a healthy state.

1. Introduction

The dietary intake pattern, and specifically the nutritional profile, are essential for the regulation of metabolism and also fundamental for correct functioning of the immune system and its capacity to sense harmful stimuli. Thus, depending on the nutritional profile the immune response could be improved or compromised.

A deeper understanding of how our dietary patterns and nutritional status affect the composition and the dynamic interactions between metabolism and the immune system could be considered an opportunity and a powerful tool for developing nutrition-based immune therapies to target metabolic diseases, as well as to modulate the ability of the metabolic system to regulate immune function.

Immunotherapy refers to the treatment of pathologies through the improvement of the functionality of the host immune system, based on the activation, enhancement or suppression of the immune response. Therefore, nutritional immunotherapy is focused on the nutritional profile, and specifically in natural bioactive food compounds, as tools to target the immune system and, through the promotion of its proper function, promote and/or restore homeostasis.

1.1. Immunometabolism

Immunometabolism refers to the interface between the immune and metabolic systems, two closely integrated disciplines, as the proper functioning of one is dependent upon the other [1]. A greater interest in immunometabolism has recently emerged as a consequence of the obesity epidemic and because obesity affects not only metabolism but also the functionality of the immune system [2].

Two central mechanisms are essential for the survival of multicellular organisms: the ability to store energy to prevent starvation and the ability to fight infection. Both immune and metabolic pathways are highly conserved and a delicate balance has evolved. The activation of the immune response is highly energy-demanding and shifts energy away from nonessential functions [3]. Conversely, infection often results in metabolic disruptions such as insulin resistance [4].

The immune system monitors and responds to the metabolic state of tissues and organisms [2]. Therefore, although obesity is not classically considered an immunopathology, it results

in the mobilisation of the innate and adaptive immune systems and is characterised by a state of low-grade inflammation. On the other hand, the metabolic state is able to control the function of immune cells, and deregulation of the immune system is also an etiopathological event in obesity. Thus, the proper functioning of the immunometabolic axis is central for the promotion and maintenance of body homeostasis and therefore health.

1.2. Immune system

The immune system is an interactive network of lymphoid organs, immune cells, humoral factors and a wide array of molecules, with the main goal of defending the host against endogenous or exogenous noxious stimuli.

Immunity results from the interaction between two branches: innate and adaptive immune responses (Figure 1). Innate immunity is the first line of host defence and is mediated mainly by leukocytes, including mast cells, granulocytes, monocytes, macrophages and natural killer cells. These immune cells are involved in the phagocytosis of pathogens, free radical production (the oxidative burst), cytokine production and antigen presentation to lymphocytes.

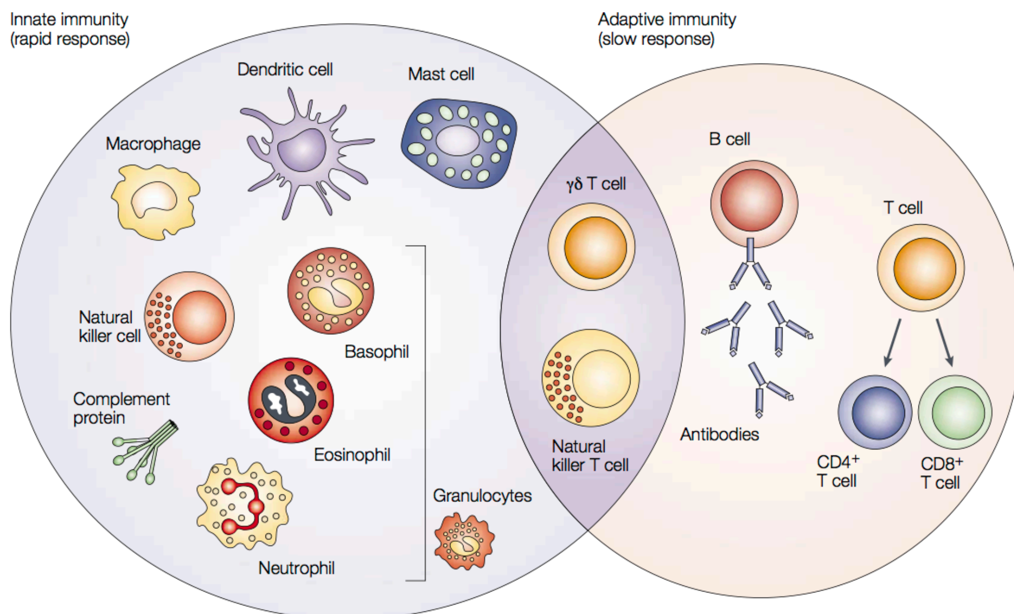

Figure 1. Simplified overview of the innate and adaptive immune systems. Adapted from [7].

In contrast, the adaptive immune response is involved in the elimination of pathogens in the late phase as well as in the generation of immunological memory. Adaptive immunity depends on the production of antibodies (immunoglobulins (Igs)) by B-lymphocytes directed against specific antigens present on pathogens (also termed the humoral immune

response). The adaptive immune response also depends on the attack of infected body cells by cytotoxic ($CD8^+$) and helper ($CD4^+$) T-lymphocytes (also termed the cell-mediated immune response). In addition, due to the presence of memory cells, the adaptive immune response is characterised by enhanced and fast responses after repetitive contact with the same antigen.

Thus, after leukocytes of the innate system sense a harmful stimuli they become activated, inducing the production and secretion of cytokines as well as presenting antigens to T- and B-lymphocytes, which in turn activates the adaptive immune system [5,6].

However, the innate immune response is not completely nonspecific, as was originally thought, but rather is able to discriminate between harmful and non-harmful stimuli. The innate immune system recognises microorganisms via a limited number of germline-encoded receptors, in contrast to the large repertoire of receptors generated via genetic recombination through the adaptive immune system.

1.2.1. Signalling in innate immunity and inflammation

Inflammation is defined as the body's immediate response, and it is triggered by innate immune cells after sensing molecular patterns from noxious stimuli to combat them and restore the system back to the homeostasis [8]. Thus, to neutralise the causative agent, the innate immune system orchestrates a coordinated action involving innate immune cells and inflammatory mediators, whose roles will depend on the nature of the triggering stimuli. The inflammatory response underlies a wide variety of physiological and pathological processes [9].

Innate immune cells recognise pathogen invasion or cell stress through highly conserved germline-encoded pattern recognition receptors (PRRs). These receptors bind to common pathogen-associated molecular patterns (PAMPs) such as microbial nucleic acids, lipoproteins and carbohydrates, or damage-associated molecular patterns (DAMPs) released from injured cells. Once activated, PRRs oligomerise and assemble large multi-subunit complexes that initiate cell-signalling cascades to trigger the release of factors that promote the recruitment of leukocytes to the region [8,10].

There are several families of PRRs, including the Toll-like receptors (TLRs) that sense conserved molecules from bacteria, viruses and parasites, and the NOD-like receptors (NLRs) that sense bacterial products and endogenous stress-induced molecules [8].

PAMPs and DAMPs are also involved in the activation of adaptive immunity, as T cells can be activated either indirectly, through the production of pro-inflammatory cytokines by innate immune cells, or directly by binding of PRRs on T cells [11].

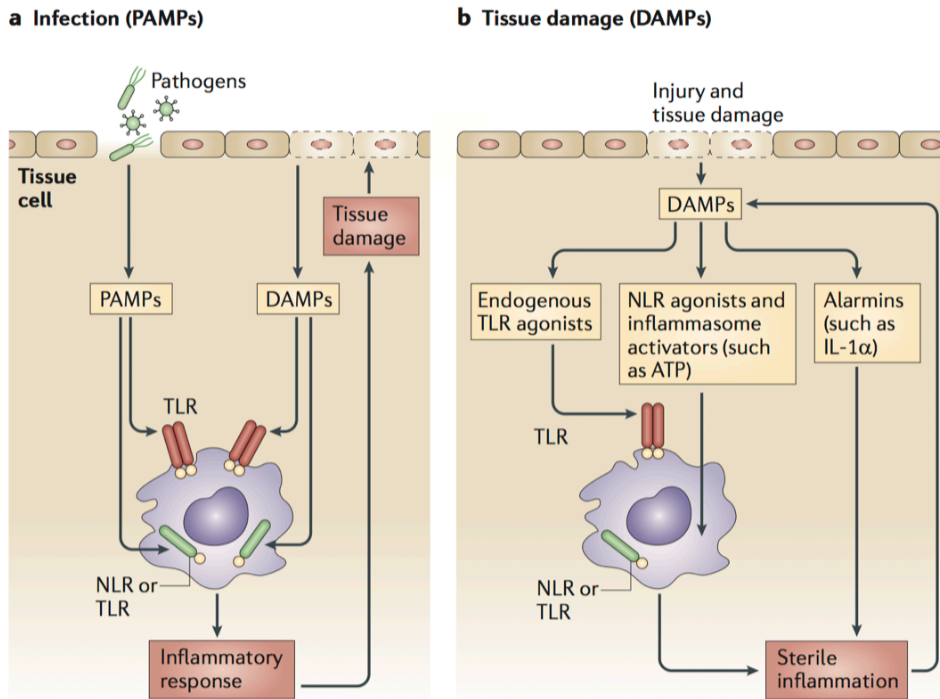

Figure 2. PAMP- and DAMP-mediated activation of innate immune response. Adapted from [11].

Inflammation can also be classified depending on the duration of the response. Acute inflammation is a short-term response, mainly initiated by tissue-resident macrophages and mast cells that recruit leukocytes, acute-phase proteins and adhesion molecules to the damaged region. This acute response leads to the activation of several transcription factors such as mitogen-activated protein kinase (MAPK), janus kinase/signal transducer and activator of transcription (JAK/STAT) and nuclear factor kappa B (NF-κB). Chronic inflammation, by contrast, is a non-resolved, dysregulated and maladaptive inflammatory response that involves not only the activation of the innate but also the adaptive immune response. Additionally, distinct from the classically described inflammatory response is metabolic inflammation, also termed metaflammation (Figure 3).

Metaflammation is defined as a low-grade chronic inflammation and is orchestrated by metabolic cells in response to an excess of nutrients and energy. Furthermore, metaflammation is distinctive and is outside the paradigm of classical inflammation defined by the cardinal signs of redness, swelling, heat and pain. Additionally, metaflammation is not accompanied by increased basal energy expenditure.

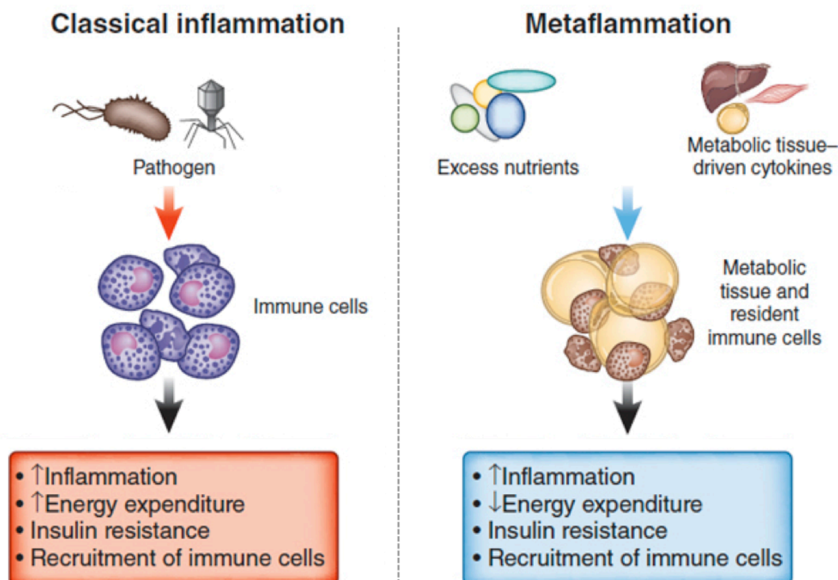

Figure 3. Signalling pathways involved in classical inflammation and in metabolic inflammation. Adapted from [12].

1.2.1.1. Toll-like receptors and NF-κB

Toll-like receptors are type I membrane glycoproteins that contain an extracellular leucine-rich repeat (LRR) domain, a transmembrane domain and a cytoplasmic Toll/IL-1 receptor (TIR) domain required for downstream signal transduction. Based on their primary sequences, 9 different TLRs have been identified in humans and 13 in mice; they are further divided into several subfamilies, each of which recognises specific PAMPs.

The expression of TLRs is not static but rather is modulated rapidly in response to pathogens, a variety of cytokines and environmental stress. TLRs may be expressed extra- or intra-cellularly. Thus, whereas certain TLRs (TLR1, 2, 4, 5 and 6) are expressed on the surface, others (TLR3, 7, 8 and 9) are found almost exclusively within endocytic compartments in the cytoplasm, and their ligands, mainly nucleic acids, require internalisation to the endosome before signalling [13] can occur. TLRs are expressed on immune cells, including macrophages, dendritic cells and B cells, but also in non-immune cells such as epithelial cells, endothelial cells or adipocytes. Although TLRs are essential for protective immunity against infection, TLR signalling must be tightly regulated to avoid inappropriate TLR responses, which result in acute and chronic inflammation as well as autoimmune diseases.

The TLR4 signalling pathway has a pivotal role in the initiation and amplification of innate immune response through the activation of the inducible transcription factor NF-κB. TLR4 recognises a very divergent collection of ligands, of which the gram-negative bacteria lipopolysaccharide (LPS) is the best characterised. The LPS-TLR4 interaction is mediated by

the lipid moiety constituent of LPS, Lipid A. This is of great relevance, because the key for LPS toxicity is the shape and conformation of this lipid, particularly the number and length of the fatty acid chains, which determine the toxicity of a given pathogen [14]. The recognition and subsequent interaction between the PAMP and TLR4 result in the triggering of downstream signalling cascades that lead to the activation of the inducible transcription factor NF-κB.

The NF-κB family is comprised of dimeric transcription factors that contain Rel- N-terminal Rel homology domains (RHD) that are responsible for binding to discrete DNA sequences, known as κB sites, present in promoter and enhancer regions of target genes. In mammalian cells, there are five members of the NF-κB family: p65 (RelA), c-Rel, RelB, p105 (the precursor of p50) and p100 (the precursor of p52). Although the NF-κB proteins form several homo- and hetero-dimers, the most frequently activated form of NF-κB is a heterodimer composed of the p50 and p65 subunits. The IκB family is characterised by ankyrin repeats and consists of several members: IκBα, IκBβ, IκBγ, IκBε, Bcl-3 and IκBζ. IκBα, IκBβ, IκBγ and IκBε, can mask the nuclear localisation signal of NF-κB in the cytoplasm through direct interactions to prevent translocation of NF-κB [15]. There are two described NF-κB signalling pathways: the canonical pathway and the alternative route. The main difference between the pathways is the specific members from IκB kinase (IKK) that are involved [16].

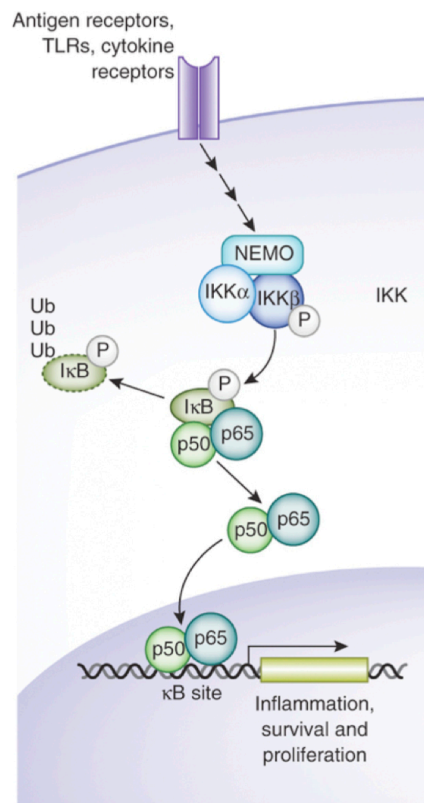

Figure 4. The canonical NF-κB signalling pathway. Adapted from [18].

Activation of NF-κB signalling is initiated by extracellular stimuli (Figure 4). These stimuli are recognised by receptors and transmitted into the cell, where adaptor-signalling proteins initiate a signalling cascade that culminates in the activation of IκB kinase (IKK). IKK is a multi-protein complex composed of two catalytic components, IKKα and IKKβ, and a regulatory component, NEMO. IKK phosphorylates the inhibitory subunit of the NF-κB-IκB complex, which is sequestered in the cytoplasm as an inactive form during non-stimulatory conditions. This phosphorylation marks IκB for proteasomal degradation and releases NF-κB from the inhibitory complex. The freed NF-κB proteins are then transported into the nucleus where, by binding to specific DNA sequences, they promote the transcription of target genes. These genes are involved in innate and adaptive immunity, inflammation, anti-apoptosis, proliferation, the stress response and cancer progression [17].

1.2.1.2. Nod-like receptors and the Inflammasome

The inflammasome is considered an immune sensor and part of a signalling pathway that integrates metabolism and inflammation, exerting pro-inflammatory functions not only locally but also on systemic metabolic parameters. Inflammasome activation has been implicated in a wide variety of metabolic processes (Figure 5), including hepatocyte function, the development of atherosclerotic lesions, inflammation and β-cell death in the pancreas, and adipose tissue homeostasis [19].

The inflammasome is a cytosol-localised protein complex of the host innate immune system that is composed of Nod-like receptor protein 3 (NLRP3), apoptosis associated speck-like protein (ASC) and caspase-1, which mediate the maturation process and secretion of the cytokines interleukin (IL)-1β and IL18 [20]. The inflammasome can sense a wide variety of danger signals, including pathogen-derived molecular patterns, noxious foreign substances and host-derived endogenous signals (Figure 6).

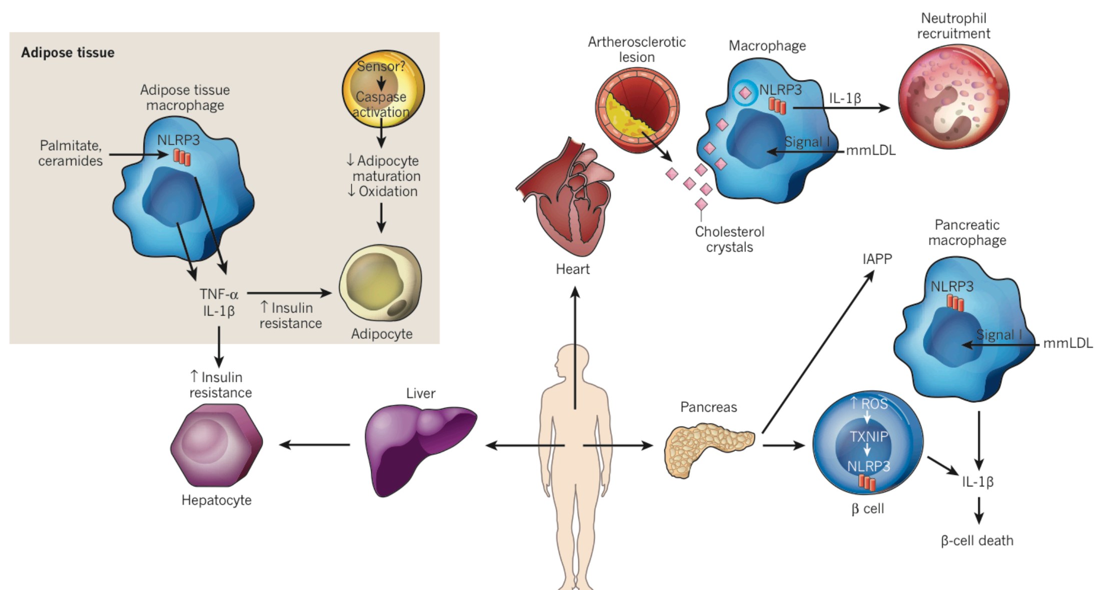

Figure 5. The role of inflammasomes in metabolic syndrome. Adapted from [19].

IL-1β is a multifunctional and pivotal inflammatory cytokine that is known as an endogenous pyrogen. It is mainly synthesised by monocytes and macrophages and is capable of affecting almost all cell types, including acting as a lymphocyte-activating factor through stimulation of T-cell proliferation [21]. IL-1β has local effects (leukocyte infiltration and lymphocyte activation) and distant effects (fever and acute phase protein induction) by binding to IL-1 receptors (IL1Rs) and IL1R AcP (accessory protein) on immune cells. Upon IL1R activation, MAPK and NF-κB pathways can amplify the inflammatory response by promoting the secretion of cytokines and chemokines that may activate the adaptive immune response [22].

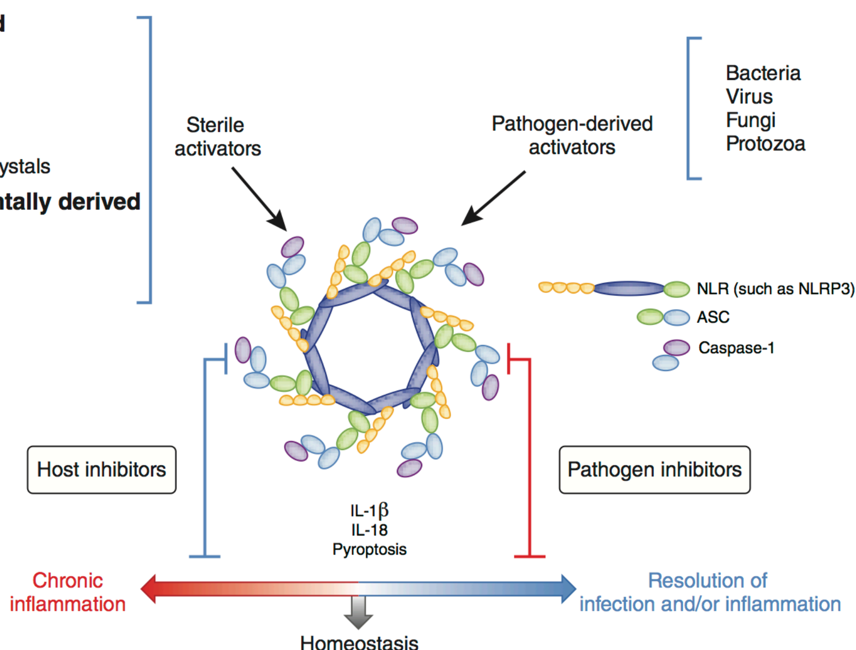

Figure 6. Inflammasome activity regulates homeostatic processes and inflammation. Adapted from [23].

The inflammasome-mediated maturation and secretion of IL-1β is the result of a two-step process (Figure 7). A first signal, known as the priming signal, is responsible for priming the cell, via TLR4 downstream signal transduction, to induce the activation of NF-κB. This mediates the transcription of the NLRP3 and the immature form of IL-1β, pro-IL-1β, which are both indispensable factors for inflammasome activation. Then, a second stimulus, or the activating signal, promotes NLRP3 inflammasome assembly into a platform that results in the processing of pro-caspase-1 into active caspase-1. Caspase-1 mediates the cleavage of pro-IL-1β into the secreted cytokines IL-1β and IL-18.

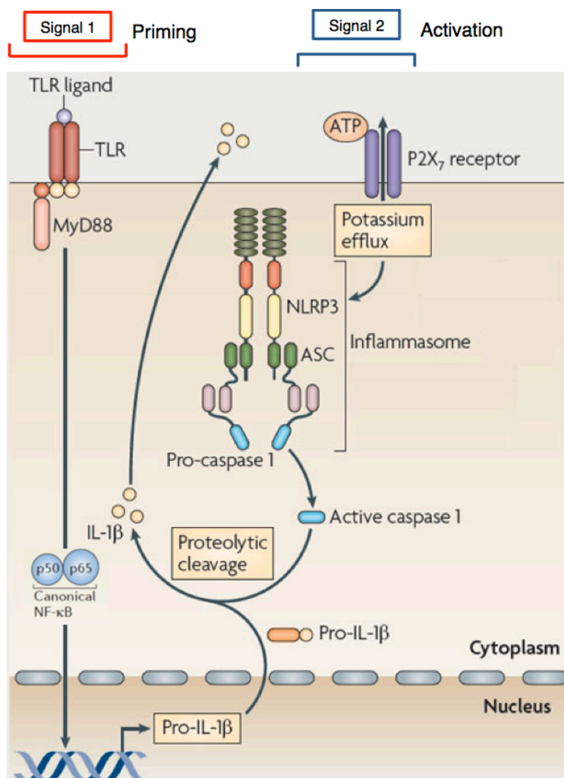

Figure 7. NLRP3 inflammasome-mediated maturation and secretion of IL-1β. Adapted from [24].

1.2.1.3. Prostaglandin synthesis and metabolism: role of cyclooxygenases

Lipids can be metabolised through a variety of lipid biosynthetic pathways, such as the eicosanoid-signalling pathway, that connect inflammation and lipid metabolism. Eicosanoids can be divided into four subclasses including the prostaglandins, thromboxanes, leukotrienes and other oxidised products.

Prostaglandins are generated from n-6 arachidonic acid, the most abundant fatty acid present in cell membranes, through the sequential actions of phospholipase A2 (PLA_2) and cyclooxygenase enzymes (COX) (Figure 8). Both enzymes are controlled directly by signalling pathways that respond to PAMPs and inflammatory cytokines, enabling rapid and robust increases in prostaglandin generation. The resulting prostaglandin metabolites have been involved in different biological roles, including the amplification of inflammatory responses [25]. Three COX isoforms have been described, COX1 (or prostaglandin endoperoxidase 1), COX2 (or prostaglandin endoperoxidase 2) and COX3 (or prostaglandin endoperoxidase 3). COX1 is constitutively expressed in most cells, as it is a housekeeping enzyme involved in the mediation of different physiological roles, including the protection of the gastric epithelium and platelet aggregation. COX2 is undetectable in basal conditions, although it can be rapidly activated by several pro-inflammatory stimuli, leading to the

synthesis of prostaglandin E_2 (PGE_2). COX3 is a splice variant of COX1, however, its biological role is poorly understood [26,27]. Remarkably, the COX1 and COX2 isoforms are the major targets of NSAIDs (non-steroidal anti-inflammatory drugs).

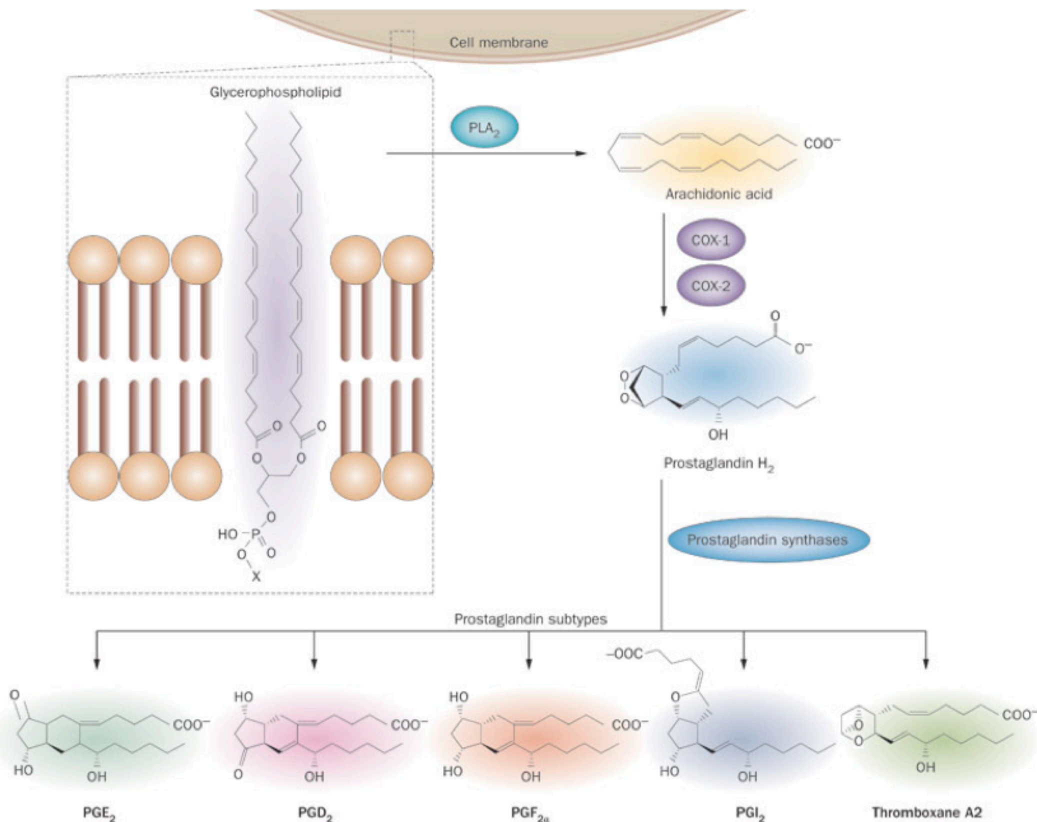

Figure 8. Cyclooxygenase (COX) mediated biosynthesis of prostaglandins (PGs), including PGE_2, PGD_2, $PGF_2\alpha$, PGI_2 and thromboxane A_2 (TxA_2). Adapted from [28].

Additionally, two different series of eicosanoids can be generated, E_2 and E_3, depending on the chemical nature of the fatty acid converted. Whereas utilisation of n-6 results in the E_2 series of eicosanoids, eicosanoid generation from n-3 fatty acids leads to the E_3 series of eicosanoids [29].

1.2.2. Immune cells

For appropriate functioning of the immune system, cell-mediated immunity must be highly regulated and sequentially and temporally orchestrated. Both innate and adaptive immune responses are orchestrated by the complex interactions and activities of a diverse group of cell types, where leukocytes or white blood cells are the most important.

Leukocytes mature in primary lymphoid organs and circulate in the blood within the pool of immune cells, known as peripheral blood mononuclear cells (PBMCs), and interact with

other cells, pathogens and pathogen-derived products. The presentation of host and foreign antigens in secondary lymphoid organs and other tissues is a pivotal event in the induction of adaptive immune responses and the development of immunological memory. The bone marrow and thymus are primary lymphoid organs, whereas spleen and lymph nodes are secondary lymphoid organs. Most leukocytes are generated from committed hematopoietic stem cells located in the bone marrow that give rise myeloid cells, platelets, erythrocytes and lymphoid stem cells. Myeloid cells are derived from a granulocyte-monocyte common precursor, a process mediated by the expression of diverse cytokines, including the granulocyte colony-stimulating factors (GCSF) and macrophage colony-stimulating factors (MCSF). Lymphoid stem cells are the precursors of T and B lymphocytes. However, whereas T cell precursors undergo selection and mature into the repertoire of T cells within the thymus, B cells mature in the bone marrow and in the spleen [30]. In addition, whereas phagocytic cells are involved in innate immunity, the adaptive immune response includes antigen-specific defence mechanisms mediated mainly by lymphocytes.

PBMCs, as cells that circulate in the bloodstream, are continuously exposed to both exogenous and endogenous stimuli from tissues throughout the body, including nutrients and metabolism-derived hormones. In this sense, PBMCs are of high interest because modulation of gene expression in PBMCs has been suggested to reflect the changes of gene expression in many of the solid tissues, including liver, adipose tissue and muscle [31,32].

1.2.2.1. The mononuclear phagocytic system: monocytes and macrophages

The mononuclear phagocytic system represents a subgroup of leukocytes defined as myeloid cells derived from the bone marrow. Macrophage precursors are released into the bloodstream as monocytes, and within a few days they populate tissues during both the steady state and inflammation.

Monocytes are considered sentinel immune effector cells that can detect and respond to invading pathogens and dietary components. Upon chemokine secretion, tissue injury, or pathogen signalling, monocytes are recruited to the affected area and diapedese across the blood vessel wall into the inflamed tissue. Once within the tissue, monocytes differentiate into macrophages or dendritic cells (DCs) under the instruction of a wide variety of proteins, including cytokines, matrix proteins, and growth factors [30,33,34]. Thus, the primary role of monocytes is to replenish the pool of tissue-resident macrophages and DCs in both healthy conditions and in response to inflammation. Monocytes, DCs and macrophages, along with neutrophils and mast cells, are the ‘professional’ phagocytic cells due to the expression of a multitude of receptors on their surfaces that detect exogenous or endogenous signals which are not normally found in healthy tissues [35]. Within the mononuclear phagocytic pool, macrophages are often distinguished form DCs by the differential expression of surface markers, such as the macrophage marker F4/80 [36].

Tissue macrophages are involved in important functions for homeostasis. As resident phagocytic cells in lymphoid and non-lymphoid tissue, they monitor constantly the immediate milieu for early signs of infection or tissue damage. In this role, macrophages are responsible for sensing, integrating and appropriately responding to a sizeable array of stimuli from the local environment [35,37]. Macrophages express a broad range of pathogen-recognition receptors, including TLRs, NLRs and the interleukin-1 receptor (IL-1R), that make them efficient at phagocytosis and the production of inflammatory cytokines.

Macrophages have remarkable plasticity that allows them to efficiently respond to environmental signals and change their phenotype, and their physiology allows them to encompass both innate and adaptive immune responses [38]. Macrophages can be divided into different subpopulations based on their anatomical location and functional phenotype (Figure 9) [39]. Included within the specialised tissue-resident macrophages are osteoclasts (bone), alveolar macrophages (lung), histiocytes (interstitial connective tissue) and Kuppfer cells (liver). Secondary lymphoid organs also have distinct populations of macrophages that perform unique functions, including marginal zone macrophages in the spleen, which are involved in the suppression of innate and adaptive immunity against apoptotic cells [40].

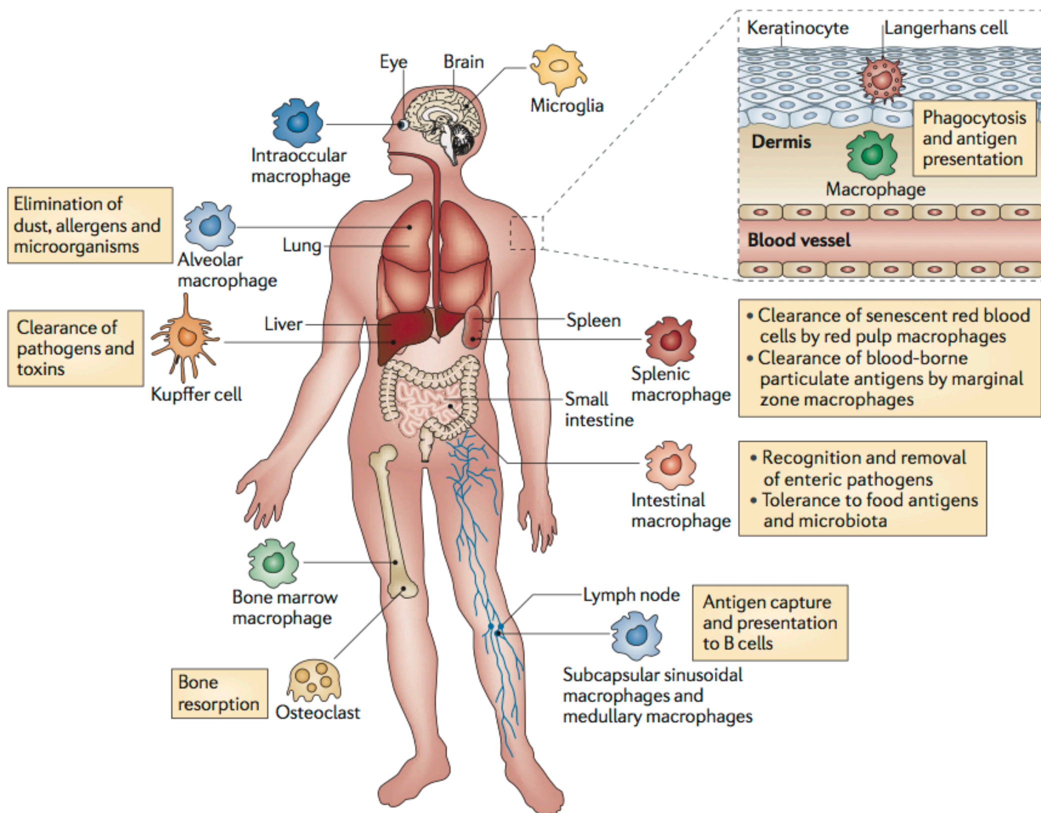

Figure 9. Macrophages are located throughout the body and perform important surveillance activities such as phagocytosis, antigen presentation and immune suppression. Extracted from [35].

Different macrophages subsets with distinct functions have been described; classically activated macrophages (M1 macrophages) mediate defence of the host against a variety of bacteria, protozoa and viruses and also have roles in antitumor immunity. Alternatively activated macrophages (M2 macrophages) have an anti-inflammatory function and regulate wound healing. Numerous studies have documented flexibility in macrophage programming, where macrophages switch from one functional phenotype to another in response to the variable microenvironmental signals of the local milieu [41,42].

1.2.2.2. Cell-mediated adaptive immunity: lymphocytes

Lymphocytes are key cellular components of the adaptive immune system. The bone marrow-generated lymphocytes mature in lymphoid organs and circulate through cardiovascular and lymphatic circulatory systems to peripheral tissues of specialised secondary lymphoid organs, such as the lymph nodes and the spleen (Figure 10). Antigen-mediated triggering of T and B cells, the main lymphocyte subsets, initiates specific cell-mediated and humoral adaptive immune responses.

The adaptive immune system relies on the specificity of antigen recognition. Lymphocytes have a wide range of antigen receptors, known as the TCR and BRC (T and B cells receptors, respectively), which are generated by somatic recombination.

T cells are categorised into two principal subsets, the CD4^{+} T cells and the CD8^{+} T cells. Naïve CD4^{+} and CD8^{+} T cells result from thymus maturation and must come in contact with antigen-presenting cells (APCs) to become activated. In this way, the interaction between a TCR and its peptide-major histocompatibility complex (MHC) ligand plays a critical role in determining the activity and specificity of the T cell. MHC class I present peptides to the TCR on CD8^{+} T cells, whereas MHC II engages with the TCR on CD4^{+} T cells [43].

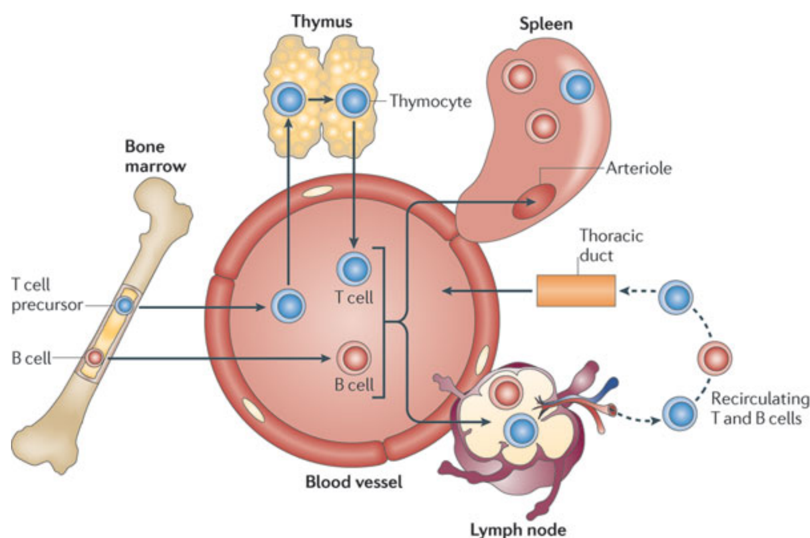

Figure 10. Generation, differentiation and peripheral circulation of T (blue) and B (red) lymphocytes. Adapted from [44].

$CD4^+$ T cells play a central role in immune protection by their capacity to help B cells to produce antibodies; to induce macrophages to develop enhanced microbial activity; to recruit neutrophils eosinophils and basophils to sites of inflammation; and through their production of cytokines and chemokines that orchestrate the spectrum of immune responses. Moreover, $CD4^+$ T cells act as T helper (T_H) cells and, after being activated, can differentiate into distinct effector fates, including T_H1, T_H2, T_H17 and regulatory T (Treg) cells, which differ from each other in their pattern of cytokine production and related functions, their distinctive expression of cell surface receptors and their characteristic transcription factors [45]. T_H1 produce pro-inflammatory cytokines such as IFN-γ to support cell-mediated immunity, whereas T_H2 cells produce anti-inflammatory cytokines, including IL-4 to support humoral immunity. Furthermore, T_H17 cells are characterised by expression of IL-17 and play a critical function in protection against microbial challenges, particularly extracellular bacteria and fungi. Treg are characterised by the expression of the forkhead-winged-helix transcription factor 3 (Foxp3), can secrete anti-inflammatory signals, inhibit macrophage migration and induce M2-like macrophage differentiation [46].

Upon engagement with antigen, naïve $CD8^+$ T cells rapidly expand and differentiate into effector $CD8^+$ T cells, also referred as cytotoxic T cells (Tc), which produce cytokines, including IFN-γ, and effector molecules that mediate a local inflammatory response and effect target cell apoptosis [47].

On the other hand, B cells are central to humoral immunity and for the maintenance of immune homeostasis. B cells can modulate immunity by releasing immunomodulatory cytokines that influence a variety of T cell, dendritic cell and antigen presenting cell functions, and regulate lymphoid tissue organisation and neogenesis. B cells can also function as polarised cytokine-producing effector cells that influence T-cell differentiation [48].

1.2.3. Lymphoid organs

1.2.3.1. Thymus

The thymus has a central role for the immune system. As a primary lymphoid organ, the thymus is crucially required for T cell differentiation and repertoire selection. Bone marrow-generated progenitor T lymphocytes circulate within the thymus where they will undergo maturation by progression through several phenotypic stages (the T cell maturation process is described in Figure 11). T cell development involves an obligatory interaction of the TCR of immature thymocytes with MHC-peptide complexes. Immature thymocytes become first double negative (DN) cells and then double positive (DP). Finally, mature lymphocytes are single positive (SP) and are selected based on two main phenotypic categories: $CD4^+$ and $CD8^+$ T cells [44,49].

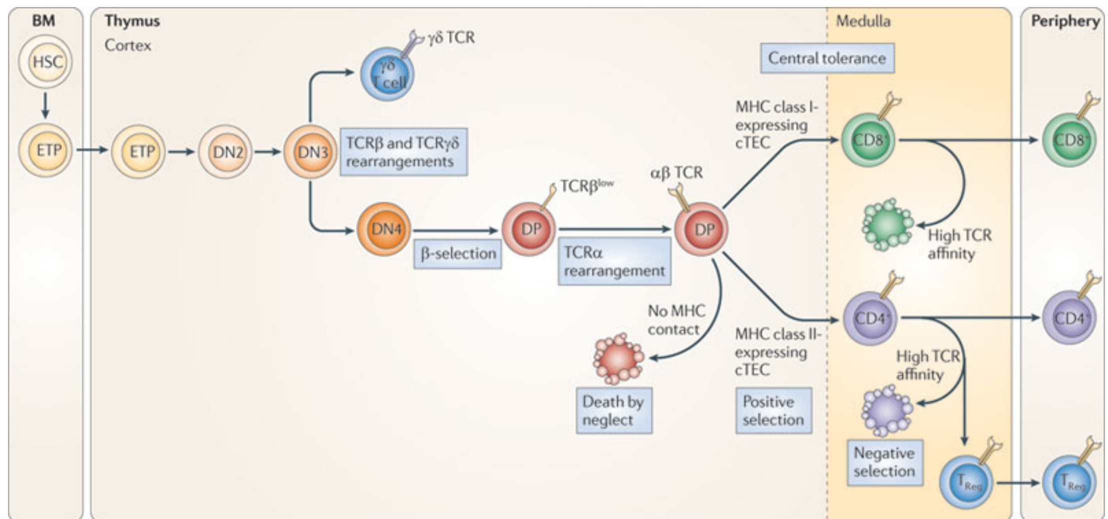

Figure 11. Major events in T cell differentiation in the thymus. Haematopoietic stem cells (HSCs) from the bone marrow give rise to early thymic progenitors (ETPs). First, ETP cells become double negative (DN) cells, which undergo crucial rearrangements of TCR genes. TCRβ gene selection leads to the generation of double positive ($CD4^{+}CD8^{+}$ or DP) cells. This is followed by the rearrangement of the TCR α-chain locus and expression of the αβ TCR. Then, by positive selection (PS), cells that have TCRs that bind to MHC class I molecules retain expression of CD8, whereas the cells that bind to MHC class II molecules retain CD4. A small percentage of the $CD4^{+}$ cells up-regulate the transcription factor FOXP3 and exert regulatory T (T_{Reg}) cell functions. γδ T cells originate from DN cells that have not yet undergone β-selection. Extracted from [44].

1.2.3.2. Spleen

The spleen, the largest secondary lymphoid organ in the body, besides filtering the blood to remove and destroy the non-functional erythrocytes and other blood cells, plays a pivotal role for the host immune system. The spleen is a key location for phagocytosis, the storage reservoir for immature monocytes, as well as a key place for T cell and B cell mediated immunity. The spleen also integrates the immune system, metabolism and the endocrine network [50].

1.3. Metabolism

Metabolism is involved in the regulation of food intake, nutrient uptake, catabolic and anabolic pathways, the distribution of nutrients, and the accumulation and mobilisation of stored energy within the body.

Obesity can be defined as a consequence of the maladaptation to the current dietary pattern, which is rich in energy density mainly due to the high content of fats and sugars [51]. As a result of this lifestyle, circulating free fatty acids (FFAs) are elevated, hindering glucose oxidation and interfering with the response to insulin. The incidence of obesity and its related metabolic disorders have increased dramatically throughout the last quarter

century, representing the greatest public health challenge of our time. The rise of worldwide obesity has resulted in the explosion of obesity-related health problems, including type 2 diabetes, coronary arterial disease, fatty liver disease and some cancers and degenerative diseases.

1.3.1. The adipose tissue as an immunological organ

Adipose tissue is a metabolically dynamic organ that, besides being the primary site of storage for an excess of energy, acts as an endocrine organ capable of synthesising a number of biologically active compounds, including a wide variety of hormones, cytokines, chemokines and growth factors, that regulate metabolic homeostasis [52–54]. The adipose tissue is composed not only of adipocytes but also of other cell types called the stromal-vascular fraction, comprised of blood cells, endothelial cells, macrophages and lymphocytes.

1.3.1.1. The role of immune cells in adipose tissue

Cell-mediated immunity plays a pivotal role in the orchestration of the metabolic response to obesity. Indeed, obesity is associated with an increased number of leukocytes within adipose tissue and adipocytes within the lymphoid microenvironment, both of which are considered part of the adaptive response to the impairment of energy balance. Therefore, an impaired immune-metabolic cell crosstalk underlies the development of obesity-related pathologies.

Adipose tissue accumulates the excess of energy intake as triglycerides, but the continuous overflow of adipocyte-storage capacities endangers its functionality causing hypertrophy, hypoxia and acidosis. As obesity develops, qualitative changes in the expanding adipose tissue can promote the transition from normal metabolic function to a metabolically dysfunctional phenotype. Thereby, compromised adipocytes trigger an adipocytokine-driven response characterised by the secretion of pro-inflammatory chemokines resulting in an obesity-induced local inflammatory response. These factors favour the apoptosis of adipocytes and cause the recruitment and infiltration of immune cells within the adipose tissue, which underlies the amplification of the inflammatory response up to systemic levels and disturbs the immunometabolic homeostasis [55].

Adipose tissue-associated macrophages switch from an M2-like phenotype to a classically activated M1-like phenotype with potent pro-inflammatory activity, with the NLRP3 inflammasome serving as the molecular switch by sensing obesity-associated danger signals [56]. Therefore, metabolically dysfunctional adipose tissue can be associated with high levels of necrotic adipocytes, and the M1 macrophages arrange around these dead cells forming crown-like structures to phagocytose the released lipids (Figure 12). Indeed, the recruitment of M1 activated macrophages into the adipose tissue coincides in time with the appearance of necrotic adipocytes and the onset of insulin resistance.

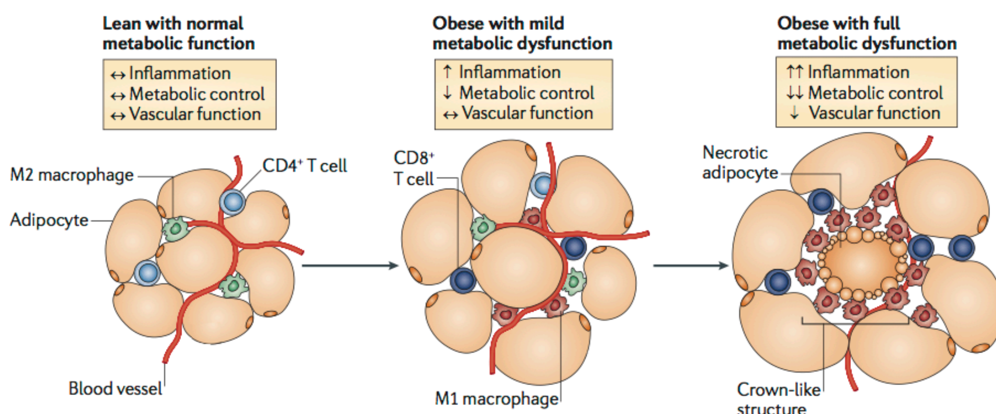

Figure 12. Obesity-induced structural and functional changes of adipose tissue. Adapted from [57].

Lean adipose tissue in mainly enriched by Tregs and M2 activated macrophages, which secrete IL-10 to enhance insulin action and glucose disposal in adipocytes. The M2 macrophage phenotype is regulated by peroxisome-activated receptor-γ (PPAR-γ) signalling and has been found to regulate important metabolic functions, including the maintenance of adipocyte function, insulin sensitivity and glucose tolerance, preventing the development of diet-induced obesity and type 2 diabetes [37,58].

Nevertheless, during obesity energy storage increases proportionally to the increase of nutrient intake, reaching adipocyte maximal storage capacity and undergoing necrotic cell death, which promotes macrophages recruitment. With the persistence of increased nutrient intake, more adipocytes undergo cell death, thereby providing the antigenic stimulus for macrophages to activate the adaptive immune system. Adipose tissue M1 macrophages are potentially capable of presenting antigens to B and T cells to promote the adaptive immune response. This is postulated to promote clonal expansion of $CD4^+$ T helper 1 (T_H1) cells and an increased infiltration by $CD8^+$ T cells. In a feed-forward loop, interferon-γ (IFNγ) production by $CD4^+$ T_H1 and the secretion of pro-inflammatory cytokines and chemotactic factors by $CD8^+$ T cells results in increased recruitment and classical activation of macrophages. Concomitant with this, the obesity-induced decrease of immunosuppressive Tregs cell numbers in adipose tissue further contributes to adipose tissue inflammation and insulin resistance. B cells, which infiltrate obese adipose tissue, can present antigens on MHC class I and II molecules to naïve T cells. IgG2 autoantibodies produced by mature B cells further amplify adipose tissue inflammation and insulin resistance (Figure 13) [58].

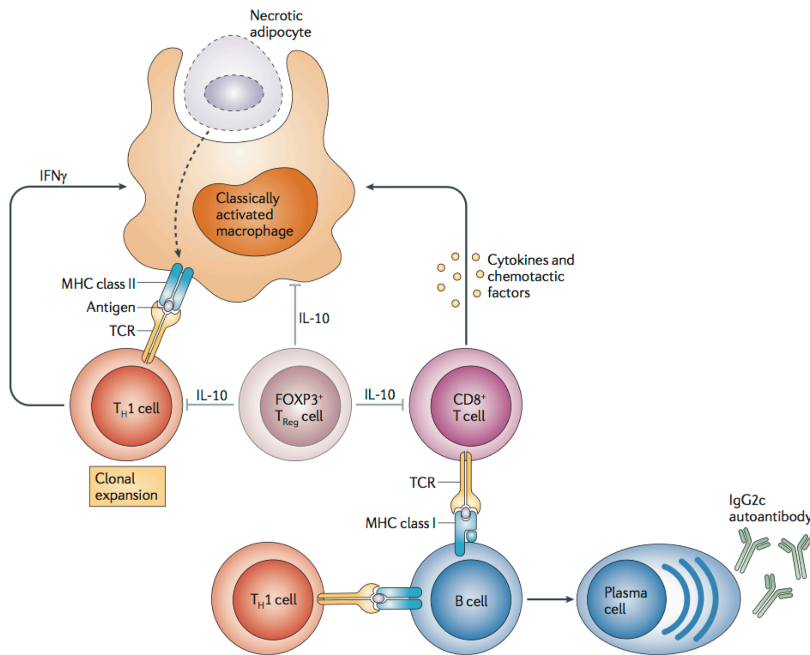

Figure 13. Crosstalk between innate and adaptive immune cells in adipose tissue. Extracted from [58].

1.3.2. Immunometabolism and obesity: the non-resolved inflammation

Changes in dietary patterns as well in lifestyle underlie the exposure of the immunometabolic axis to a constant excess of nutrients and energy, threatening the delicate balance between immunity and metabolism, even underlying some pathologies.

Normally, in response to a fixed insult, classical inflammation mediates its removal or neutralisation and the inflammation is finally resolved. However, the inflammatory response found in the obese state is of a different nature and is characterised by an array of hallmarks (Figure 14) that differentiate it from the classical inflammatory response [59].

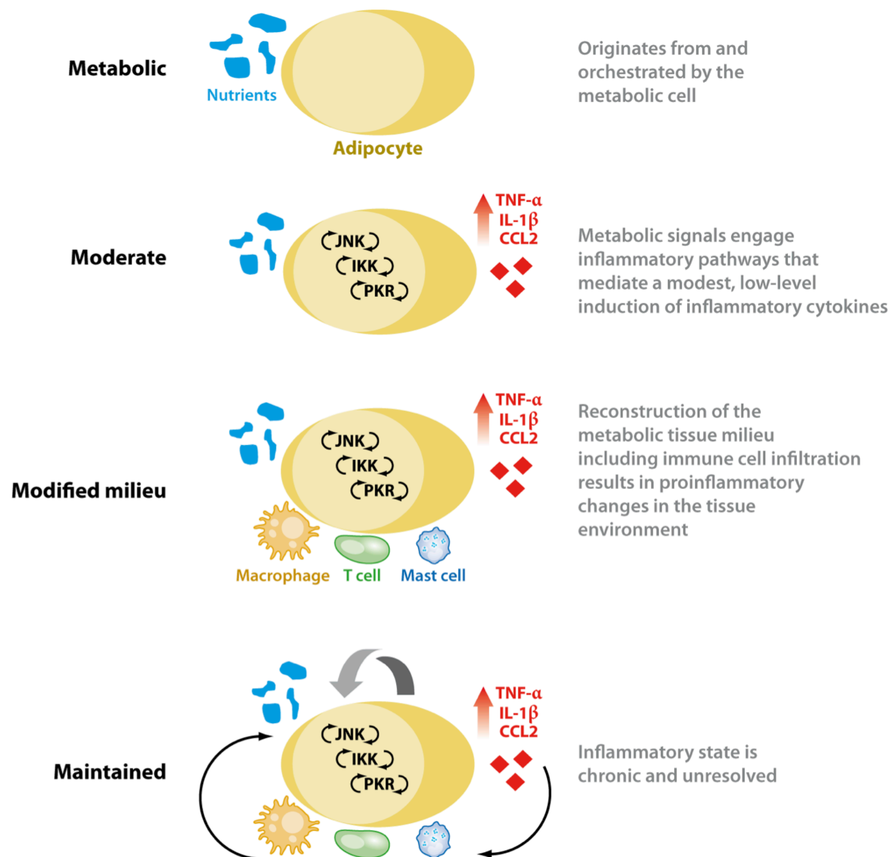

Figure 14. Hallmarks of metaflammation [60].

I. Obesity-induced inflammation is triggered by nutrients and orchestrated by metabolic cells.

The inflammatory trigger in obesity is metabolic, and inflammation is mainly induced as consequence of an energy imbalance promoted by an excess of nutrient consumption. Thereby, not only is metabolism the nature of the inducing stimuli but also metabolic cells, such as adipocytes, are the cells that sustain the insult and whose response begins the inflammatory cascade, thus mediating the interface between the metabolic input and the inflammatory output. Therefore, metabolic signals emerging from metabolic cells trigger the inflammatory response and impair metabolic homeostasis.

II. The inflammatory response is moderate, there is a low-grade and local expression of inflammatory mediators induced by stress sensors.

The first evidence regarding the implication of inflammatory pathways in metabolic diseases was based on the expression of a pro-inflammatory cytokine, TNF-α, in the adipose tissue, and in adipocytes themselves, of obese rodents and humans [61]. It is now considered that not only TNF-α but also a wide array of inflammatory cytokines are

increased in obese tissue, including IL-6, IL-1β and monocyte chemotactic protein-1 (MCP-1) [9,62]. In addition, while adipose tissue is a predominant site for cytokine expression in obesity, other metabolic tissues such as the liver [63], muscle [64], pancreas [65], and brain [66], have been also postulated as targets of the obesity-induced inflammatory response [60]. Furthermore, obesity-induced inflammation increases the systemic levels of cytokines, acute-phase reactants and adhesion molecules [67].

III. The milieu is modified, altering the composition of immune cells to favour a pro-inflammatory tissue environment.

Obesity-induced metabolic tissue inflammation is associated with an increased number of infiltrated immune cells that contribute to increased tissue cytokine expression. The state of activation of the adipose tissue resident macrophage population, pro-inflammatory (M1) or anti-inflammatory (M2), is determined by metabolic factors. Although multiple types of immune cells are involved in the response to the metabolic overload of obesity, particularly in the adipose tissue, the role of immune cells in metaflammation are unclear.

IV. Metabolic inflammation induced by obesity is chronic.

The pulsatile nature of the trigger stimulus is one of the most important aspects to understanding the intricacies of obesity-induced inflammation (Figure 15). In obesity, as a consequence of the fasting-feeding cycle a pulsatile inflammatory response is chronically induced in metabolic cells. Under homeostatic conditions, low-level inflammatory peaks resulting from feeding are resolved once the nutrients are metabolised. However, in overfeeding or obesity conditions, a constant stimulus from nutrient intake results in a more consistently active inflammatory response. Over time, these low-level signals accumulate and amplify with each nutrient exposure, begin to impair normal metabolic pathways and cause the activation of immune cells.

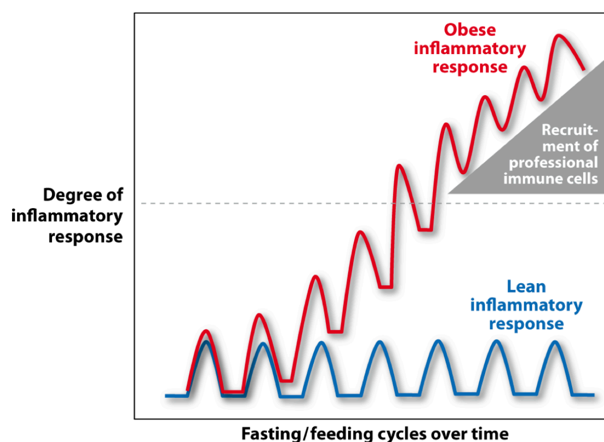

Figure 15. Pulsatile nature of diet-induced inflammatory responses. Extracted from [60].

The occurrences of inflammatory cytokine expression and immune cell infiltration associated with the onset of the metabolic inflammation appear to happen gradually but are unresolved over time. This deficiency of resolution could be associated with the new emergence of obesity as pathology and the lack of evolutionary selection to develop such a response to metabolic signals.

1.3.3. Leptin links the crossroad between immunity and metabolism

Leptin, the product of the obese (*ob* or *Lep*) gene, is an adipocyte-derived cytokine that belongs to the family of long-chain helical cytokines and is structurally similar to IL-6. The leptin signalling pathway is one of the most important networks that senses and manages nutrients, playing a key role in linking the nutritional status with neuroendocrine immune functions, influencing directly the physiological and pathological metabolic states in the body [68]. Once leptin is secreted into the bloodstream it has central and peripheral effects. In the hypothalamus, leptin regulates appetite, basal metabolism, autonomic nervous system outflow and bone mass [69]. In the periphery, leptin increases basal metabolism, influences reproductive function, regulates pancreatic β-cell function and insulin secretion, is pro-angiogenic for epithelial cells, regulates bone marrow haematopoiesis, and affects thymic generation of T cells and the differentiation of T_H1 cells in lymph nodes (Figure 16).

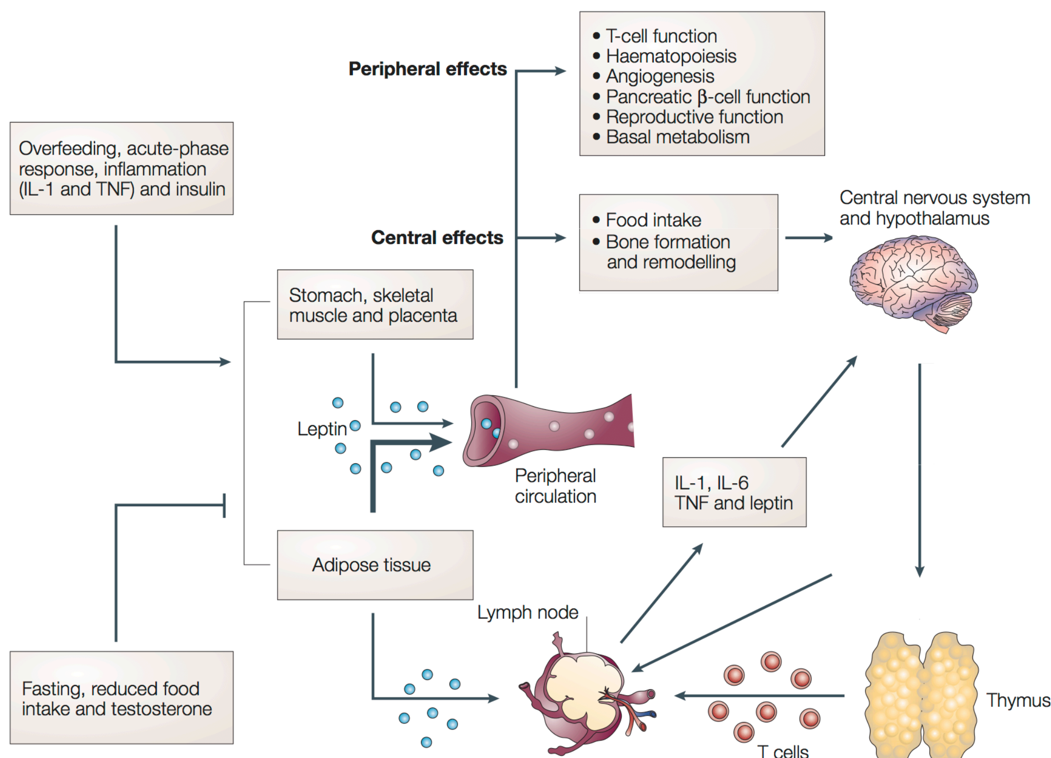

Figure 16. Central and peripheral effects of leptin. Extracted from [68].

In addition, leptin modulates both the innate and adaptive immune systems. In innate immunity, leptin promotes the activation of monocytes and macrophages [70,71]. In PBMCs, leptin increases the production of growth hormone, a cytokine-like hormone that influences the immune response by controlling the survival and proliferation of immune cells [72]. The adaptive immune response is also modulated by leptin, which modulates the proliferation and cytokine production of $CD4^{+}$ T cells. Leptin induces the production of adhesion molecules, such as intercellular adhesion molecule 1 (ICAM1) and very late antigen 2 (VLA2), which are responsible for the induction of clustering, activation and migration of immune cells to sites of inflammation. Interestingly, thymic homeostasis is also affected by leptin, including the generation, maturation and survival of thymic T cells. In memory T cells, leptin promotes switching towards T_H1-cell immune responses by increasing IFN-γ and TNF-α secretion, production of IgG2a by B cells and delayed-type hypertensive (DTH) responses. Furthermore, leptin has anti-apoptotic effects on mature T cells and on haematopoietic precursors (Figure 17).

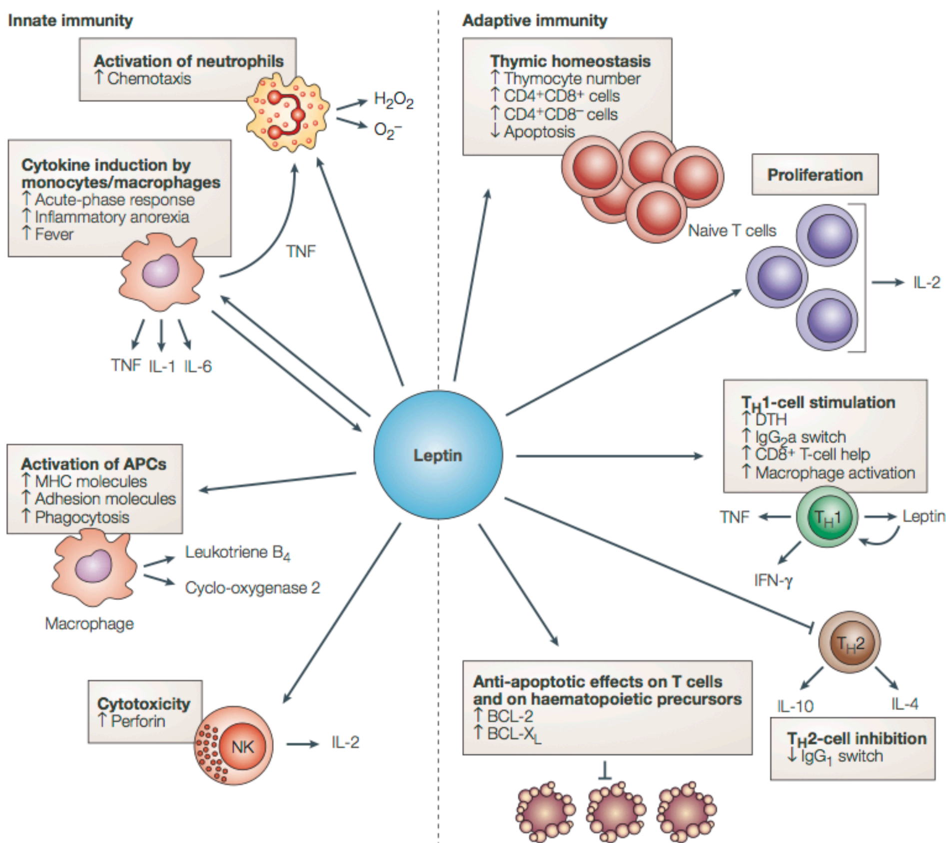

Figure 17. Effects of leptin on innate and adaptive immune responses. Extracted from [68].

1.3.3.1. Leptin receptor signalling

The leptin receptor, which plays a pivotal role in the transduction of leptin signalling, is a member of the class I cytokine receptor (gp130) superfamily and has at least six splice forms as a result of alternative splicing, with cytoplasmic domains of different lengths (LepRa-LepRf). The short forms of the leptin receptors are expressed by several non-immune tissues and are involved in the transport and degradation of leptin. The long form of the leptin receptor, leptin receptor B (LepRb), is the only variant with full signalling capability and is expressed by the hypothalamus as well as by immune cells, including lymphocytes, monocytes and macrophages, and is fundamental for the transduction of the intracellular leptin signalling pathway [73].

Therefore, upon leptin binding to its extracellular domain, LepRb undergoes a conformational change that activates Janus-family tyrosine kinase 2 (JAK2) (Figure 18). JAK2 becomes activated by cross-phosphorylation, and tyrosine phosphorylates the cytoplasmic domain of the receptor. Four of the phosphorylated tyrosine residues (Tyr_{974}, Tyr_{985}, Tyr_{1077} and Tyr_{1138}) function as docking sites for cytoplasmic adaptors.

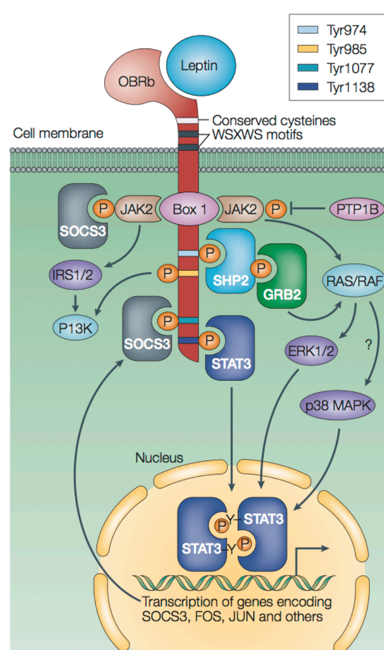

Figure 18. Signalling pathways activated by the leptin receptor. Extracted from [68].

The phosphorylation of the distal membrane Tyr_{1138} serves as a docking site for the signal transducer and activator of transcription 3 (STAT3), which is a substrate of JAK2. STAT3 is a latent transcription factor that, after subsequent recruitment, is phosphorylated by JAK2 and dimerises, leading to nuclear translocation and inducing the expression of suppressor of cytokine signalling 3 (SOCS3) and other genes. SOCS3 takes part in a feedback loop that inhibits leptin signalling by binding to phosphorylated tyrosine (Tyr_{1077}) [74–76]. Similarly,

Tyr_{1077} recruits and mediates the phosphorylation of a related transcription factor, STAT5 [77]. Tyr_{985} and Tyr_{974} in turn recruit the SRC homology 2 (SH2) domain-containing phosphatase 2 (SHP2) and activate extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) pathways through the adaptor protein growth factor receptor-bound protein 2 (GRB2), ultimately inducing the expression of FOS and JUN [78]. In addition, after leptin binding, JAK2 can induce phosphorylation of the insulin receptor substrate (IRS) proteins, which are responsible for the activation of phosphatidylinositol 3-kinase (PI3K) [79]. In contrast, the phosphotyrosine phosphatase 1B (PTP1B) is involved in negative regulation of LepRb signalling through the dephosphorylation of JAK2.

However, the transduction of leptin signalling can be attenuated in consequence of homeostatic alterations such as those that happen in obesity. This event is known as leptin resistance and underlies the pathogenesis of several immunometabolic disorders.

1.3.3.2. Leptin resistance and obesity

Obesity leads to the development of leptin resistance through the attenuation of hypothalamic LepRb signal transduction. Two potential mechanisms have been hypothesised to explain the failure of circulating leptin to reach its targets in the brain and inhibit intracellular LepRb signalling [80]. These mechanisms include an impaired leptin transport across the blood-brain barrier as well as the inability of leptin to activate hypothalamic-signalling, a consequence of the presence of intracellular inhibitors such as SOCS3, PTP1B and SHP-2 [81–84].

Leptin can access the brain by different mechanisms, including a specific transport across the blood-brain barrier, diffusion from the circumventricular organs or direct access from the blood to neuroendocrine neurons that project to the circulation [76]. These leptin transport mechanisms can be saturated. In fact, it has been described that in diet-induced obesity leptin transport to the brain is decreased [85]. In addition, a physical interaction between leptin and CRP, an acute-phase protein overexpressed in obesity, may also impede the transmission of leptin across the blood-brain barrier [86,87].

Moreover, specific molecular inhibitors can also down-regulate hypothalamic leptin signalling. In a physiological state, when circulating leptin concentrations are low, STAT3 activation and the expression of SOCS3 are modest. In this basal condition, an increase of leptin secretion is almost fully translated into increased central LepRb signal transduction. However, in obesity, where leptin is continuously overexpressed, the activation of STAT3 is promoted, which results in an increase in SOCS3 expression. Thus, chronic high levels of LepRb activation induce its own feedback inhibition, limiting the downstream leptin signalling pathway [76,88].

1.4. Nutritional profile

Foods are more than just a combination of nutrients intended to cover the energetic and metabolic requirements, but foods are also a complex network of molecular signals able to modulate the functionality of the body. Thus, the effects of nutrition on health and disease cannot be understood without the knowledge of how nutrients act at the molecular level [89].

There is strong evidence supporting the fact that the nutritional profile affects both metabolism and the immune system [3,90,91]. The interactions between food composition and the immune-metabolic interface can occur at many levels, ranging from endocrine signalling to the direct sensing of food components by immune cells [90,92].

Thus, a greater understanding of the critical parts of immunometabolism that are susceptible to targeting through the nutrition profile may provide opportunities to break the close connection existing between the defects in metabolism and immunity with the onset and propagation of diseases.

1.4.1. Immunometabolism and nutrient sensing

On one hand, the ability to use macronutrients is essential for the generation and maintenance of a protective effector immune response [90]. On the other, the capacity of the immune system to sense metabolic stress is a pivotal event linked to the progression of obesity and its related pathologies. Therefore, the proper functioning of the pathways that are involved in the sensing and management of nutrients is pivotal for the correct functioning of immunometabolism and the maintenance of homeostasis.

The immune system is able to respond against peripheral signals, including pathogenic and food-derived molecular signals. Indeed, abnormalities of lipid metabolism impair immunometabolic homeostasis. Immune and lipid abnormalities are not completely separate processes, and these two systems are closely intertwined and can feed back to each other. Circulating free fatty acids (FFAs) are typically elevated in diet-induced obesity, and the dietary fat intake has an important influence on the composition of these FFAs [93]. In addition to circulating FFAs, immune cell types in adipose tissue are exposed to high local concentrations of FFAs released from adipocytes by lipolysis. Therefore, these cells exist within a lipid-rich environment that could amplify the effects of fatty acids within adipose tissue [94].

1.4.2. Targeting immunometabolism through nutrition: from a weak to boosted immune system

There is evidence that an inflammatory response may be acutely evoked by nutrients. For example, the administration of an acute dose of lipids was associated with the activation of inflammation in skeletal muscle and liver tissues from mice [95], or after the ingestion of a

high-fat, high-carbohydrate meal in humans, resulted in the secretion of reactive oxygen species (ROS) and NF-κB activation in circulating blood mononuclear leukocytes [96].

In this way, classical innate immune recognition pathways have evolved to survey the nutrient environment. TLR4 can sense the presence of free fatty acids [97], whereas ATP is an important activator of the NLRP3 inflammasome [98]. Several other immune cell-associated sensors couple information about the local nutrient or metabolite environment to the coordination of local immune responses.

Taking then into account the extensive evidence that connects metabolic and immune pathways, added to the fact that metabolism controls function in immune cells, leads to the conclusion that it is possible to modulate immunometabolism through nutrition. Indeed, nutrition has to be considered a two-sided coin: on the one hand, diets based on energy-dense foods or containing high levels of fat or carbohydrates promote an imbalance in energy metabolism as well as induce obesity and its related pathologies. On the other hand, immunometabolism could also be improved through nutrition, based on nutritional immunotherapy; the immunometabolism can be targeted with nutrition for treating immune disorders and related pathologies.

Thereby, immunometabolic homeostasis could be improved through the promotion of healthy eating habits, including the diminution of energy-dense foods and the increase of foods containing immunologically active compounds such as grape seed procyanidin extract (GSPE) or docosahexaenoic acid (DHA).

1.4.2.1. The Western dietary pattern as model of obesity-induced immune impairment: the cafeteria diet

The pandemic increase of obesity is in accordance with the change of the dietary pattern in Western societies, where the prevalence of highly palatable, energy dense foods has exponentially increased. In this sense, the cafeteria diet (CAF) refers to a robust model for human metabolic syndrome induced by mimicking the western dietary pattern [99]. Using this model, animals are allowed free access to standard chow while being concurrently offered highly palatable, energy-dense, unhealthy human foods. Through feeding the cafeteria diet, voluntary hyperphagia is promoted, which results in rapid weight gain as well as in the increase of adiposity.

1.4.2.2. In immunometabolism the type of fat matters: dietary fat and fatty acids

The typical Western-type diet contains around 23% of the total energy intake as fat [100,101]. Fat is present naturally in foods such as red meat, fish, dairy, eggs and oils, and aside from its principal role as energy source, dietary fat also influences many physiological processes. In this way, the fatty acid composition of cell membranes modulates its structure,

fluidity and function [102], as well as the production of eicosanoids and other secondary messengers, the function of receptors and the activity of several enzymes.

In chemical terms, fats are acylglycerols, which are defined as compounds with a fatty acid molecule (acyl group) linked to a glycerol by an ester bond. The broad majority of dietary and storage fats are triglycerides, compounds in which 3 fatty acid molecules are bound to a single glycerol molecule [101]. Fatty acids differ in respect to the length of their chain and in the number and position of double bonds in the chain. Therefore, fatty acids are classified as saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). SFAs are straight chain fatty acids with no double bonds within the carbon chain of variable length, MUFAs are fatty acids with one double bond along its chain and PUFAs contain more than one double bond within the fatty acid chain. Additionally, PUFAs are divided into two biologically important families, the n-6 PUFAs, which have their first double bond located on the sixth carbon atom from the methyl end, and the n-3 PUFAs, which have their first double bond on the third carbon atom [103].

In the 1950's, it was first recognised that dietary fat could contribute to cardiovascular disease in Western cultures. However, the Greenland Eskimos ate large amounts of fat but had a very low incidence of cardiovascular diseases, along with low levels of cholesterol and lipoproteins. Therefore, the analysis of the nutritional profile of the Eskimo diet revealed that they intake high amounts of marine PUFAs, suggesting that the quality of dietary fat rather than quantity is fundamental for the development of cardiovascular diseases. In this way, the long-chain n-3 PUFAs are thought to promote beneficial effects on metabolic functions as a consequence of their anti-inflammatory properties. Immune cells normally contain large quantities of the n-6 PUFA arachidonic acid that, as has been explained previously (see; 1.2.1.3. Prostaglandin synthesis and metabolism: role of cyclooxygenases), can be converted to eicosanoids by cyclooxygenase. Increased dietary intake of long-chain n-3 PUFAs leads to its incorporation into the phospholipids of cells membranes, replacing arachidonic acid and repressing the production of eicosanoids from arachidonic acid. Furthermore, n-3 PUFAs from fish, such as docosahexaenoic acid (DHA), can modulate inflammation by interfering with the activation and transcriptional activity of transcription factors such as the peroxisome proliferator-activated receptors (PPARs) or NF-κB.

1.4.2.3. Procyanidins as bioactive food compounds: improving health through the diet

Foods such as fruits and vegetables contain a largely array of bioactive compounds not among the essential nutrients. Included in these bioactive food compounds are the polyphenols, which are secondary metabolites of plants characterised by at least one aromatic ring and one hydroxyl group. Polyphenols are classified according to their chemical structure. In this way, procyanidins, or flavan-3-ols, belong to the flavan group, which is part of the flavonoid compounds within the polyphenols.

The consideration of polyphenols as bioactive molecules derives from the xenohormesis theory. Xenohormesis (Figure 19) is a hypothesis that some stress-signalling molecules synthesised by plants, such as the polyphenols, might also provide their functional activity to their consumer, also known as interspecies communication.

In this sense, the most basic purpose of the growth of the grape and its seed in the vine, as in the vast majority of plants, is the propagation of its species. Therefore, the grape seed contains the most essential components required for the formation of a new plant, among which are the polyphenols. Polyphenols in plants play important physiological functions, including their function as chemical messengers within the plant and their protective activity. Polyphenols defend the plant against microbes, fungi, insects and UV radiation, chelate potentially harmful metals and act as antioxidants to the free radicals derived from photosynthesis [104]. Furthermore, it has been described that grapes, under nutritionally stressful conditions, produce and accumulate greater amounts of polyphenols.

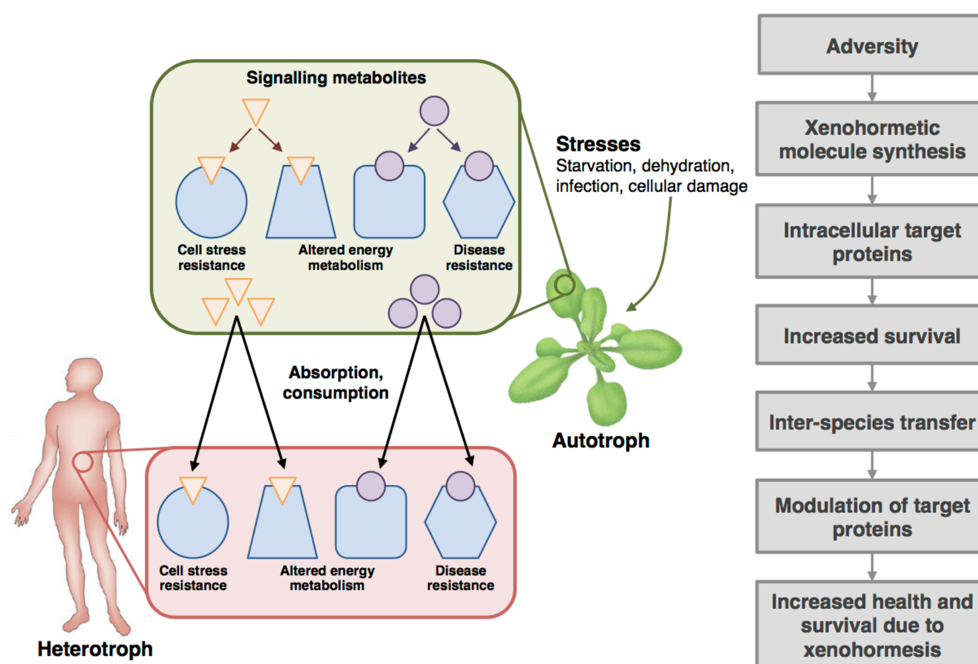

Figure 19. The xenohormesis hypothesis. Adapted from [105].

A compendium about the effects of procyanidins in inflammation, regarding their molecular targets and health implications, is enclosed at the end of this introduction.

1.5. The obese phenotype, a complex interplay between the genotype and the environment

So far, we have noted that the onset and development of obesity is linked to a sedentary lifestyle and high intake of energy-dense and micronutrient-poor foods, such as diets rich in fat or sugar. However, in addition to these environmental factors, genetics also plays a key role in the resulting obese phenotype. The obesity phenotype is, therefore, the result of complex interactions between genetic and environmental factors [106].

Nutrigenomics, a study of the genome-wide influences of nutrition, considers nutrients as dietary signals that, once they are detected by the cellular sensor systems of immunometabolism, influence gene and protein expression and, subsequently, metabolite production. So, the modulation of gene, protein or metabolite expression patterns in response to particular food compounds or different nutritional profiles are considered dietary signatures [89].

The genetic component has a large impact on susceptibility to obesity; therefore, the phenotypic response to diet is partly determined by genetic factors [107]. For instance, studies of monozygotic twins have shown that differences in obesity-related traits, such as adiposity, are strongly influenced by genetic background, although the genes that are responsible for the individual differences in the sensitivity to diet remain to be fully identified [108].

Thus, in obesity the interaction between the genotype and the environment, that is, the response or the adaptation to an environmental factor, such as the nutritional profile, is dependent upon the genotype of the individual [109]. However, this interaction can be at two different levels. First, as nutrients can act at the molecular level, the nutritional profile of the diet or the presence of bioactive compounds might differentially modulate the expression of key genes involved in the regulation of immunometabolism. Second, individual genetic predisposition also plays a pivotal role in the molecular management of these food-derived signals [106].

CHAPTER 1

Procyanidins and inflammation: Molecular targets and health implications

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Review Article

Procyanidins and inflammation: Molecular targets and health implications

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Abstract.
The inflammatory response has been implicated in the pathogenesis of many chronic diseases. Thus, the modulation of the inflammatory response by the consumption of bioactive food compounds, such as procyanidins, is a powerful tool to promote health. Procyanidin-mediated anti-inflammatory molecular mechanisms include, among others, the modulation of the arachidonic acid pathway, the inhibition of the gene transcription, protein expression and enzymatic activity of eicosanoid generating enzymes, the production and secretion of inflammatory mediators (such as cytokines and nitric oxide), the inhibition of mitogen-activated protein kinase (MAPK) pathway activation, and the modulation of the nuclear factor-κB (NF-κB) pathway. The NF-κB pathway can be regulated by procyanidins at several levels. During early events in NF-κB signaling, procyanidins modulate Iκκ activity, and the cytoplasmic retention of p65:p50 NF-κB by the inhibition of IκB phosphorylation and proteasomal degradation, while at late stages, they affect the nuclear translocation of pro/anti-inflammatory NF-κB homo/hetero dimers and their subsequent binding to the promoter regions of target genes. To identify and understand the value of procyanidins in the modulation of the inflammatory response, the molecular mechanisms underlying the anti-inflammatory activities and prohomeostatic effects of procyanidins need to be investigated further.

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1. Introduction

The human diet consists largely of plant-derived products, such as vegetables, fruits, and cereals. Polyphenols represent a large and diverse group of secondary plant metabolites that are commonly found in fruits, vegetables, legumes, dark chocolate, tea, and red wine [1–3].

The main classes of polyphenols can be classified as phenolic acids, flavonoids, and the less common stilbenes and lignans [4]. In turn, flavonoids can be divided into flavanols, flavones, anthocyanidins, isoflavonoids, and neoflavonoids. Flavanols (or flavan-3-ols) can exist as monomers, such as catechin, epicatechin, and its gallated forms, or as monomeric and oligomeric forms, which are referred to as proanthocyanidins [5]. The proanthocyanidins that consist exclusively of (epi)catechin units linked together are called procyanidins, and these are the most abundant type of proanthocyanidins in plants. Procyanidins are structurally diverse, and this diversity is based on the number of monomer units involved (Fig. 1). Procyanidins usually occur together with monomeric (+)-catechin and (−)-epicatechin; thus, it is difficult to attribute the observed effects to the procyanidins, the monomeric forms or both [6].

In recent years, the role of dietary procyanidins as health protective agents has become an important area of human nutrition research [7–10]. Epidemiological studies have indicated that populations that consume procyanidin-rich foods have lower incidences of inflammatory disease and diseases of multifactorial pathogenesis, including metabolic syndrome, atherosclerosis, and cancer [11,12].

Inflammation is a defense mechanism that can be triggered by a trauma or by an array of proinflammatory mediators. These mediators may be external pathogens, such as bacterial lipopolysaccharides, or intrinsic mediators, such as

Abbreviations: THP-1, human monocytic cell line; B_1 and B_2, procyanidin dimers; C_1, procyanidin trimer; LPS, lipopolysaccharide; COX_1, cyclooxygenase-1; COX_2, cyclooxygenase-2; PGE_2, prostaglandin E_2; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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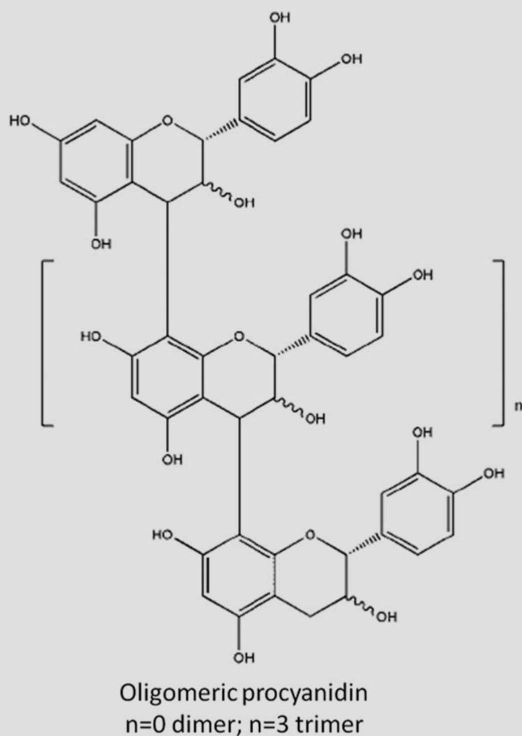

Fig. 1. Structures of oligomeric procyanidins.

some cytokines, eicosanoids, and fatty acids [13,14]. The inflammatory response represents a complex network of cellular and molecular interactions that are common to a variety of proinflammatory triggers and pathways; these shared inflammatory mediators include specific patterns of eicosanoid and cytokine production [15]. Therefore, the modulation of the inflammatory response by the dietary intake of bioactive food compounds, such as procyanidins, is a powerful tool for promoting health and homeostasis and for preventing disease [16,17]. In this review, we summarize these findings regarding the prohomeostatic effects of procyanidins in the modulation of the inflammatory response.

2. Metabolism, absorption, and bioavailability of procyanidins

Flavanols (and, in turn, procyanidins) are the most ubiquitous class of molecules in plants that are consumed by humans. Procyanidins are present as bioactive compounds in a wide range of foods, such as fruits, legume seeds, and cereal grains, and in beverages such as red wine, tea, or cocoa; cocoa has the highest known procyanidin content among all foods (Table 1).

The prohomeostatic biological properties of procyanidins depend on both the amount consumed and their bioavailability. The average polyphenol intake in a healthy human diet has been estimated to be ~ 1 g per day [18], and procyanidins represent over 50% of the total daily intake. The bioavailability of various procyanidins differs according to their chemical structure. The degree of polymerization and the presence of galloyl moieties are critical for bioavailability, as are the nature of the metabolites circulating in plasma and their subsequent biological effects [5]. For these reasons, the most common procyanidins in the human diet are not necessarily the most biologically active.

The metabolism and absorption of polyphenols and procyanidins have been studied intensively [18–22]. The procyanidins found in foods vary significantly in their degree of polymerization. The bioavailability of procyanidins depends on their absorption and on their metabolism at the gastrointestinal tract, tissue, and cellular levels after absorption [23]. Although Spencer et al. [24] used *in vitro* experiments to demonstrate that procyanidins with high levels of polymerization are degraded to mixtures of epicatechin monomers and dimers under conditions similar to those in gastric juices, Rios et al. [25] demonstrated that procyanidins are stable during gastric transit in humans *in vivo*. Tsang et al. [26] found that the small and large intestines of grapeseed-fed rats contained (−)-epicatechin dimers, trimers, tetramers, and higher molecular weight procyanidins. More recent studies have demonstrated that monomers, dimers, and trimers are absorbed intact in the gastrointestinal tract [21,27]. Once absorbed, procyanidins are conjugated to glucuronide, sulfate, and methyl groups, mainly in the gut mucosa and liver. In the plasma, monomers are present extensively as conjugated metabolites and to a lesser extent as nonconjugated molecules [23]. The polyphenols that reach the colon can be metabolized by the microflora into a wide array of phenolic acids [28].

3. Procyanidin modulation of the inflammatory response

Although the inflammatory response can be beneficial, managing the host defense against infectious agents and trauma, inflammation also contributes to the pathophysiology of many chronic diseases. The interactions between cells in the innate and acquired immune response systems and inflammatory mediators orchestrate the acute and chronic inflammation underlying many diseases [13].

The inflammatory response involves both local and systemic events. Although the inflammatory response may vary depending on the triggering stimuli, there is a common spectrum of mediators involved. If the normal, healthy state is not restored (*i.e.*, homeostasis is disturbed), the inflammation persists and becomes chronic.

Procyanidins, as bioactive food compounds, exert physiological and cellular activities that promote homeostasis. There is a large body of scientific evidence describing the broad effects of procyanidins on inflammation [5,23]. The molecular mechanisms targeted by procyanidins include, but are not limited to, the modulation of various mediators of

Table 1
Procyanidin concentrations in commonly consumed foods

Food	Total procyanidins	Oligomeric forms	Food	Total procyanidins	Oligomeric forms
Carrot	0	0	Almond	184.02	176.25
Chestnut	0.05	0.03	Apple (Gala)	92.42	86.48
Coffee	0.11	0	Barley	99.24	96.11
Fig	0.04	0.01	Blueberries	176.49	173.03
Pomegranate	1.01	0.29	Cranberries	418.77	411.51
White wine	0.81	0.22	Currant	158.03	157.13
Apple juice	12.22	7.26	Curry	74.16	74.16
Apricot	11.32	10	Grape seed	373.4	202.11
Avocado	7.39	6,43	Hazelnut	500.66	490.83
Banana	3.37	3.24	Hops	293.16	135.63
Beans	8.1	5.2	Peach	71.75	67.27
Beer	2.03	1.4	Pecans	494.05	476.83
Blackberries	23.31	19.58	Pistachio	237.34	226.4
Grape juice	48.59	47.26	Black plums	247.27	238.91
Kiwi	3.16	2.65	Red wine	61.63	44.99
Lentils	1.84	1.31	Strawberries	65.89	62.18
Mango	12.7	10.4	Walnut	67.25	60.32
Nectarine	29.18	23.61	Cacao beans	9481.75	8100.47
Peanut	10.51	5.14	Chocolate	1635.94	1437.4
Pears	42.3	40.27	Cinnamon	8108.14	8084.22
Tee	13.42	0	Sorghum	1902.38	1893.29

Four groups of foods have been classified according to their procyanidin contents, expressed as mg/100 g of edible portion. The data are expressed as total monomeric and oligomeric procyanidin content and as oligomeric content only. Group 1 (■) contains foods with low procyanidin contents ($<$ 50 mg/100 g of edible portion). Group 2 (■) contains foods with moderate procyanidin contents (50–100 mg/100 g of edible portion). Group 3 (■) contains foods with high procyanidin contents (100–1000 mg/100 g of edible portion). Group 4(■) contains foods with very high procyanidin contents ($>$ 1000 mg/100 g of edible portion). Data source: USDA Database for the Proanthocyanidin Content of Selected Foods.

inflammation (eicosanoids, cytokines, and nitric oxide (NO) production) as well as the NF-κB and MAPK pathways.

This review will focus mainly on the effects of procyanidins on cell signaling and other molecular mechanisms related to inflammation.

3.1. Procyanidins and inflammatory mediators

3.1.1. Procyanidin modulation of arachidonic acid pathway. In 1972, Flower and Vane [29] found that aspirin, a nonsteroidal anti-inflammatory drug, is an inhibitor of cyclooxygenase (COX) activity. Aspirin inhibits the transformation of arachidonic acid (AA) molecules into prostaglandins (PGs), a class of compounds that mediate physiological and pathophysiological inflammation. These findings have made the AA pathway the subject of intense research.

The potential of flavonoids as inhibitors of AA-metabolizing enzymes was initially reported in 1980 [30]. Since then, the modulation of the AA pathway has been considered one of the most important anti-inflammatory mechanisms of dietary flavonoids, including procyanidins. This modulation involves the inhibition of eicosanoid-generating enzymes, including phospholipase A2, cyclooxygenases, and lipoxygenases (LOXs), thereby reducing the secretion of prostanoids and leukotrienes (LTs) [30–34].

COX_2 is considered a pivotal enzyme in inflammation; therefore, the modulation of COX_2 plays a key role in the regulation of the inflammatory response. COX_2 is regulated at the transcriptional level by proinflammatory stimuli, such as LPS, proinflammatory cytokines, and growth factors, which activate the MAPK and NF-κB pathways that ultimately promote the transcription of the COX_2 gene [35]. Procyanidins have been found to inhibit COX_2 in several ways, including through COX_2 gene transcription, COX_2 protein expression, and COX_2 enzyme activity. Four transcription factors, NF-κB, CCAAT/enhancer-binding protein (C/EBP), activator protein 1 (AP-1), and CRE-binding protein (CREB), can modulate the transcription of COX_2 by binding to the *cis*-acting elements in the COX_2 promoter region [36]. The pretreatment of mouse or human macrophages with procyanidin-rich extracts or with pure procyanidin compounds inhibits the transcription of COX_2 mRNA [36,37]. Procyanidins inhibit COX_2 transcription by downregulating the pathways upstream of COX_2 transcription factors [36]. COX_2 protein expression can also be downregulated by procyanidins in different cell types. In LPS-activated human THP-1-derived

macrophages and mouse RAW264.7 macrophages, a decrease in COX_2 protein expression was observed on pretreatment with procyanidin-rich extract or B_2 procyanidin dimer [36,37]. Procyanidin-rich extracts and the B_2 procyanidin dimer are also dose-dependent inhibitors of the COX_2 protein expression in other cell inflammation models in both mice and humans [38–40]. Using a cell-free assay, Martinez-Micaelo et al. [17] found that pure procyanidin dimers (B_1 and B_2) and trimers (C_1) selectively altered COX_1 and COX_2 activity. Specifically, they found that B_1 is a selective and competitive inhibitor of COX_1 activity, with an IC_{50} value of 8.0 μM, and that B_2 and C_1 are selective and competitive inhibitors of COX_2, with IC_{50} values of 9.7 and 3.3 μM, respectively. Procyanidins apparently compete with AA for binding to the active site of COX proteins, decreasing the catalytic efficiency of PG biosynthesis. The potential of procyanidins as strong inhibitors of COX activity, gene, and protein expression is consistent with the decrease of PG secretion observed in primary human macrophages [17,41].

On the other hand, LOXs are involved in the metabolism of AA into several inflammatory mediators, including LTs, and 5-, 12-, and 15-LOX are critical enzymes in the synthesis of proinflammatory eicosanoids [42] (Fig. 2). The effects of procyanidins on LT patterns were demonstrated by Schramm et al. [43], who found that the intake of procyanidin-rich chocolate in humans led to significant decreases in the plasma concentrations of cysteinyl LTs, which are metabolites of the degradation of AA by the 5-lipoxygenase pathway, as well as an increase of (−)-epicatechin metabolites in the plasma. Moreover, Sies et al. [44] found that oligomeric procyanidins can inhibit both the dioxygenase and LTA_4 synthase activities of human recombinant 5-lipoxygenase in a dose-dependent manner. In this way, these molecules modulate the first two steps in the conversion of AA to various proinflammatory LTs [42]. Although dietary procyanidins have been identified as modulators of LOX activity, the interactions between procyanidins and LOXs have not been fully elucidated.

Even though several pure procyanidin compounds have been described to be strong COX and LOX inhibitors, some studies of the structure–activity relationship of procyanidins suggest that the presence of galloyl moieties may be important for this inhibitory activity [36,38,45].

In conclusion, the COX and LOX enzymes play key roles in the modulation of the AA pathway and thus the inflammatory response. Therefore, the prohomeostatic modulation of the AA pathway by procyanidins is a potential mechanism by which they may modulate the inflammatory response.

3.1.2. Modulation of cytokine production by procyanidins. Cytokines are intercellular mediators that modulate a broad range of cellular and physiological responses, including the activation of several proinflammatory pathways and transcription factors [46]. Therefore, an imbalance of proinflammatory/anti-inflammatory cytokine secretion is considered a hallmark of the activation/repression of an inflammatory response. Cytokines are often used as biomarkers of inflammation, and their transcription and secretion have been directly associated with several human diseases [47].

Several *in vitro* and *in vivo* studies have shown that procyanidins (extracts as well as monomers, dimers, or trimers) can downregulate the transcription and secretion of proinflammatory cytokines, including the interleukins (ILs) IL-1β, IL-2, Il-6, and IL-8, tumor necrosis factor-α (TNF-α) and interferon-γ (INF-γ), and can upregulate the secretion of anti-inflammatory cytokines such as IL-10, IL-4, or transforming growth factor-β (TGF-β) in peripheral blood mononuclear cells, macrophages, or lymphoid cell lines. This behavior of procyanidins has also been observed using *in vivo* models of inflammation [48–52]. Miyake et al. [53] reported that the oral administration of highly oligomeric procyanidins purified from a South American herb jatoba dramatically reduced the secretion of IFN-γ and arthritis symptoms in mice.

3.1.3. No-mediated effects of procyanidins. NO, a gaseous free radical, is synthesized from L-arginine by nitric oxide synthases (NOS), including endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) isoforms. NO is considered to be a pivotal signaling molecule in endothelial macrophages [54]. At physiological concentrations, NO exerts several anti-inflammatory, antioxidant, antiatherosclerotic, and vasodilatory activities; however, when produced in excessive amounts under oxidative and inflammatory conditions, NO can be a precursor of potent pro-oxidant and nitrating compounds, such as peroxynitrite and nitrogen dioxide [44]. Procyanidins have been found to be strong *in vitro* inhibitors of NO synthesis, downregulating iNOS mRNA and protein expression, and therefore reducing NO production and secretion in murine macrophages [41,46,54].

3.2. Procyanidins and signaling pathways

3.2.1. The nuclear factor-κb pathway and procyanidins. The transcription factor NF-κB is responsible for the transcriptional regulation of numerous genes underlying immune response control [55,56]. NF-κB plays an essential role in normal physiology, and its constitutive activation is associated with several diseases [57]. The NF-κB signaling pathways can be differentiated as classical/canonical pathways or alternative pathways based on the specific IkappaB kinase (Iκκ) family members that are involved in their activation [58]. In mammals, the NF-κB family comprises five proteins, including p65 (RelA), RelB, c-Rel, p105/p50 (NF-κB1), and p100/p52 (NF-κB2), which associate with each other to form transcriptionally distinct homo- and heterodimeric complexes; the p65:p50 heterodimer is the most abundant and the most relevant in inflammation compared with the other Rel dimers [57]. In resting cells, the p65:p50 NF-κB heterodimer is sequestered in the cytoplasm by the binding of its inhibitory protein, IkappaB (IκB), a member of the IκB family of proteins. In response to an inflammatory stimulus, such as LPS from a bacterial cell wall, the classical pathway of NF-κB activation leads to the activation of the IκB kinase

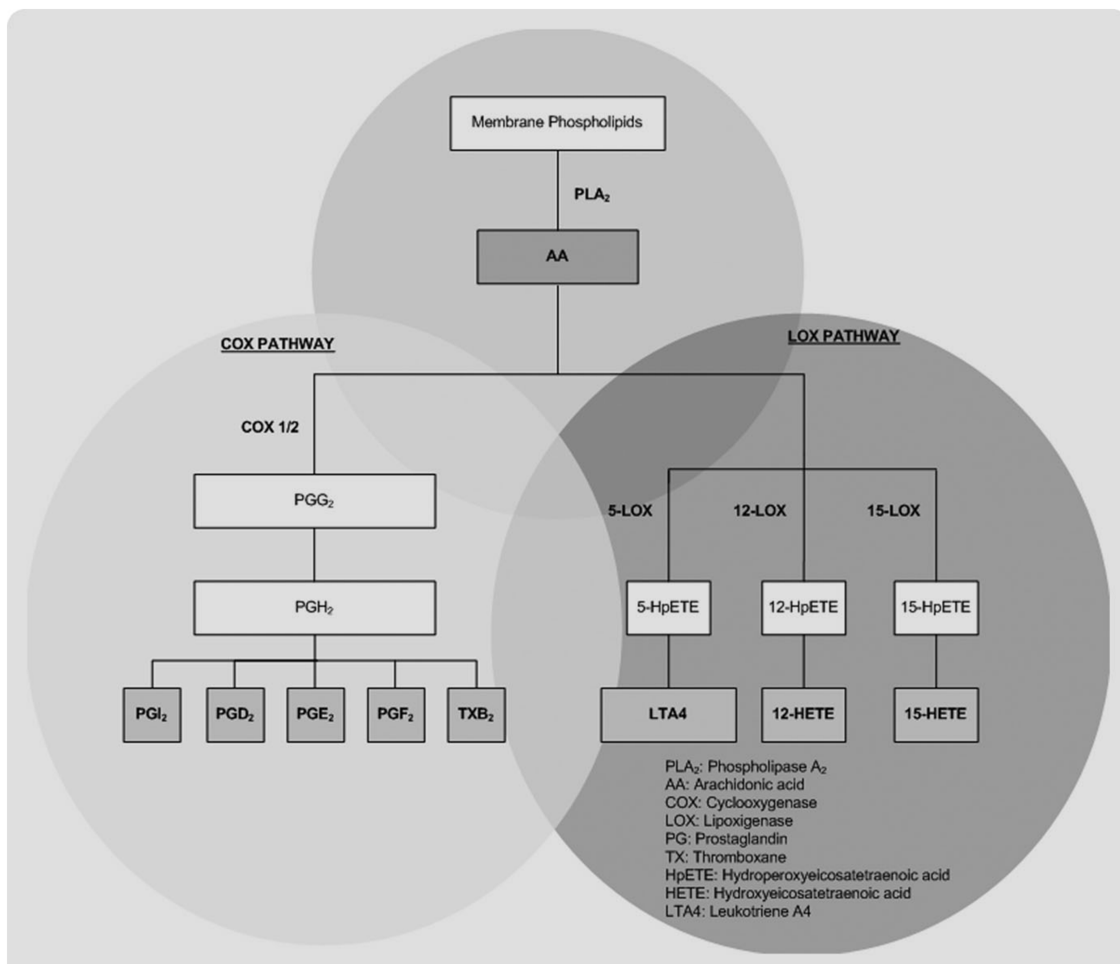

Fig. 2. AA pathways. AA can be converted to either PGs or LTs by COXs or LOXs. AA, an eicosanoid precursor, is released from cell membrane phospholipids through phospholipase A_2 (PLA_2) cleavage. AA can then be metabolized by the COX pathway into PGs and thromboxane A2 (TXA2) or by the LOXs pathway to hydroperoxyeicosatetraenoic acids (HpETEs), hydroxyeicosatetraenoic acids (HETEs) and LTs. COX_1 is a housekeeping enzyme that is constitutively expressed in most cells and is involved in the modulation of many physiological responses, whereas COX_2 is an inducible enzyme that produces large amounts of PGs. COX_2 is highly expressed in inflammatory-related cell types, including macrophages and mast cells, and its expression is triggered by proinflammatory stimuli such as lipopolysaccharides (LPS) or cytokines. LOXs metabolize the AA-generating hydroxyl acid and LTs. Three different LOX isoenzymes (5-, 12-, and 15-LOX) have been found in different cells and tissues. 5-LOX produces proinflammatory 5-HETE and LTs, 12-LOX synthesises 12-HETE and is involved in the induction of the inflammatory response, and 15-LOX produces anti-inflammatory 15-HETE.

(IκκB), a member of the Iκκ complex, triggering IκB-α phosphorylation (pIκB-α). Then, pIκB-α is recognized by the ubiquitin ligase machinery, causing its polyubiquitination and subsequent proteasomal degradation. After pIκB-α degradation, the p65:p50 heterodimers are able to translocate to the nucleus, where they bind to the κB motif found in the promoter or enhancer regions of numerous proinflammatory genes and induce their expression [56]. The alternative activation pathway of nuclear factor NF-κB is not activated by LPS, and it is independent of IκκB and the Iκκγ kinase [59].

NF-κB plays a critical role in the transcriptional regulation of a wide range of genes that are involved in the regulation of inflammation and also underlying inflammatory diseases [60]. NF-κB transcriptional regulation include genes encoding for cytokines (IL-1-α and β, IL-2, IL-6, IL-8, IL-9, IL-11, IL-12, IL-13, IL-15, TNF-α, lymphotoxin α and β, and

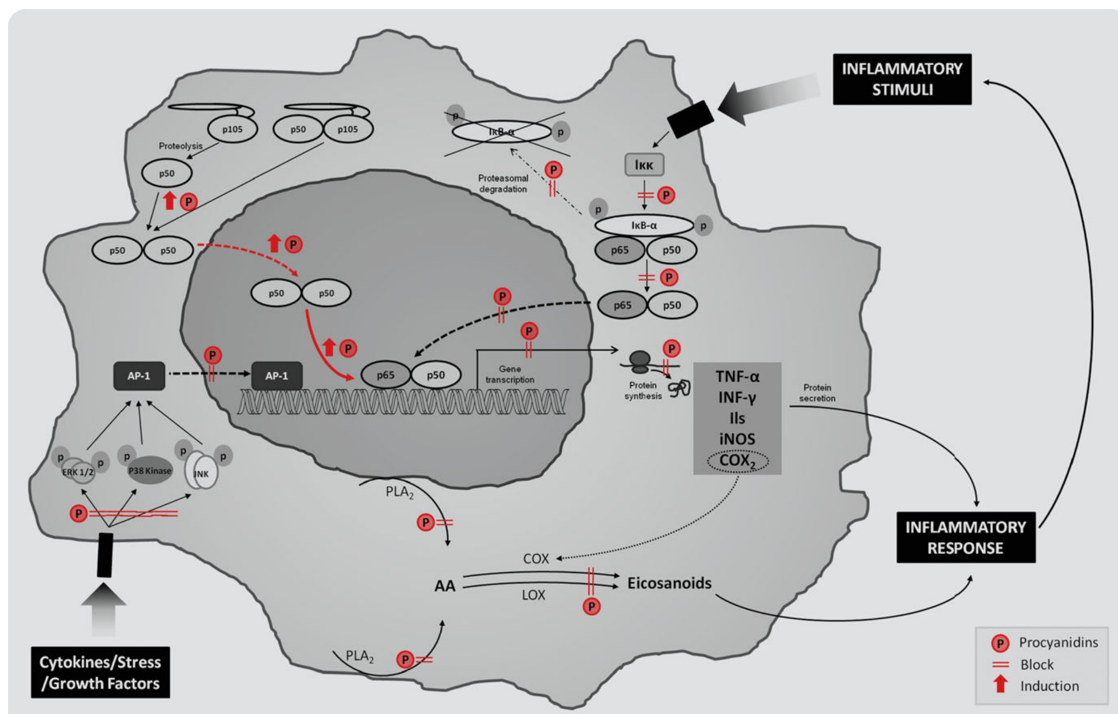

Fig. 3. The proposed anti-inflammatory mechanisms of procyanidins. The mechanisms underlying the anti-inflammatory effects of procyanidins involve, among others, the modulation of AA pathway, the production and secretion of inflammatory mediators and the modulation of the MAPK and NF-κB pathways. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

granulocyte-macrophage colony-stimulating factor (GM-CSF)), adhesion molecules (*e.g.*, intercellular adhesion molecule (ICAM-1), vascular cell adhesion molecule (VCAM-1), and endothelial leukocyte adhesion molecule (ELAM-1)), acute phase proteins (serum amyloid A (SAA) and acute-phase protein (CRP)), inducible enzymes (iNOS and COX_2) and cytokine receptors (interleukin-2 receptor (IL-2R), chemokine receptor transporter 5, IL-1 receptor antagonist (IL-1RA), and TNF receptor member 6 (TNFSR6)). All of these genes contain verified NF-κB binding sites in their sequences, providing strong experimental evidence of their direct control by NF-κB [61].

Procyanidins have been described as strong and selective inhibitors of NF-κB activation in both *in vitro* and animal models [17,52,62–65]. One finding supporting this assumption is that procyanidins (extracts, dimers, or trimers) downregulate IκκB activity, thus decreasing the phosphorylation of pIκB-α and its subsequent ubiquitination and proteasomal degradation [52,62]. Thus, procyanidins can inhibit NF-κB activation by promoting the retention of the p65:p50 heterodimer in the cytoplasm as an inactive complex bound to IκB (Fig. 3).

A previous investigation [62] reported that in Jurkat T cells, treatment with procyanidin dimers might regulate NF-κB activation through the accumulation of B_2 in the nucleus and its direct interaction with the p65 and p50 NF-κB subunits. The B_2 procyanidin, due to its molecular structure, can establish hydrogen bonds with the arginine residues in the DNA binding regions of p50 and p65, thus mimicking the guanine pairs present in the κB DNA sequence [23].

Recently, a novel p65:p50 cytosolic sequestering mechanism has been described [17]. p105 is a NF-κB family member with dual functions in the NF-κB signaling system. First, due to the ankyrin repeats in its structure, p105 can act as an IκB family member and thus inhibit the nuclear translocation of p65. Second, p105 is a precursor of the p50 subunit, so the cytoplasmic over-expression of p105 is associated with a decrease in NF-κB and MAPK pathway activation. Procyanidins, especially B_2, can also promote the formation of p50:p50 homodimers and their nuclear translocation, which is associated with a repression of NF-κB transcriptional activity. In addition, procyanidins downregulate the binding of NF-κB p65 to the corresponding κB-consensus sequences in the promoter and enhancer regions of several proinflammatory genes [36,17,64].

3.2.2. MAPK pathways. MAPKs are Ser/Thr kinases that are involved in the regulation of several cellular processes [46]. MAPKs are part of the three-tiered signaling pathways,

including the extracellular signal related kinase (ERK), p38 MAPK and Jun N-terminal kinase (JNK) pathways, that regulate NF-κB and activator protein-1 (AP-1) DNA binding [33] (Fig. 3). Mitogens, growth factors, stress, and inflammatory stimuli are activators of the ERK, JNK, and p38 cascades. MAPK signaling pathway activation can be regulated by protein phosphorylation, and procyanidin extracts and pure procyanidin compounds such as B_1 dimer and C_1 trimer markedly suppressed the phosphorylation of ERK, JNK, and p38 in immune cells [36,46,52]. The inhibition of MAPK activation elicits a decrease in AP-1 activity and thus suppresses the production of several proinflammatory cytokines [46].

4. Conclusions

Procyanidins, which are plant polyphenols formed by the polymerization of flavan-3-ols, comprise a vast array of biologically active compounds that are common in human diets all over the world. Procyanidins possess anti-inflammatory activities both *in vitro* and *in vivo*.

The mechanisms underlying the anti-inflammatory effects of procyanidins involve, among others, the modulation of several pivotal pathways in the regulation of cellular homeostasis, such as the AA pathway (through the modulation of eicosanoid-generating enzymes), the production and secretion of inflammatory mediators (*e.g.*, cytokines or nitric oxide), and the modulation of MAPKs and NF-κB pathways.

The modulation of the inflammatory response by the dietary intake of bioactive food compounds may be a powerful tool for promoting health and homeostasis and for preventing disease development. However, there is a lack of human intervention studies on the effects of individual procyanidins or combinations of procyanidins on inflammation.

The beneficial effects promoted by bioactive foods are not exclusive to polyphenols or procyanidins but are also exerted by other bioactive food compounds present in food; for example, several fatty acids have been described as bioactive prohomeostatic compounds. In this context, there is a growing area of functional food science devoted to studying and understanding the possible additive or synergistic interactions between different bioactive compounds and their potential benefits [10,17,66,67].

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References

[1] Erdman, J. W., Balentine, D., Arab, L., Beecher, G., Dwyer, J. T., Folts, J., Harnly, J., Hollman, P., Keen, C. L., Mazza, G., Messina, M., Scalbert, A., Vita, J., Williamson, G., and Burrowes, J. (2007) Flavonoids and heart health: Proceedings of the ILSI North America Flavonoids Workshop, May 31–June 1, 2005, Washington, DC. J. Nutr. **137**, 718S–737S.

[2] Landberg, R., Sun, Q., Rimm, E. B., Cassidy, A., Scalbert, A., Mantzoros, C. S., Hu, F. B., and Van Dam, R. M. (2011) Selected dietary flavonoids are associated with markers off inflammation and endothelial dysfunction in U.S. Women. J. Nutr. **141**, 618–625.

[3] Perez-Jimenez, J., Fezeu, L., Touvier, M., Arnault, N., Manach, C., Hercberg, S., Galan, P., and Scalbert, A. (2011) Dietary intake of 337 polyphenols in French adults. Am. J. Clin. Nutr. **93**, 1220–1228.

[4] Scalbert, A., Manach, C., Morand, C., Remesy, C., and Jimenez, L. (2005) Dietary polyphenols and the prevention of diseases. Crit. Rev. Food Sci. Nutr. **45**, 287–306.

[5] De La Iglesia, R., Milagro, F. I., Campion, J., Boque, N., and Martinez, J. A. (2010) Healthy properties of proanthocyanidins. Biofactors **36**, 159–168.

[6] Williamson, G. and Manach, C. (2005) Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. Am. J. Clin. Nutr. **81**, 243S–255S.

[7] Auger, C., Al-Awwadi, N., Bornet, A., Rouanet, J. M., Gasc, F., Cros, G., and Teissedre, P. L. (2004) Catechins and procyanidins in Mediterranean diets. Food Res. Int. **37**, 233–245.

[8] Yilmaz, Y. and Toledo, R. T. (2004) Health aspects of functional grape seed constituents. Trends Food Sci. Technol. **15**, 422–433.

[9] Kenny, T. P., Keen, C. L., Schmitz, H. H., and Gershwin, M. E. (2007) Immune effects of cocoa procyanidin oligomers on peripheral blood mononuclear cells. Exp. Biol. Med. **232**, 293–300.

[10] Martinez-Micaelo, N., Gonzalez-Abuin, N., Pinent, M., Ardevol, A., Blay, M. (2011) Anti-inflammatory effects of procyanidins and docosahexanoic acid in human macrophages THP-1. Inflamm. Res. **60**, 258–259.

[11] Engler, M. B. and Engler, M. M. (2006) The emerging role of flavonoid-rich cocoa and chocolate in cardiovascular health and disease. Nutr. Rev. **64**, 109–118.

[12] Khan, N., Monagas, M., Llorach, R., Urpi-Sarda, M., Rabassa, M., Estruch, R., and Andres-Lacueva, C. (2010) Targeted and metabolomic study of biomarkers of cocoa powder consumption effects on inflammatory biomarkers in patients at high risk of cardiovascular disease. Agro Food Industry Hi-Tech **21**, 51–54.

[13] Libby, P. (2007) Inflammatory mechanisms: the molecular basis of inflammation and disease. Nutr. Rev. **65**, S140–S146.

[14] Olefsky, J. M. and Glass, C. K. (2010) Macrophages, inflammation, and insulin resistance. Annu. Rev. Physiol. **72**, 219–246.

[15] Selmi, C., Mao, T. K., Keen, C. L., Schmitz, H. H., and Gershwin, M. E. (2006) The anti-inflammatory properties of cocoa flavanols. J. Cardiovasc. Pharmacol. **47**, S163–S171.

[16] Gonzalez, R., Ballester, I., Lopez-Posadas, R., Suarez, M. D., Zarzuelo, A., Martinez-Augustin, O., and Sanchez De Medina, F. (2011) Effects of flavonoids and other polyphenols on inflammation. Crit. Rev. Food Sci. Nutr. **51**, 331–362.

[17] Martinez-Micaelo, N., Gonzalez-Abuin, N., Terra, X., Richart, C., Ardevol, A., Pinent, M., and Blay, M. (2012) Omega-3 docosahexaenoic acid and procyanidins inhibit cyclo-oxygenase activity and attenuate NF-kappaB activation through a p105/p50 regulatory mechanism in macrophage inflammation. Biochem. J. **441**, 653–663.

[18] Scalbert, A. and Williamson, G. (2000) Dietary intake and bioavailability of polyphenols. J. Nutr. **130**, 2073S–2085S.

[19] Baba, S., Osakabe, N., Natsume, M., and Terao, J. (2002) Absorption and urinary excretion of procyanidin B2 epicatechin-(4 beta-8)-epicatechin in rats. Free Radic. Biol. Med. **33**, 142–148.

[20] Holt, R. R., Lazarus, S. A., Sullards, M. C., Zhu, Q. Y., Schramm, D. D., Hammerstone, J. F., Fraga, C. G., Schmitz, H. H., and Keen, C. L. (2002) Procyanidin dimer B2 epicatechin-(4 beta-8)-epicatechin in human plasma after the consumption of a flavanol-rich cocoa. Am. J. Clin. Nutr. **76**, 798–804.

[21] Gonthier, M. P., Donovan, J. L., Texier, O., Felgines, C., Remesy, C., and Scalbert, A. (2003) Metabolism of dietary procyanidins in rats. Free Radic. Biol. Med. **35**, 837–844.

[22] Stoupi, S., Williamson, G., Viton, F., Barron, D., King, L. J., Brown, J. E., and Clifford, M. N. (2010) In vivo bioavailability, absorption, excretion, and pharmacokinetics of (14)C procyanidin B2 in male rats. Drug Metab. Dispos. **38**, 287–291.

[23] Fraga, C. G. and Oteiza, P. I. (2011) Dietary flavonoids: role of (-)-epicatechin and related procyanidins in cell signaling. Free Radic. Biol. Med. **51**, 813–823.
[24] Spencer, J. P., Chaudry, F., Pannala, A. S., Srai, S. K., Debnam, E., and Rice-Evans, C. (2000) Decomposition of cocoa procyanidins in the gastric milieu. Biochem. Biophys. Res. Commun. **272**, 236–241.
[25] Rios, L. Y., Bennett, R. N., Lazarus, S. A., Remesy, C., Scalbert, A., and Williamson, G. (2002) Cocoa procyanidins are stable during gastric transit in humans. Am. J. Clin. Nutr. **76**, 1106–1110.
[26] Tsang, C., Auger, C., Mullen, W., Bornet, A., Rouanet, J. M., Crozier, A., and Teissedre, P. L. (2005) The absorption, metabolism and excretion of flavan-3-ols and procyanidins following the ingestion of a grape seed extract by rats. Br. J. Nutr. **94**, 170–181.
[27] Deprez, S., Mila, I., Huneau, J. F., Tome, D., and Scalbert, A. (2001) Transport of proanthocyanidin dimer, trimer, and polymer across monolayers of human intestinal epithelial Caco-2 cells. Antioxid. Redox. Signal. **3**, 957–967.
[28] Han, X. Z., Shen, T., and Lou, H. X. (2007) Dietary polyphenols and their biological significance. Int. J. Mol. Sci. **8**, 950–988.
[29] Flower, R. J. and Vane, J. R. (1972) Inhibition of prostaglandin synthetase in brain explains the anti-pyretic activity of paracetamol (4-acetamidophenol). Nature **240**, 410–411.
[30] Baumann, J., Von Bruchhausen, F., and Wurm, G. (1980) Flavonoids and related compounds as inhibition of arachidonic acid peroxidation. Prostaglandins **20**, 627–639.
[31] Welton, A. F., Tobias, L. D., Fiedler-Nagy, C., Anderson, W., Hope, W., Meyers, K., and Coffey, J. W. (1986) Effect of flavonoids on arachidonic acid metabolism. Prog. Clin. Biol. Res. **213**, 231–242.
[32] Hong, J., Bose, M., Ju, J., Ryu, J. H., Chen, X., Sang, S., Lee, M. J., and Yang, C. S. (2004) Modulation of arachidonic acid metabolism by curcumin and related beta-diketone derivatives: effects on cytosolic phospholipase A(2), cyclooxygenases and 5-lipoxygenase. Carcinogenesis **25**, 1671–1679.
[33] Kim, H. P., Son, K. H., Chang, H. W., and Kang, S. S. (2004) Anti-inflammatory plant flavonoids and cellular action mechanisms. J. Pharmacol. Sci. **96**, 229–245.
[34] Hamalainen, M., Nieminen, R., Asmawi, M. Z., Vuorela, P., Vapaatalo, H., and Moilanen, E. (2011) Effects of flavonoids on prostaglandin E(2) production and on COX-2 and mPGES-1 expressions in activated macrophages. Planta Med. **77**, 1504–1511.
[35] Kang, Y. J., Wingerd, B. A., Arakawa, T., and Smith, W. L. (2006) Cyclooxygenase-2 gene transcription in a macrophage model of inflammation. J. Immunol. **177**, 8111–8122.
[36] Hou, D. X., Masuzaki, S., Hashimoto, F., Uto, T., Tanigawa, S., Fujii, M., and Sakata, Y. (2007) Green tea proanthocyanidins inhibit cyclooxygenase-2 expression in LPS-activated mouse macrophages: molecular mechanisms and structure-activity relationship. Arch. Biochem. Biophys. **460**, 67–74.
[37] Zhang, W. Y., Liu, H. Q., Xie, K. Q., Yin, L. L., Li, Y., Kwik-Uribe, C. L., and Zhu, X. Z. (2006) Procyanidin dimer B2 epicatechin-(4 beta-8)-epicatechin suppresses the expression of cyclooxygenase-2 in endotoxin-treated monocytic cells. Biochem. Biophys. Res. Commun. **345**, 508–515.
[38] Kundu, J. K., Na, H. K., Chun, K. S., Kim, Y. K., Lee, S. J., Lee, S. S., Lee, O. S., Sim, Y. C., and Surh, Y. J. (2003) Inhibition of phorbol ester-induced COX-2 expression by epigallocatechin gallate in mouse skin and cultured human mammary epithelial cells. J. Nutr. **133**, 3805S–3810S.
[39] Kang, N. J., Lee, K. W., Lee, D. E., Rogozin, E. A., Bode, A. M., Lee, H. J., and Dong, Z. (2008) Cocoa procyanidins suppress transformation by inhibiting mitogen-activated protein kinase kinase. J. Biol. Chem. **283**, 20664–20673.
[40] Meeran, S. M., Vaid, M., Punathil, T., and Katiyar, S. K. (2009) Dietary grape seed proanthocyanidins inhibit 12-O-tetradecanoyl phorbol-13-acetate-caused skin tumor promotion in 7,12-dimethylbenz[a]anthracene-initiated mouse skin, which is associated with the inhibition of inflammatory responses. Carcinogenesis **30**, 520–528.
[41] Terra, X., Valls, J., Vitrac, X., Merrillon, J. M., Arola, L., Ardevol, A., Blade, C., Fernandez-Larrea, J., Pujadas, G., Salvado, J., and Blay, M. (2007) Grape-seed procyanidins act as antiinflammatory agents in endotoxin-stimulated RAW 264.7 macrophages by inhibiting NFkB signaling pathway. J. Agric. Food Chem. **55**, 4357–4365.
[42] Samuelsson, B., Rouzer, C. A., and Matsumoto, T. (1987) Human leukocyte 5-lipoxygenase: an enzyme possessing dual enzymatic activities and a multicomponent regulatory system. Adv. Prostaglandin Thromboxane Leukot. Res. **17A**, 1–11.
[43] Schramm, D. D., Wang, J. F., Holt, R. R., Ensunsa, J. L., Gonsalves, J. L., Lazarus, S. A., Schmitz, H. H., German, J. B., and Keen, C. L. (2001) Chocolate procyanidins decrease the leukotriene-prostacyclin ratio in humans and human aortic endothelial cells. Am. J. Clin. Nutr. **73**, 36–40.
[44] Sies, H., Schewe, T., Heiss, C., and Kelm, M. (2005) Cocoa polyphenols and inflammatory mediators. Am. J. Clin. Nutr. **81**, 304S–312S.
[45] Seeram, N. P., Zhang, Y., and Nair, M. G. (2003) Inhibition of proliferation of human cancer cells and cyclooxygenase enzymes by anthocyanidins and catechins. Nutr. Cancer **46**, 101–106.
[46] Santangelo, C., Vari, R., Scazzocchio, B., Di Benedetto, R., Filesi, C., and Masella, R. (2007) Polyphenols, intracellular signalling and inflammation. Ann. Ist. Super. Sanita **43**, 394–405.
[47] Mack, C. L. (2007) Serum cytokines as biomarkers of disease and clues to pathogenesis. Hepatology **46**, 6–8.
[48] Mao, T. K., Powell, J., Van De Water, J., Keen, C. L., Schmitz, H. H., Hammerstone, J. F., and Gershwin, M. E. (2000) The effect of cocoa procyanidins on the transcription and secretion of interleukin 1 beta in peripheral blood mononuclear cells. Life Sci. **66**, 1377–1386.
[49] Mao, T. K., Van De Water, J., Keen, C. L., Schmitz, H. H., and Gershwin, M. E. (2002) Effect of cocoa flavanols and their related oligomers on the secretion of interleukin-5 in peripheral blood mononuclear cells. J. Med. Food **5**, 17–22.
[50] Al-Hanbali, M., Ali, D., Bustami, M., Abdel-Malek, S., Al-Hanbali, R., Alhussainy, T., Qadan, F., and Matalka, K. Z. (2009) Epicatechin suppresses IL-6, IL-8 and enhances IL-10 production with NF-kappaB nuclear translocation in whole blood stimulated system. Neuroendocrinol. Lett. **30**, 131–138.
[51] Terra, X., Montagut, G., Bustos, M., Llopiz, N., Ardevol, A., Blade, C., Fernandez-Larrea, J., Pujadas, G., Salvado, J., Arola, L., and Blay, M. (2009) Grape-seed procyanidins prevent low-grade inflammation by modulating cytokine expression in rats fed a high-fat diet. J. Nutr. Biochem. **20**, 210–218.
[52] Terra, X., Palozza, P., Fernandez-Larrea, J., Ardevol, A., Blade, C., Pujadas, G., Salvado, J., Arola, L., and Blay, M. T. (2011) Procyanidin dimer B1 and trimer C1 impair inflammatory response signalling in human monocytes. Free Radic. Res. **45**, 611–619.
[53] Miyake, M., Ide, K., Sasaki, K., Matsukura, Y., Shijima, K., and Fujiwara, D. (2008) Oral administration of highly oligomeric procyanidins of Jatoba reduces the severity of collagen-induced arthritis. Biosci. Biotechnol. Biochem. **72**, 1781–1788.
[54] Yoon, J. H. and Baek, S. J. (2005) Molecular targets of dietary polyphenols with anti-inflammatory properties. Yonsei Med. J. **46**, 585–596.
[55] Ghosh, S., May, M. J., and Kopp, E. B. (1998) NF-kappa B and rel proteins: evolutionarily conserved mediators of immune responses. Annu. Rev. Immunol. **16**, 225–260.
[56] Hayden, M. S., West, A. P., and Ghosh, S. (2006) NF-kappa B and the immune response. Oncogene **25**, 6758–6780.
[57] Li, Q. T. and Verma, I. M. (2002) NF-kappa B regulation in the immune system. Nat. Rev. Immunol. **2**, 725–734.
[58] Hayden, M. S. and Ghosh, S. (2004) Signaling to NF-kappa B. Genes Dev. **18**, 2195–2224.
[59] Oeckinghaus, A. and Ghosh, S. (2009) The NF-kappa B family of transcription factors and its regulation. Cold Spring Harb. Perspect. Biol. **1**: a000034.
[60] Gilmore, T. D. (1999) The Rel/NF-kappa B signal transduction pathway: introduction. Oncogene **18**, 6842–6844.
[61] Pahl, H. L. (1999) Activators and target genes of Rel/NF-kappa B transcription factors. Oncogene **18**, 6853–6866.
[62] Mackenzie, G. G., Carrasquedo, F., Delfino, J. M., Keen, C. L., Fraga, C. G., and Oteiza, P. I. (2004) Epicatechin, catechin, and dimeric

procyanidins inhibit PMA-induced NF-kappaB activation at multiple steps in Jurkat T cells. FASEB J. **18**, 167–169.

[63] Chacon, M. R., Ceperuelo-Mallafre, V., Maymo-Masip, E., Mateo-Sanz, J. M., Arola, L., Guitierrez, C., Fernandez-Real, J. M., Ardevol, A., Simon, I., and Vendrell, J. (2009) Grape-seed procyanidins modulate inflammation on human differentiated adipocytes in vitro. Cytokine **47**, 137–142.

[64] Mackenzie, G. G., Delfino, J. M., Keen, C. L., Fraga, C. G., and Oteiza, P. I. (2009) Dimeric procyanidins are inhibitors of NF-kappaB-DNA binding. Biochem. Pharmacol. **78**, 1252–1262.

[65] Terra, X., Pallares, V., Ardevol, A., Blade, C., Fernandez-Larrea, J., Pujadas, G., Salvado, J., Arola, L., and Blay, M. (2011) Modulatory effect of grape-seed procyanidins on local and systemic inflammation in diet-induced obesity rats. J. Nutr. Biochem. **22**, 380–387.

[66] Pallares, V., Calay, D., Cedo, L., Castell-Auvi, A., Raes, M., Pinent, M., Ardevol, A., Arola, L., and Blay, M. (2012) Additive, antagonistic, and synergistic effects of procyanidins and polyunsaturated fatty acids over inflammation in RAW 264.7 macrophages activated by lipopolysaccharide. Nutrition **28**, 447–457.

[67] Pallares, V., Calay, D., Cedo, L., Castell-Auvi, A., Raes, M., Pinent, M., Ardevol, A., Arola, L., and Blay, M. (2012) Enhanced anti-inflammatory effect of resveratrol and EPA in treated endotoxin-activated RAW 264.7 macrophages. Br. J. Nutr. 1–12. Available on CJO 2012 doi:10.1017/S0007114511007057.

2. Hypothesis and Objectives

The metabolism and immune systems are among the most fundamental requirements for survival, and the resulting interface from its multilevel interactions, immunometabolism, is a central mechanism for the promotion and maintenance of homeostasis. Therefore, the improper regulation of immunometabolism underlies the onset and progression of several diseases, such as the obesity, as well as offers a substantial therapeutic target to maintain and improve health.

The importance of the nutritional profile in the maintenance of homeostasis and thus in health is well established and there are many diseases that result from chronic metabolic imbalances related to diet, including obesity, type 2 diabetes, inflammatory diseases and cancer. However, nutrition can also be considered as a source of bioactive compounds.

From a molecular perspective, nutrition can be defined as a set of dietary signals sensed by immune and metabolic cells and capable of modulating its functionality through the regulation of gene expression, protein synthesis, and metabolite production. Therefore, the nutritional profile might play a crucial role for the proper regulation of immunometabolism and thus health. Based on these concepts is nutritional immunotherapy, which is intended to promote homeostasis through optimising the functionality of the body's own immune system. Taking this into account, we hypothesised that **the nutritional profile and, more specifically, food-derived molecular signals, could modulate immunometabolism**.

Because immunometabolism involves complex genetic, molecular, cellular and environmental interactions, we have analysed the nutritional regulation of immunometabolism based on the integration of different disciplines, including nutrition, metabolism, physiology, immunology, transcriptomics and bioinformatics. Different objectives have been proposed to assess this hypothesis:

1. **To determine the ability of food compounds to modulate the activation of the inflammatory response against a pathogen in innate immune effector cells (Chapter 2).**

To achieve this objective, LPS-stimulated human macrophages were challenged with different bioactive food compounds, including the procyanidins B_1, B_2 and C_1, as well as

the n-3 fatty acid DHA alone or in combination with individual procyanidins. The immunomodulatory effects were determined by the modulation of eicosanoid production and COX activity, the activation of the NF-κB signalling pathway and the secretion of pro-inflammatory mediators.

2. To determine whether innate immune cells can sense food-derived molecular signals.

After assessment of the role of food compounds in the modulation of innate immunity, the sensing capacity of different nutritional profiles by the NLRP3 inflammasome, the signalling pathway that integrates metabolism and inflammation, was evaluated. This main objective was separated into two parts, depending upon the nature of the dietary signals:

2.1. To evaluate whether B_2 procyanidin might modulate the activation of the inflammasome (Chapter 3).

To determine if bioactive food compounds could interfere in the bacterial activation of the NLRP3 inflammasome, LPS-induced macrophages were treated with procyanidin B_2, one of the most immunologically active molecules within the procyanidins. The capacity of B_2 to modulate LPS-mediated inflammasome activation was measured based on the modulation of activation of the NF-κB signalling pathway (the priming step), the assembly of NLRP3 (the activation step) and the secretion of IL-1β (the main goal of inflammasome activation).

2.2. To analyse the effect of the dietary fatty acid composition on inflammasome activation (Chapter 4).

The nutritional profile sensing capacity of the inflammasome was assessed through the immunomodulatory effects of four dietary fatty acids differentiated by chemical structure: palmitate and stearic acid (saturated fatty acids), docosahexaenoic acid (n-3 PUFA) and arachidonic acid (n-6 PUFA). The inflammasome sensing capacity was assessed through the supplementation of the different fatty acids as priming signals for ATP-activated macrophages, in the ability of fatty acids to interfere with LPS-induced inflammasome activation and in the role of dietary fatty acids in the activation of inflammasome.

3. To analyse the effect of chronic overfeeding of highly palatable and energy-dense foods in the regulation of immunometabolism in rats (Chapter 5).

To assess the role of diet-induced obesity in immunometabolic function, rats were fed the cafeteria diet, which is a robust model of human metabolic syndrome. The capacity of the immune system to recognise and respond to changes in metabolic function was determined based on the adipose tissue-triggered inflammatory response as well as through the functionality of the thymus and the spleen.

4. **To evaluate whether homeostasis could be improved by targeting immunometabolism through supplementing the diet with bioactive food compounds (Chapter 6).**

To determine the role of the nutritional profile in the promotion and maintenance of homeostasis and therefore health, rats with an impaired immunometabolism, as a consequence of diet-induced obesity, were challenged with bioactive food compounds, including a procyanidin extract, oil rich in DHA or the combination of both, to improve homeostasis by targeting immunometabolism through the diet.

5. **To determine if genetic and environmental factors can interfere with the link between metabolic and immunological abnormalities in the context of diet-induced obesity (Chapter 7).**

The influence of genetic background on the phenotypic response to diet-induced obesity was analysed in two inbred rat strains subjected to a metabolic challenge based on dietary energy content. The role of genotype was evaluated by comparing phenotypic and genetic adaptation, as well as by comparing the obesity-induced modulation of the monocyte transcriptome in both genetic backgrounds.

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3. Results

CHAPTER 2

Omega-3 docosahexaenoic acid and procyanidins inhibit cyclo-oxygenase activity and attenuate NF-κB activation through a p105/p50 regulatory mechanism in macrophage inflammation

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Omega-3 docosahexaenoic acid and procyanidins inhibit cyclo-oxygenase activity and attenuate NF-κB activation through a p105/p50 regulatory mechanism in macrophage inflammation

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The inflammatory response has been implicated in the pathogenesis of many chronic diseases. Along these lines, the modulation of inflammation by consuming bioactive food compounds, such as $\omega-3$ fatty acids or procyanidins, is a powerful tool to promote good health. In the present study, the administration of DHA (docosahexaenoic acid) and B_1, B_2 and C_1 procyanidins, alone or in combination, prevented the inflammatory response induced by the LPS (lipopolysaccharide) endotoxin in human macrophages and brought them to the homoeostatic state. DHA and B_1 were strong and selective negative regulators of cyclo-oxygenase 1 activity, with IC_{50} values of 13.5 μM and 8.0 μM respectively. Additionally, B_2 and C_1 were selective inhibitors of pro-inflammatory cyclo-oxygenase 2 activity, with IC_{50} values of 9.7 μM and 3.3 μM respectively. Moreover, DHA and procyanidins prevented the activation of the NF-κB (nuclear factor κB) cascade at both early and late stages with shared mechanisms. These included inhibiting IκBα (inhibitor of NF-κB α) phosphorylation, inducing the cytoplasmic retention of pro-inflammatory NF-κB proteins through p105 (NF-κB1) overexpression, favouring the nuclear translocation of the p50–p50 transcriptional repressor homodimer instead of the p50–p65 pro-inflammatory heterodimer, inhibiting binding of NF-κB DNA to κB sites and, finally, decreasing the release of NF-κB-regulated cytokines and prostaglandins. In conclusion, DHA and procyanidins are strong and selective inhibitors of cyclo-oxygenase activity and NF-κB activation through a p105/p50-dependent regulatory mechanism.

Key words: cyclo-oxygenase, docosahexaenoic acid (DHA), nuclear factor κB (NF-κB), omega-3 fatty acid, procyanidin, prostaglandin.

INTRODUCTION

The inflammatory response is a body defence mechanism that is triggered by an external aggression, such as trauma or infection by external pathogens, and it represents a complex network of cellular and molecular interactions that is responsible for facilitating tissue repair and for returning the cell to physiological homoeostasis [1,2]. The cellular response is composed of local and systemic activation processes and is mediated by transcription factors, such as NF-κB (nuclear factor κB), that modulate the synthesis and secretion of cytokines such as IL (interleukin)-6 or prostanoids formed by the metabolism of arachidonic acid by the COX (cyclo-oxygenase) pathway [3].

Macrophages can be considered as the main co-ordinators of the innate immune response, as they are the first to recognize exogenous pathogens. Through a series of receptors based on pattern recognition, including TLR (Toll-like receptor) types and complement receptors, macrophages are able to identify the most common pathogens that trigger the inflammatory response [1,4].

IL-6 is one of the most important pro-inflammatory cytokines secreted by macrophages in response to an inflammatory stimulus. IL-6 is involved in the modulation of a broad range of cellular and physiological responses [5]. COX, or PGHS (prostaglandin H synthase), is responsible for the metabolic conversion of $\omega-6$ AA (arachidonic acid), the most abundant fatty acid present in the cell membrane, into PGE_2 (prostaglandin E_2) and TX (thromboxane) [5–7]. Additionally, prostanoids can also be synthesized from $\omega-3$ fatty acids, such as DHA (docosahexaenoic acid). The $\omega-6$ and $\omega-3$ fatty acids lead to two different sets of eicosanoids, the E_2 and E_3 series respectively [8]. These eicosanoids act as chemical messengers for the immune system, and their principal function involves regulating the inflammatory response. Three isoforms of COX (EC number 1.14.99.1) responsible for the biosynthesis of eicosanoids have been described, which include COX1 (or prostaglandin endoperoxide synthase 1), COX2 (or prostaglandin endoperoxide synthase 2) and COX3 (or prostaglandin endoperoxide synthase 3) [7,9]. COX1 is constitutively expressed in most cells and is therefore regarded as a housekeeping molecule and is involved in the mediation of different physiological responses (e.g. the protection of the gastric epithelium or platelet aggregation). COX2 remains undetectable in the majority of mammalian tissues under basal conditions, although its expression is inducible by several inflammatory stimuli [e.g. LPS (lipopolysaccharide) or inflammatory cytokines], leading to an increase in PGE_2 synthesis. Additionally, the activation of COX2 is implicated in numerous inflammation-associated diseases and instances of tumorigenesis [6,9]. Therefore COX1 and COX2 are of particular interest because they are the major targets of NSAIDs (non-steroidal anti-inflammatory drugs). COX3 has been described as a splice

Abbreviations used: AA, archidonic acid; COX, cyclo-oxygenase; DHA, docosahexaenoic acid; ERK, extracellular-signal-regulated kinase; HRP, horseradish peroxidase; IκB, inhibitor of nuclear factor κB; IKK, IκB kinase; IL, interleukin; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB; NSAID, non-steroidal anti-inflammatory drug; PBMC, peripheral blood mononuclear cell; PGE_2, prostaglandin E_2; PUFA, polyunsaturated fatty acid; TLR, Toll-like receptor.

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variant of COX1; however, its biological role is still poorly studied [10].

The transcription factor NF-κB regulates the transcription of numerous genes that control the immune response [11,12]. Although NF-κB plays an essential role in normal physiology and in the inflammatory immune response, the constitutive activation of NF-κB is associated with different diseases [11–14]. There are two NF-κB signalling pathways: the classical, or canonical, pathway and the alternative route. The main difference between these two activation pathways includes the specific IKK [IκB (inhibitor of NF-κB) kinase] family members that are involved [13]. In mammals, the NF-κB family consists of five proteins, including p65 (RelA), RelB, c-Rel, p105/p50 (NF-κB1) and p100/p52 (NF-κB2), that associate with each other to form transcriptionally distinct homo- and hetero-dimeric complexes [15], although the p65–p50 heterodimer represents the most abundant of the Rel dimers. NF-κB family proteins are characterized by the presence of the RHD (Rel homology domain), a 300 amino acid N-terminal segment responsible for DNA binding, dimerization, nuclear translocation, interaction with IκB family proteins and transcriptional regulation [12–14]. In resting cells, the NF-κB heterodimer is retained in the cytoplasm and is bound to its inhibitory protein, IκB, a member of the IκB family of proteins. In response to an inflammatory stimulus, such as LPS from the bacterial cell wall, the classical pathway of NF-κB activation leads to the activation of IKKβ, a member of the IKK complex, and triggers the phosphorylation of IκBα protein (pIκBα). pIκBα is recognized by the ubiquitin ligase machinery, causing its polyubiquitination and subsequent proteasomal degradation. After pIκBα degradation, the NF-κB heterodimer is able to translocate to the nucleus, where, bound to the κB motif found in the promoter or enhancer regions, it induces the expression of numerous pro-inflammatory genes [12]. The alternative activation pathway of NF-κB is not activated by LPS, and it is independent of IKKβ and IKKγ [11–13,16].

Procyanidins are phenolic compounds that are present in a variety of vegetables, fruits and cereals, but are mainly found in cocoa, grapes and apples. Procyanidins are polymers that are composed of flavan-3-ol units, such as catechin and epicatechin. Procyanidins can be defined as bioactive food compounds, owing to their influence on physiological status and cellular homoeostasis, as well as their beneficial effects on health, such as inhibition of the release of pro-inflammatory mediators [17–20]. The mechanisms involved in the modulation of the inflammatory response by procyanidins are not yet well understood and are currently a subject of intense study.

PUFAs (polyunsaturated fatty acids) are important constituents of cells that are mainly involved in preserving the fluidity of the cell membrane, regulating cellular signals or modulating gene expression [21]. $\omega-3$ fatty acids that are present as bioactive food compounds can replace the $\omega-6$ fatty acids located in the cell membranes of macrophages, triggering a competition between $\omega-3$ and $\omega-6$ fatty acids during prostanoid formation [22]. In general, the eicosanoids from the 3 series that are derived from $\omega-3$ fatty acids have less inflammatory activity than the 2 series that are derived from $\omega-6$ fatty acids. In some cases, the eicosanoids derived from the $\omega-3$ fatty acids do not have the biological activities of their counterparts [23]. DHA is present in certain aquatic organisms, such as fish (fish oil) or marine algae. Studies have indicated that the supplementation of diets with $\omega-3$ fatty acids, such as DHA, produces beneficial effects on symptoms in diseases characterized by chronic inflammation, such as atherosclerosis, rheumatoid arthritis or obesity [24,25]. The mechanisms by which $\omega-3$ fatty acids, specifically DHA, exert anti-inflammatory effects remain unclear.

The present study is focused on the regulation of the inflammatory response by DHA and the procyanidins B_1, B_2 and C_1, alone or in combination, in LPS-stimulated human macrophages. This modulation of the inflammatory response was based upon the secretion of inflammatory mediators, such as IL-6 and PGE_2, as well as modulation of the activity of the COX pathway. Additionally, the modulation effects on the expression of NF-κB family proteins and their implications in the NF-κB signalling pathway were investigated.

MATERIAL AND METHODS

Reagents

Procyanidin dimers B_1 [epicatechin-(4$\beta\rightarrow$8)-catechin] and B_2 [epicatechin-(4$\beta\rightarrow$8)-epicatechin] were obtained from ExtraSynthese. Procyanidin trimer C_1 [epicatechin-(4$\beta\rightarrow$8)-epicatechin-(4$\beta\rightarrow$8)-epicatechin] was obtained from TransGmbH. *cis*-4-,7,10,13,16,19-DHA was obtained from Sigma–Aldrich. RPMI 1640 culture medium was purchased from Gibco. Cell culture reagents were provided by BioWhittaker, except for the differentiating agent PMA, which was purchased from InvivoGen, and LPS (*Escherichia coli* 0111:B4), which was purchased from Sigma–Aldrich. The anti-p65, anti-p50/p105, anti-pIκBα and FITC-conjugated goat anti-rabbit IgG were acquired from Santa Cruz Biotechnology, and the anti-COX2 polyclonal antibody was purchased from Bioworld. The HRP (horseradish peroxidase)-conjugated monoclonal anti-rabbit IgG antibody was obtained from GE Healthcare, and the advanced ECL (enhanced chemiluminescence) Western blotting detection kit was provided by GE Healthcare. The NSAIDs nimesulide, sc-560 and indomethacin were purchased from Cayman Chemicals. Bradford reagent and Histopaque®-1077 were obtained from Sigma–Aldrich.

Cell lines and cell culture

Human THP-1 monocytes were purchased from the European Collection of Animal Cell Cultures (ECACC number 88081201). Cells were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin, 25 mM Hepes and 10 % FBS (fetal bovine serum) at 37 °C in a humidified incubator with 5 % CO_2. The differentiation of THP-1 monocytes into macrophages was induced by growing the cells in fresh medium containing 0.5 μg/ml PMA for 24 h. After differentiation, the medium containing PMA was removed and adherent THP-1 macrophages were incubated in RPMI medium for 24 h. Later, macrophages were treated with 25 μM DHA, 17.3 μM B_1, 17.3 μM B_2 or 11.5 μM C_1, or a combination of DHA with one of the procyanidins in serum-free RPMI 1640-supplemented medium. For all experiments, control cells treated with ethanol alone (vehicle of the DHA and procyanidins) were included (final concentration in the medium was $\leqslant$0.1 %). Finally, all of the macrophages, except for the unstimulated control, were stimulated with 1 μg/ml LPS. The experimental design is shown in Figure 1. Cell viability was assessed as better than 97 % under all experimental conditions using Trypan Blue staining (results not shown).

Isolation of primary human monocyte-derived macrophages

Five healthy donors were recruited from the University Hospital *Joan XXIII*. The study was approved by the Institutional Review Board. All participants gave written informed consent for participation in medical research. Human PBMCs (peripheral

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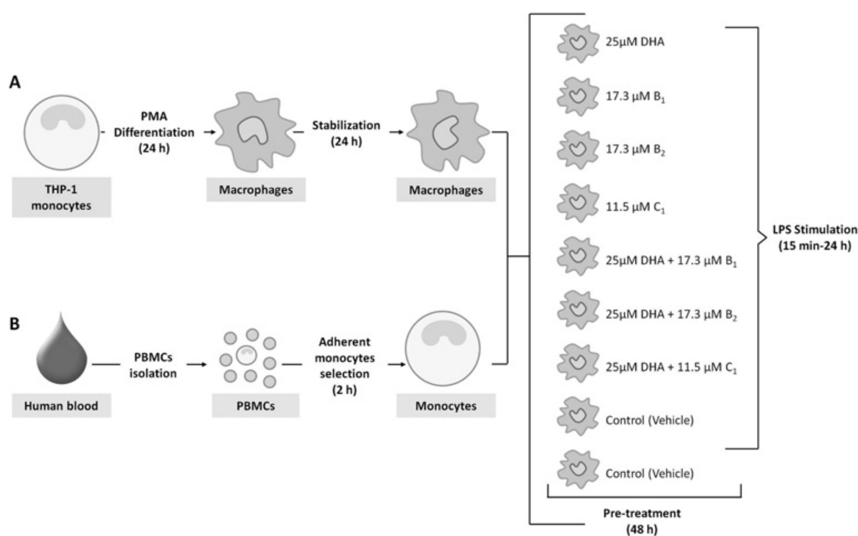

Figure 1 Schematic diagram of the experimental design

Macrophages used in the present study were obtained from two origins: THP-1 monocytes and primary human macrophages. (**A**) THP-1 monocytes were differentiated into macrophages in the presence of 0.5 μg/ml PMA for 24 h. After differentiation, the macrophages were stabilized with PMA-free medium for 24 h prior to DHA and/or procyanidin pre-treatment. (**B**) PBMCs were isolated from healthy blood donors using a density gradient. Monocytes were purified and activated by adherence to plastic after 2 h. THP-1 macrophages and primary human macrophages were pre-treated with 25 μM DHA, 17.3 μM B_1, 17.3 μM B_2 or 11.5 μM C_1, or a combination of DHA and one of the procyanidins for 48 h. Additionally, control cells treated with just the vehicle (ethanol) were used. Finally, pre-treated macrophages were stimulated with 1 μg/ml LPS, except for the control group (without LPS stimulation), for 15 min to 24 h, depending on the subsequent analysis.

blood mononuclear cells) were isolated from healthy blood donors by density gradient centrifugation using Histopaque® -1077, according to the manufacturer's protocol (Sigma–Aldrich). Histopaque®-1077 is a solution of polysucrose (5.7 g/dl) and sodium diatrizoate (9 g/dl), adjusted to a density of 1.077 ± 0.001 g/ml. Monocytes were purified by adherence to plastic in serum-free RPMI 1640-supplemented medium inducing monocyte activation. Briefly, 2×10^6 cells/ml were seeded into 12-well plates and, after 2 h, non-adherent cells were removed by several washes with warm PBS. Freshly isolated primary activated monocytes were treated with DHA and/or procyanidins and were stimulated with LPS following the same protocol as previously described for THP-1 macrophages, which is shown in Figure 1.

IL-6 secretion

The effect of DHA/procyanidin pre-treatments on IL-6 concentration was measured with an ELISA kit following the manufacturer's protocol (BioLegend). The secretion of IL-6 was normalized according to the protein content measured using a Bradford assay.

PGE_2 secretion

The effect of the DHA/procyanidin pre-treatments on PGE_2 in LPS-stimulated macrophage supernatants was determined by a specific competitive immunoassay (EIA) following the manufacturer's protocol (Cayman Chemicals). The secretion of PGE_2 in culture supernatants was normalized according to the protein concentration, which was determined using a Bradford assay.

COX1 and COX2 cell-free assay

The kinetic effects of the treatments (DHA, B_1, B_2 and C_1) on the activity of COX1 and COX2 enzymes were determined using a cell-free inhibition assay, on the basis of the fact that the rate-limiting step in prostaglandin synthesis is catalysed by COXs. Then, the modulation of the synthesis of PGE_2 from AA by DHA, B_1, B_2 and C_1 was determined. Briefly, the cell-free assay was performed using purified COX1 from ram seminal vesicles and COX2 from sheep placental cotyledons (Cayman Chemicals). The COX isoforms were incubated with various concentrations of AA (0.15–25 μM) for 20 min (pH 8.0), at 37 °C in the presence of 18 mM adrenaline (epinephrine), 5 μM haematin and concentrations of DHA, B_1, B_2, and C_1 that ranged from 0.01 to 500 μM, as well as commercial inhibitors (indomethacin, nimesulide and sc-560). The reactions were stopped by the addition of 10 μl of 10 % formic acid. The PGE_2 concentration was determined using a specific competitive immunoassay (EIA, Cayman Chemicals) [26]. IC_{50} values were expressed as the concentration of compound required to inhibit 50 % of the PGE_2 biosynthesis. The kinetic constants were analysed from double-reciprocal plots of velocity against AA concentration using GraphPad Prism (version 5.0). Kinetic parameters were obtained by fitting the points to the Michaelis–Menten kinetic equation:

$$V = \frac{V_{\max} \cdot [S]}{K_m + [S]}$$

where V is the initial rate, $V_{\max}$ represents the maximum enzyme rate at saturating substrate concentrations, [S] is the substrate concentration and K_m is the substrate concentration at which the reaction rate is half that of $V_{\max}$. The turnover number, or catalytic constant (k_{cat}), was calculated from the following equation:

$$k_{cat} = \frac{V_{\max}}{[E]_t}$$

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where $[E]_t$ represents the total active enzyme concentration. Additionally, the dissociation constant for inhibitor binding (K_i) was obtained from the following equation:

$$K_i = \frac{[\text{inhibitor}]}{\frac{K_{m,obs}}{K_m} - 1}$$

where $K_{m,obs}$ represents the K_m value specific for each inhibitor, and K_m is the value obtained under control conditions.

Nuclear and cytoplasm extract preparation

Pre-treated LPS-stimulated THP-1 cytoplasmic and nuclear proteins were extracted using methods that have been described previously [25,27]. Briefly, 2×10^6 cells were washed twice with ice-cold PBS. Cells were lysed in ice-cold buffer A [10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM PMSF, 0.5 mM DTT (dithiothreitol), 0.1 % Nonidet P40 and 10 μg/ml protease and phosphatase inhibitors]. Subsequently, cells were centrifuged, and the supernatant, i.e. the cytoplasmic fraction, was removed and stored at -80 °C. The nuclear pellet was washed once with PBS and resuspended in ice-cold buffer B [20 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 25 % glycerol, 0.5 mM PMSF and 10 μg/ml protease and phosphatases inhibitors]. The nuclear extract was obtained by centrifugation, and the supernatant, i.e. the nuclear fraction, was removed and stored at -80 °C. The protein concentrations in the cytoplasmic and nuclear fractions were quantified with a Bradford assay using BSA as the standard.

Western blot analysis

Cytoplasmic and nuclear proteins were electrophoresed by SDS/PAGE, and separated proteins were transferred on to a PVDF membrane. The blots were blocked and incubated overnight at 4 °C with the appropriate primary antibody. After washing, the blots were incubated with HRP-conjugated secondary antibody, developed with a chemiluminescent reagent (GE Healthcare) and exposed using the Alpha Innotech FluorChem FC2 Imager. A semi-quantitative analysis of the proteins was performed using ImageJ software.

Immunofluorescence staining of NF-κB p65 and confocal microscopy

THP-1-derived macrophages were cultured on sterile commercial poly-L-lysine-treated coverslips (BD BioCoat, BD Biosciences) prior to pre-treatment with DHA and/or procyanidins for 48 h. Then, THP-1 macrophages were activated with LPS for 1 h. Cells were washed, fixed in 4 % paraformaldehyde and permeabilized with 0.5 % Triton X-100. After incubation with blocking buffer, the cells were incubated with NF-κB p65 primary antibody overnight at 4 °C and were then incubated with FITC-conjugated goat anti-mouse IgG. The coverslips were washed, and the samples were then counterstained with ethidium bromide and were mounted in Sigma–Aldrich 10979 mounting medium with polyvinyl alcohol and anti-fading reagent. Confocal images were acquired with a laser confocal-scanning microscope (Nikon Eclipse TE2000-E) using EZ-C1 3.40 software.

NF-κB-binding assay

The effect of pre-treatment with DHA and/or procyanidins on NF-κB p65 binding to target DNA was determined with the TransAM NF-κB Chemi Assay (Active Motif), according to the manufacturer's protocol.

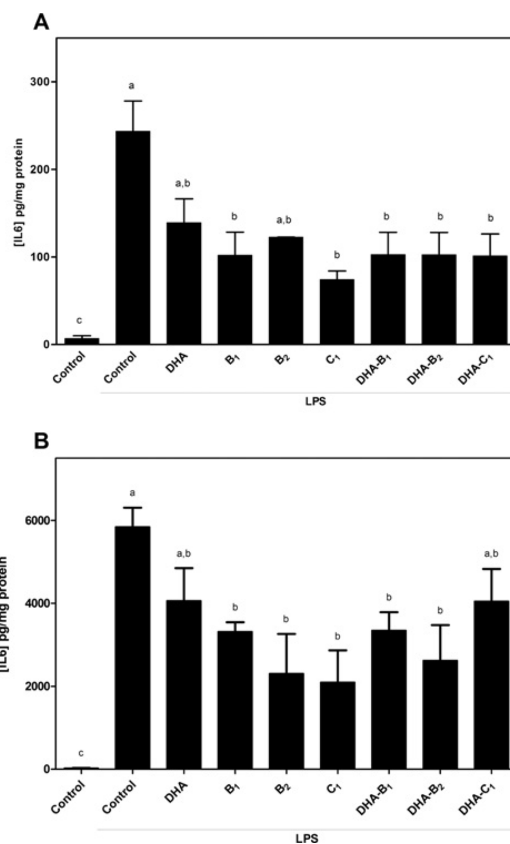

Figure 2 Modulation of IL-6 secretion in LPS-stimulated human macrophages

THP-1 and primary human macrophages were pre-treated with 25 μM DHA, 17.3 μM B_1, 17.3 μM B_2 or 11.5 μM C_1, or a combination of DHA and one of the procyanidins for 48 h. Then the macrophages were stimulated with 1 μg/ml LPS for 24 h (except for the control group, without LPS stimulation). (**A**) Inhibition of IL-6 release in THP-1 macrophages. (**B**) Inhibition of IL-6 secretion in primary human macrophages. Results are presented as means $\pm$ S.E.M. for three independent experiments for THP-1 macrophages and five donors for primary human macrophages. Letters indicate significant differences among the IL-6 secretions. Bars that share the same letters are not significantly different from one another, but bars with different letters are significantly different (one-way ANOVA, Tukey test, $P < 0.05$).

Statistical analysis

Results are expressed as the means $\pm$ S.E.M. Effects of the pre-treatments were assessed using ANOVA. We used a Tukey test to make pairwise comparisons. Differences were considered significant when the P values were <0.05. All calculations were performed using SPSS 17.0 software.

RESULTS

DHA, B_1, B_2 and C_1 inhibit cytokine production in human macrophages

The secretion of IL-6 was regulated by the pre-treatment of macrophages with DHA, B_1, B_2 or C_1, or the combination

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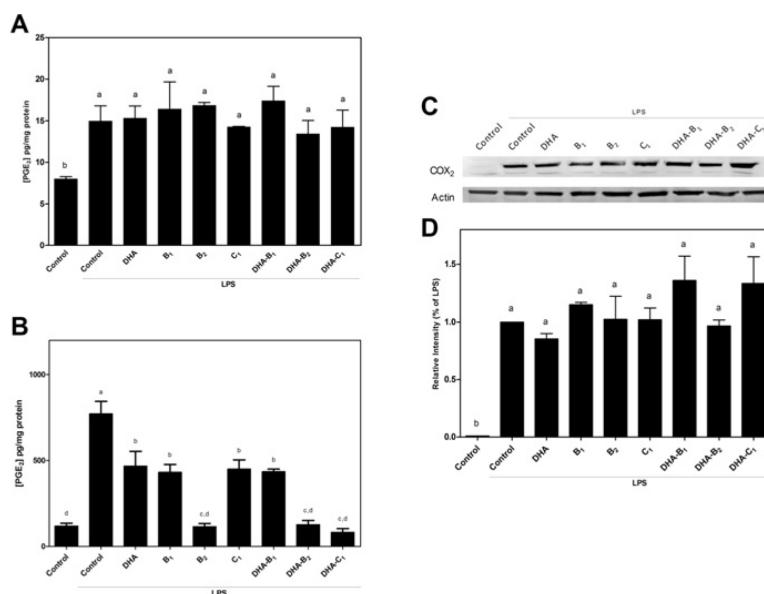

Figure 3 Effect of pre-treatment with 25 μM DHA, 17.3 μM B_1, 17.3 μM B_2 or 11.5 μM C_1, or a combination of DHA and one of the procyanidins for 48 h on the secretion of PGE_2 and COX2 protein expression in LPS-stimulated human macrophages

(**A**) Effect of pre-treatments on PGE_2 secretion in THP-1 macrophages. (**B**) Modulation effects of DHA and/or procyanidin pre-treatments on PGE_2 release in human primary macrophages. (**C**) Representative blots of COX2 expression in THP-1 macrophages. (**D**) Relative levels of COX2 expression in THP-1 macrophages quantified by densitometry; data are expressed relative to the amount of COX2 expression in vehicle LPS-stimulated control cells. Results are represented as the means $\pm$ S.E.M. for three independent experiments for THP-1 macrophages and five donors for primary human macrophages. Letters indicate significant differences (one-way ANOVA, Tukey's test, $P < 0.05$) between pre-treatments in each determination.

of DHA with a procyanidin for 48 h. In both THP-1 macrophages (Figure 2A) and primary human macrophages (Figure 2B), the release of IL-6 was inhibited by the pre-treatments when compared with the LPS-stimulated control. In THP-1 macrophages, pre-treatment with the B_1 dimer, C_1 trimer or any combination of DHA and procyanidins was more efficient in inhibiting the release of IL-6. Additionally, the secretion of IL-6 in primary human macrophages was significantly inhibited by B_1, B_2 and C_1, and any combination of DHA with procyanidin dimers.

Differential modulation of PGE_2 secretion and COX2 protein expression in human macrophages by DHA, B_1, B_2 or C_1

The effect of the pre-treatments on the modulation of PGE_2 secretion differed depending on the macrophage cell source. As shown in Figure 3(A), the pre-treatment of THP-1 macrophages with DHA and/or procyanidins did not lead to a significant decrease in PGE_2 secretion. Additionally, the post-transcriptional COX2 expression (Figure 3B) was not modulated by DHA or procyanidins. Nevertheless, in primary human macrophages, the secretion of PGE_2 was significantly inhibited with all of the pre-treatments relative to the LPS-stimulated control group. In these cases, the pre-treatments with B_2 and combinations of DHA with B_2 and C_1 were the most efficient in decreasing the secretion of PGE_2 levels to near the basal state, which corresponded to the LPS-unstimulated control group.

DHA and procyanidins are modulators of COX activity

To carefully study the modulation of COX activity during prostaglandin biosynthesis by DHA and procyanidins, a cell-free assay was performed. The concentration of inhibitor that reduced prostaglandin production by half (IC_{50}) was used to evaluate the effects of DHA and procyanidins in COX1 and COX2 activity analyses. Three commercial NSAIDs, sc-560 (selective COX1 inhibitor), nimesulide (selective COX2 inhibitor) and indomethacin (non-selective COX inhibitor) were used as controls (Table 1). DHA and procyanidins discriminated between the two COX isoforms. DHA and the B_1 dimer were strong selective COX1 inhibitors (IC_{50} value of 13.5 μM and 8.0 μM respectively), whereas the B_2 dimer and C_1 trimer were strong selective COX2 inhibitors (IC_{50} value of 9.7 μM and 3.3 μM respectively). In an effort to characterize the mechanism of inhibition of COX1 and COX2 by DHA and procyanidins, the effects of the AA concentration on the rate of enzyme inactivation were studied by using a fixed concentration of inhibitor (15 μM for DHA, and 10 μM for B_1, B_2 and C_1). Kinetic parameters were determined by constructing Michaelis–Menten curves (Figure 4) and Lineweaver–Burk transformations (shown as insets in Figure 4A for COX1 and Figure 4B for COX2 respectively). The kinetic parameters for the inhibition of COX1 by DHA and B_1 as well as the inhibition of COX2 by B_2 and C_1 are reported in Tables 2 and 3 respectively. The modulation of COX1 by DHA was a result of a decrease in the maximal reaction rate (V_{max}) and an increase in the Michaelis–Menten constant (K_m); therefore DHA acted as a mixed inhibitor of COX1 activity with a dissociation constant (K_i) of 0.73 ± 0.17 μM for the interaction of DHA with COX1. B_1 down-regulated the biosynthesis of PGE_2; additionally, it exhibited an increase in K_m, yet V_{max} was not affected. This indicated that B_1 competed with AA in the biosynthesis of PGE_2 by COX1 with a K_i value of 6.87 ± 0.66 μM. This competitive inhibition behaviour, indicated by an increase in K_m and an unaffected V_{max}, were also observed

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Table 1 Selective inhibition of COX enzymes

IC_{50} values were determined as the concentration of the compound required to inhibit 50 % of the PGE_2 production.

	IC_{50} (μM)	
Additive	COX1	COX2
DHA	13.5	>50
B_1	8.0	>500
B_2	>500	9.7
C_1	>500	3.3
Indomethacin	0.63	0.99
Nimesulide	19.0	4.9
sc-560	0.13	4.4

for the B_2 and C_1 regulation of PGE_2 biosynthesis by COX2. The dissociation constants for the interactions of B_2 and C_1 with COX2 were 7.75 ± 0.68 μM and 11.69 ± 1.25 μM respectively. The catalytic efficiencies (k_{cat}/K_m) of COX1 and COX2 were also significantly decreased by DHA and B_1, and by B_2 and C_1 respectively.

DHA and/or B_1, B_2 and C_1 procyanidins are down-regulators of NF-κB activation

The transcription factor NF-κB plays a major role in the induction of transcription of numerous pro-inflammatory genes. Thus the phosphorylation of IκBα is an essential step for the nuclear translocation of the NF-κB p65–p50 heterodimer. Figure 5(A) shows the effect of DHA and/or procyanidins on the phosphorylation of IκBα. The pre-treatment of THP-1 macrophages with DHA or any combination of DHA and procyanidins slightly decreased the phosphorylation of IκBα. The expression and intracellular localization of several members of the NF-κB family are also essential steps during the activation of the NF-κB pathway. Therefore the effects of DHA and/or procyanidins on the cytoplasmic and nuclear p105, p50 and p65 expression were studied (Figure 5). The cytoplasmic localization of p105 was induced by the B_2 dimer and the combination of DHA/B_2 pre-treatments on THP-1 macrophages. However, B_1, B_2 and DHA/B_1 pre-treatments inhibited the translocation of p50 to the nucleus through the induction of cytoplasmic p105 expression and inhibition of p50 translocation. Furthermore, all of the DHA and/or procyanidin pre-treatments inhibited the nuclear translocation of p65 in THP-1 macrophages through p65 cytoplasmic retention. Moreover, confocal microscopy was used to visualize the effects of the pre-treatments on nuclear and cytoplasmic p65 expression after 1 h of LPS activation. Figure 6 confirms the dramatic inhibition of nuclear translocation, shown in Figure 5, and demonstrates the retention of p65 in the cytoplasm rather than in the nuclei in all of the pre-treatments.

DHA, B_1, B_2 and C_1 decrease the NF-κB p65 DNA-binding activity

The functional effects of DHA and/or procyanidins on NF-κB–DNA binding was assessed based on the inhibition of NF-κB p65 binding to the κB consensus sequence, which is located in the promoter and enhancer regions of several pro-inflammatory genes. Figure 7 shows the inhibitory effects of all pre-treatments (DHA and/or procyanidins) on NF-κB–DNA binding activity.

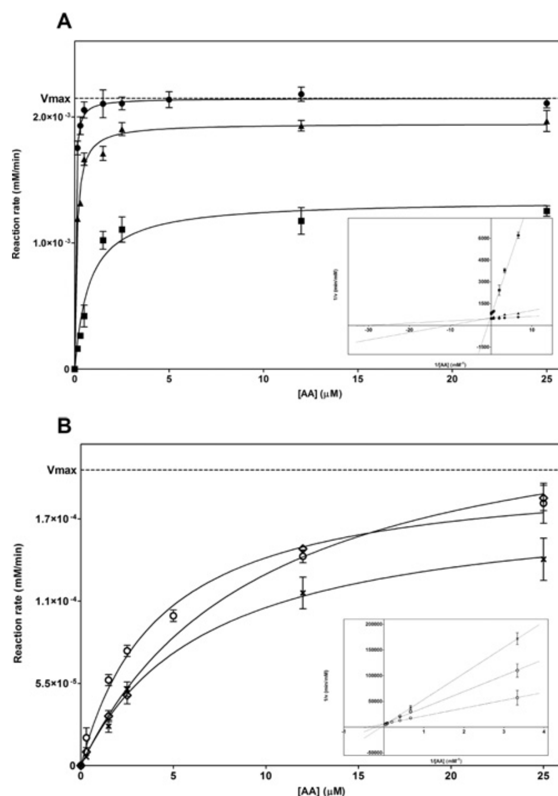

Figure 4 Determination of Michaelis–Menten and Lineweaver–Burk (insets) curves for the COX conversion of AA into PGE_2

The kinetic parameters corresponding to the selective inhibition of COX activity were determined by fitting the initial reaction rates to the standard Michaelis–Menten equation using a cell-free assay. (**A**) Representative plots for COX1 in control (●), DHA (▲) and B_1 (■) conditions. (**B**) Representative plots for COX2 in control (○), B_2 (◇) and C_1 (×) conditions. The kinetic study was determined using the mean of three determinations using eight concentrations of AA that ranged from 0.15 to 25 μM.

DISCUSSION

Bacterial LPS stimulates immune cells, such as monocytes and macrophages, to trigger the inflammatory response. This response is characterized by the release of an array of pro-inflammatory mediators, such as IL-6 or PGE_2, as well as the activation of TLR-4, which results in a signal transduction that activates the NF-κB pathway and, subsequently, the transcription of numerous pro-inflammatory genes. Therefore the modulation of the inflammatory response by the dietary intake of bioactive food compounds is a powerful tool for promoting a healthy and homoeostatic condition and for preventing disease development. The molecular mechanisms underlying the anti-inflammatory activities of bioactive food compounds, such as procyanidins and PUFAs, are poorly understood and have been under intense study. Furthermore, their combined effects are even less understood. The present study investigated the anti-inflammatory effects of the $\omega-3$ fatty acid DHA, the procyanidins B_1, B_2 and C_1, and the combination of DHA with any of the procyanidins in human macrophages. To determine whether the modulation of the inflammatory response was conserved between the THP-1 cell line and primary human macrophages, we

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Table 2 Kinetic parameters for selective COX1 inhibition by the DHA and B_1 dimers

Initial reaction rates were determined using a cell-free assay for various concentrations of AA (0.15–25 μM). Data were fitted to the standard Michaelis–Menten kinetic equation and Lineweaver–Burk transformation. The results are represented as means ± S.E.M. for three independent experiments, and the significance of the differences between the inhibitors for each kinetic parameter compared with the control condition was analysed by ANOVA (*$P < 0.05$).

Sample	V_{max} (μM/min)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$)	K_i (μM)
Control	$(2.14 \pm 0.016) \times 10^{-3}$	$(33 \pm 3.5) \times 10^{-3}$	$(0.151 \pm 1.0) \times 10^{-3}$	4.63 ± 0.47	–
DHA	$(1.34 \pm 0.05) \times 10^{-3}$*	$(762 \pm 11) \times 10^{-3}$*	$(0.094 \pm 4.1) \times 10^{-3}$	0.13 ± 0.03*	0.73 ± 0.17
B_1	$(1.95 \pm 0.026) \times 10^{-3}$	$(19 \pm 9.4) \times 10^{-3}$*	$(0.137 \pm 2.5) \times 10^{-3}$	1.32 ± 0.10*	6.87 ± 0.66

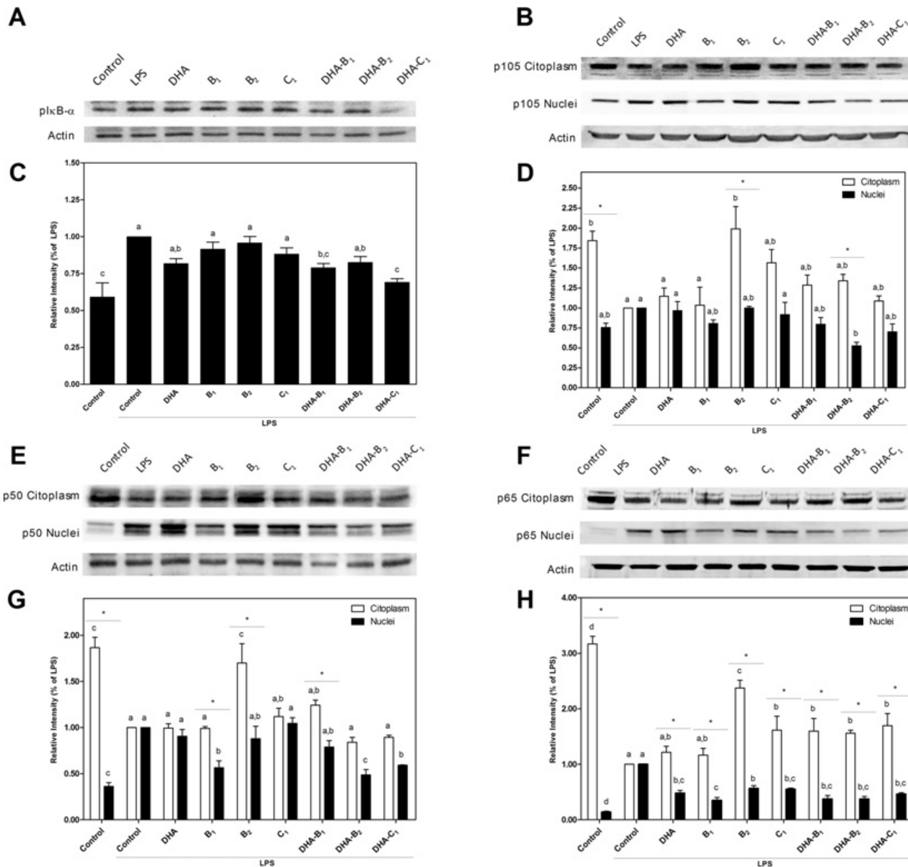

Figure 5 Inhibition of NF-κB pathway activation by DHA and/or procyanidins in THP-1 macrophages

Western blot assays of pIκBα (**A** and **C**), p105 (**B** and **D**), p50 (**E** and **G**) and p65 (**F** and **H**) protein components of the NF-κB pathway in cytoplasmic (black columns) and nuclear (white columns except for pIκBα) fractions of THP-1 macrophages. Cells were pre-treated with 25 μM DHA, 17.3 μM B_1, 17.3 μM B_2 or 11.5 μM C_1, or a combination of DHA and one of the procyanidins for 48 h following stimulation with 1 μg/ml LPS for a duration that ranged from 15 min to 1 h (except for the control group). (**A**) Representative blot for IκBα phosphorylation. (**B**, **E** and **F**) Representative blots for cytoplasmic and nuclear expression of p105, p50 and p65 respectively. (**C**, **D**, **G** and **H**) Relative protein levels of pIκBα, p105, p50 and p65 respectively, quantified by densitometry. Results are expressed relative to the amount of NF-κB family protein expression in the vehicle LPS-stimulated control cells. Results represent the means ± S.E.M. for four to five independent experiments. Letters indicate significant differences between the NF-κB protein expression by pre-treatment in the same intracellular localization (i.e. the cytoplasm or nucleus). Means that share the same letters indicate that pre-treatment was not significantly different from each other in the same intracellular localization (one-way ANOVA, Tukey test, $P < 0.05$). *Significant differences between intracellular localization (i.e. the cytoplasm and nucleus) in each pre-treatment (Student's t test, $P < 0.05$).

determined the IL-6 and PGE_2 levels for both human macrophage models.

The concentrations of DHA and procyanidins applied to the macrophage pre-treatments mimicked levels that would be typical from a Mediterranean diet [28–31]. It has previously been determined in a pharmacokinetic study that subjects who consumed fish once or twice a month had detectable plasma DHA levels of approximately 182 μM [32,33]. This concentration is higher than the 25 μM DHA tested in the present study. However, pharmacokinetic results from the administration of plant extracts rich in polyphenols, such as cocoa or grape seed extracts, concluded that plasma concentrations of unmodified

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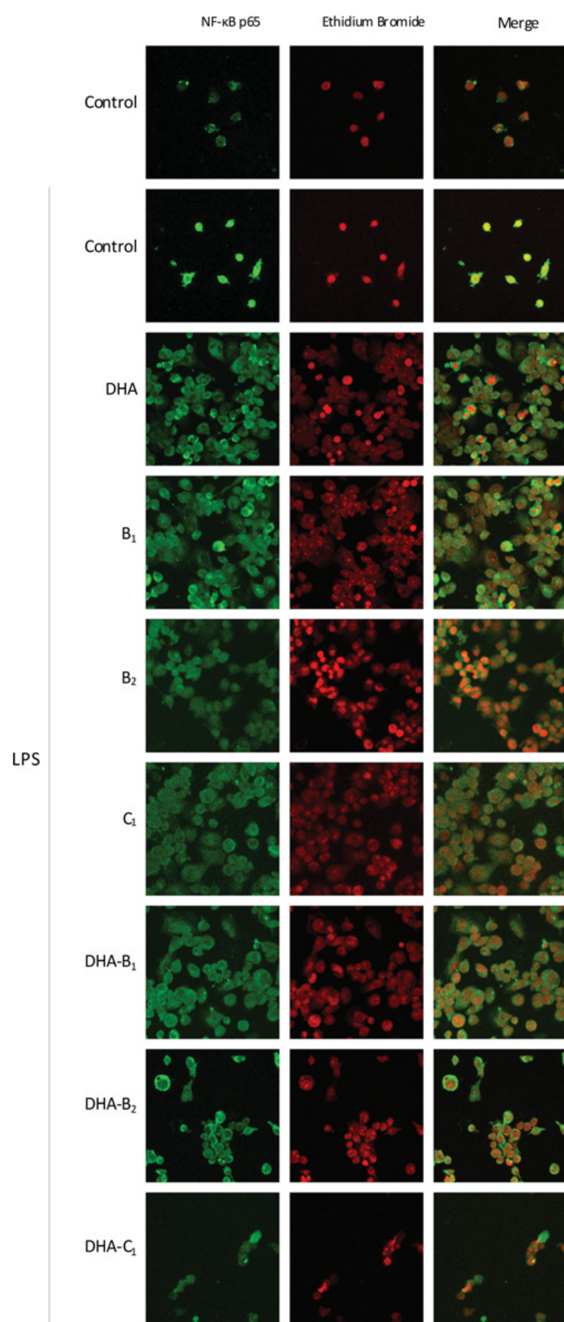

Figure 6 Inhibition of NF-κB p65 nuclear translocation by DHA and/or procyanidins (B_1, B_2 and C_1) in THP-1 macrophages

Indirect immunofluorescence and confocal microscopy analysis was used to visualize the subcellular localization of NF-κB p65. THP-1 macrophages were pre-treated with 25 μM DHA, 17.3 μM B_1, 17.3 μM B_2 or 11.5 μM C_1, or a combination of DHA and one of the procyanidins for 48 h following stimulation with 1 μg/ml LPS for 1 h (except for the control group). NF-κB p65 is represented by the green staining, nuclear DNA is revealed by ethidium bromide staining, and the combined images are presented. The results are representative of three independent experiments.

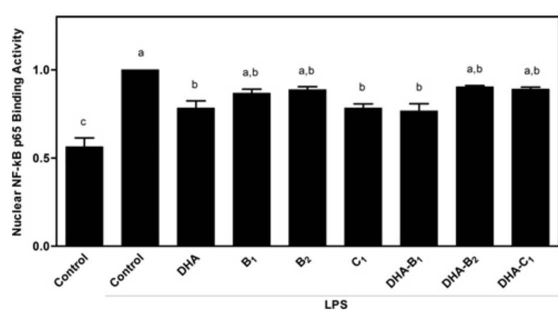

Figure 7 Inhibition of NF-κB p65 DNA-binding activity by DHA and/or B_1, B_2, and C_1 pre-treatments in THP-1 macrophages

A consensus site (5′-GGGACTTTCC-3′) binding assay was used to evaluate the effect of 25 μM DHA, 17.3 μM B_1, 17.3 μM B_2 or 11.5 μM C_1, or a combination of DHA and one of the procyanidins for 48 h following stimulation with 1 μg/ml LPS for 1 h (except for the control group) on NF-κB p65 DNA-binding activity. Results are represented as the means $\pm$ S.E.M. for three independent experiments. Letters indicate significant differences among the NF-κB p65 DNA-binding activities, which are expressed as a percentage of the LPS-stimulated control group. Bars that share the same letters are not significantly different from one another, but bars with different letters are significantly different (one-way ANOVA, Tukey's test, $P < 0.05$).

compounds depended on the polyphenol source. In this respect, the range of physiological concentrations of dimers and trimers detected was 2–10 μM in the plasma of mice models [28,29,34], although the range of dimer and trimer concentrations in human plasma can be greater. In the present study, the concentrations used were 17.3 μM for the B_1 and B_2 dimers, and 11.5 μM for the C_1 trimer, although we observed the same anti-inflammatory behaviour using half the concentration for each procyanidin (results not shown).

IL-6 is a potent pro-inflammatory cytokine involved in the modulation of a broad range of cellular and physiological responses, including the activation of JAK (Janus kinase)/STAT (signal transducer and activator of transcription) and MAPK (mitogen-activated protein kinase) family members [35–39]. Therefore an increase in IL-6 secretion is a hallmark of the activation of an inflammatory response. The pre-treatment of THP-1 macrophages and primary human macrophages with DHA with or without procyanidins B_1, B_2 and C_1 translated into a significant fall in IL-6 secretion.

COX1 and COX2 enzymes catalyse the first committed steps during the synthesis of prostaglandins from AA. Actually, both isoforms are of particular interest because they are catalogued as the pharmacological targets of NSAIDs [7].

Although the secretion of PGE_2 and LPS-inducible COX2 protein expression was not down-regulated in THP-1 macrophages by pre-treatments of DHA, procyanidins B_1, B_2 or C_1, or combinations of DHA with each of the procyanidins, all of the pre-treatments significantly inhibited PGE_2 secretion in primary human macrophages. The discrepancy between the modulation of PGE_2 secretion in THP-1 macrophages and primary human macrophages could be explained by the THP-1 differentiation process. To induce the differentiation of THP-1 monocytes into functional macrophages, mimicking the *in vivo* process, it is necessary to treat THP-1 monocytes with a differentiating agent, such as PMA [40,41]. The differentiation of cells with PMA involves the up-regulation of COX1 expression and the enhanced capacity to produce PGE_2 [42,43]. Thus the PGE_2 levels released in THP-1 macrophages are not only due to LPS induction; the PGE_2 levels could also be concealed due to the induction of COX1 by PMA.

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Table 3 Kinetic parameters for selective COX2 inhibition by the B_2 dimer and C_1 trimer

Initial reaction rates were determined as described in Table 2. Data were fitted to the standard Michaelis–Menten kinetic equation. Results are represented as the means ± S.E.M. for three independent experiments, and the significance of the differences between inhibitors for each kinetic parameter compared with the control condition was analysed by ANOVA (* $P < 0.05$).

Sample	V_{max} (μM/min)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$)	K_i (μM)
Control	$(0.198 \pm 0.077) \times 10^{-3}$	4.22 ± 0.48	$(4.11 \pm 0.16) \times 10^{-3}$	$(0.972 \pm 0.012) \times 10^{-3}$	–
B_2	$(0.252 \pm 0.001) \times 10^{-3}$	9.71 ± 1.00*	$(5.25 \pm 0.22) \times 10^{-3}$	$(0.54 \pm 0.00155) \times 10^{-3}$*	7.75 ± 0.68
C_1	$(0.177 \pm 0.015) \times 10^{-3}$	6.78 ± 1.01*	$(3.69 \pm 0.21) \times 10^{-3}$	$(0.467 \pm 0.0423) \times 10^{-3}$*	11.69 ± 1.25

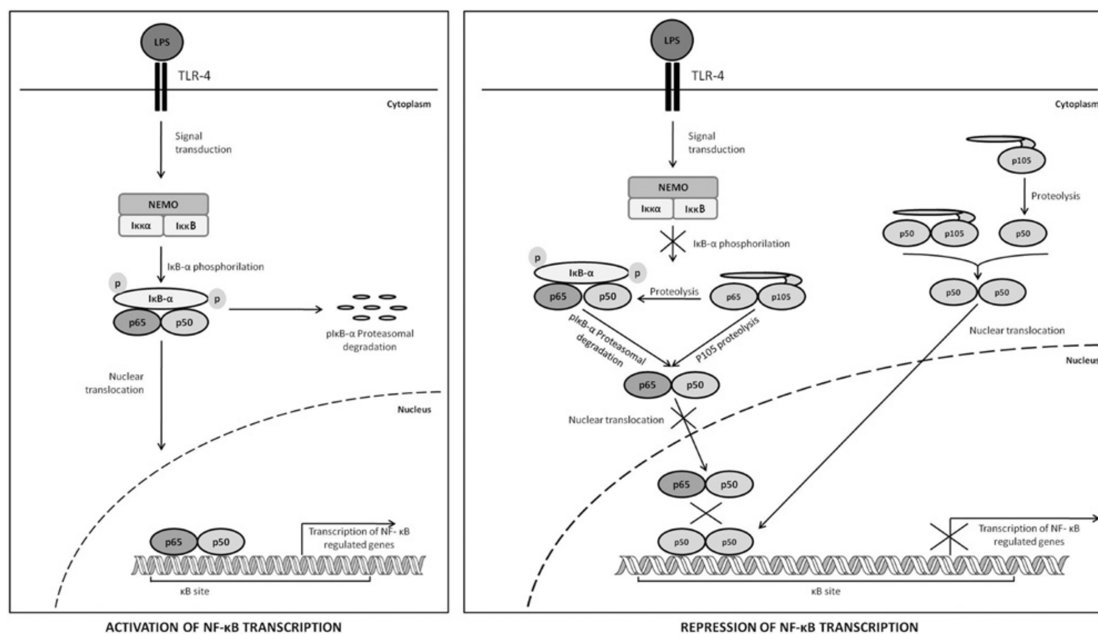

Figure 8 NF-κB activation and proposed mechanism for its inhibition by DHA and procyanidins

(Left-hand panel) An inflammatory stimulus [such as TNFα (tumour necrosis factor α) or LPS] induces the activation of the IKK complex, which phosphorylates IκBα and pIκBα is degraded by the proteasome. Then, NF-κB is able to translocate to the nucleus and activate the transcription of the pro-inflammatory genes. (Right-hand panel) DHA and procyanidins B_1, B_2 and C_1 are down-regulators of NF-κB at early and late stages with shared mechanisms through the inhibition of IκBα phosphorylation, the cytoplasmic retention of pro-inflammatory NF-κB proteins through p105 (NF-κB1) overexpression, the induction of nuclear translocation of the p50–p50 transcriptional repressor homodimer instead of the p65–p50 pro-inflammatory heterodimer, the inhibition of NF-κB DNA binding to κB sites and the secretion of NF-κB-regulated cytokines. NEMO, NF-κB essential modulator.

We set up a cell-free assay to elucidate the kinetic relationship between DHA and procyanidins with both constitutive and LPS-induced COX isoforms. Through the determination of the IC_{50} values (Table 1), we demonstrated the discriminatory capacity of DHA and procyanidins compared with the COX isoforms. The DHA and B_1 dimers were strong and selective inhibitors of COX1 activity, and the B_2 dimer and C_1 trimers were selective inhibitors of COX2 activity. Some authors have determined that, although COX1 and COX2 share 60 % primary sequence identity, there are differences between their active sites. These subtle differences could play key roles in the different affinities of COX1 and COX2 towards DHA and the procyanidins, including the procyanidin dimer isoforms B_1 and B_2. On the basis of our kinetic cell-free assay, we confirmed that DHA and procyanidins altered the substrate specificity of COX1 and COX2 to AA, as reflected by the modulation of the kinetic parameters. This change in the AA specificity was the result of the modulation of the maximal reaction rate (V_{max}) and the Michaelis–Menten constant (K_m). DHA exhibited mixed inhibitory behaviour towards COX1 through the down-regulation of V_{max} and the up-regulation of K_m; therefore DHA binds to a different location than the active site where AA binds. For the B_1 inhibition, a decrease in K_m and no change in V_{max} demonstrated that B_1 competes with AA in binding to the active site in COX1. This competitive behaviour, a decrease in K_m and no change in V_{max}, was also observed in the inhibition of COX2 activity by B_2 and C_1. The decrease in apparent K_m values from DHA and B_1 treatments for COX_1 as well as from B_2 and C_1 treatments for COX_2 was the reason for the decrease in the catalytic efficiency (k_{cat}/K_m) of PGE_2 biosynthesis. The selective inhibition of COX catalysis of PGE_2 synthesis by DHA and procyanidins in primary human macrophages was strongly supported by the kinetic parameters that were obtained from the cell-free assay. LPS is well described as a potent stimulus for PGE_2 secretion, which is predominantly performed by COX2 [44]. As shown in Figure 3(A), the inhibition of PGE_2 was greater for B_2 (a selective COX2 inhibitor) pre-treatment than

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DHA or B1 (selective COX1 inhibitors), and the combination of DHA (selective inhibitor of COX1) with B_2 and C_1 (competitive inhibitors of COX2) inhibited the secretion of PGE_2 to nearly basal levels.

The transcription factor NF-κB is one of the most important inducible transcription factors in the regulation of the expression of most genes involved in the control of the inflammatory response, cellular proliferation and cell adhesion [12,13]. As an activator and modulator of many inflammatory processes, such as pro-inflammatory cytokines and prostaglandin secretion, the modulation of the NF-κB signalling pathway by DHA and procyanidins in THP-1 macrophages was evaluated. The present study demonstrated the capacity of DHA, alone or in combination with procyanidins, to down-regulate IKKβ activity, which was expressed as a decrease in the phosphorylation of pIκBα, a key modulator of NF-κB activation (Figure 5A).

However, p105, or NF-κB1, is an NF-κB family member that is associated with a dual function in the NF-κB signalling system. LPS-induced NF-κB activation leads to the proteolytic degradation of p105 to p50; therefore p105 can be considered as a p50 precursor. Furthermore, p105 possesses ankyrin repeats in its structure, as do IκB family members. These allow for association with other members of the NF-κB family proteins in the cytoplasm and subsequently cause the inhibition of nuclear translocation and DNA binding of the NF-κB complex [45,46]. The p105 is bound and retained in the cytoplasm by all NF-κB proteins, in contrast with IκBα, which only binds to NF-κB dimers that contain a p65 subunit [16]. The degradation of p105 also liberates the p105-associated MAPK, which is responsible for the activation of the ERK (extracellular-signal-regulated kinase)/MAPK cascade [47]. Thus the effects of the presence of p105 in the cytoplasm can be related to the decreased activation of the NF-κB transcription factor [46,47] and the pro-inflammatory ERK/MAPK cascade. Our findings showed that pre-treatments with the B_2 dimer, C_1 trimer and any of the combinations of DHA with B_1, B_2 or C_1 led to an increase in the p105 cytoplasmic retention in LPS-induced THP-1 macrophages (Figure 5).

The classical and most abundant NF-κB heterodimer is composed of p50 and p65 subunits and is characterized as a potent activator of the expression of several pro-inflammatory genes. Moreover, p50 is able to form homodimers itself, and the p50–p50 homodimers can be found in the nucleus bound to DNA. Although p65 possesses a transactivation domain, p50 does not; therefore the presence of the p50–p50 homodimer in the nucleus represses the transcriptional activity of NF-κB [14,48,49]. Thus the nuclear p50 subunit could modulate the NF-κB signalling pathway, participating with the pro-inflammatory heterodimer p65–p50 or the repressor homodimer p50–p50. Therefore the B_2 dimer is the most powerful compound during the induction of p50 cytoplasmic expression and, together with B_1 and DHA/B_1, is an inhibitor of p50 nuclear translocation. However, the pre-treatment of LPS-stimulated THP-1 macrophages with any of the compounds studied (DHA and/or procyanidins) down-regulated the nuclear translocation of p65, and it favoured the cytoplasmic retention of and dramatically inhibited the nuclear translocation of p65. The stronger capacity of DHA and procyanidins to inhibit the nuclear translocation of p65 was corroborated by p65 immunostaining and confocal microscopy visualization (Figure 6). A greater nuclear expression of p50 than that of p65 was observed in p50 and p65 blots, as shown in Figure 5, for all of the pre-treatments. In keeping with the conventional NF-κB complex structure, the NF-κB heterodimer is formed by one subunit of each dimer, p50 and p65 [13,14]. The fact that the pre-treatment induced a greater nuclear expression of the p50 subunit than p65, and the fact that the inhibition of p65 nuclear translocation was more dramatic than that of p50, led us to consider that the fraction of p50 detected in the nucleus was forming p50–p50 homodimers. Therefore our bioactive molecules, procyanidins and, more significantly, DHA, not only inhibited the translocation of the p50–p65 pro-inflammatory heterodimer, but also induced the translocation of the p50–p50 transcriptional repressor homodimers.

In addition, we demonstrated that the pre-treatment of THP-1 macrophages with DHA and/or procyanidins down-regulated the binding activity of NF-κB p65 to the corresponding κB consensus sequence located in the promoter and enhancer regions of several pro-inflammatory genes (Figure 7).

In conclusion, most LPS-stimulated inflammatory responses lead to the activation of the transcription factor NF-κB signalling pathway, the secretion of cytokines and the formation of prostaglandins (Figure 8). Therefore the selective inhibition of COX activity in cell-free assays, the inhibition of IL-6 and PGE_2 secretion, and the down-regulation of the NF-κB activation signal pathway in human macrophages by food bioactive elements, such as DHA and procyanidins B_1, B_2 and C_1, lead to great interest in their use as potential anti-inflammatory compounds. Along these lines, the conserved and, in certain cases such as in PGE_2 secretion, the improvement in the anti-inflammatory response due to DHA, B_1, B_2 and C_1 pre-treatments in the THP-1 cell line and primary human macrophages is relevant proof of the capacity of bioactive food compounds to promote good health through the modulation of inflammation with diet.

AUTHOR CONTRIBUTION

Neus Martinez-Micaelo, Noemi González-Abuín, Anna Ardèvol, Montserrat Pinent and Mayte Blay designed the research. Neus Martinez-Micaelo, Ximena Terra and Cristobal Richart isolated human macrophages. Neus Martinez-Micaelo performed the experiments. Neus Martinez-Micaelo and Mayte Blay interpreted the data and wrote the paper. Mayte Blay directed the project.

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REFERENCES

1 Olefsky, J. M. and Glass, C. K. (2010) Macrophages, inflammation, and insulin resistance. Annu. Rev. Physiol. **72**, 219–246

2 Willoughby, D. A., Moore, A. R., Colville-Nash, P. R. and Gilroy, D. (2000) Resolution of inflammation. Int. J. Immunopharmacol. **22**, 1131–1135

3 Calder, P. C. (2009) Polyunsaturated fatty acids, inflammation and inflammatory diseases. J. Pharm. Pharmacol. **61**, 195

4 Linton, M. F. and Fazio, S. (2003) Macrophages, inflammation, and atherosclerosis. Int. J. Obes. **27**, S35–S40

5 Funk, C. D. (2001) Prostaglandins and leukotrienes: advances in eicosanoid biology. Science **294**, 1871–1875

6 Colville-Nash, P. R. and Gilroy, D. W. (2000) Cyclooxygenase enzymes as targets for therapeutic intervention in inflammation. Drug News Perspect. **13**, 587–597

7 Smith, W. L., DeWitt, D. L. and Garavito, R. M. (2000) Cyclooxygenases: structural, cellular, and molecular biology. Annu. Rev. Biochem. **69**, 145–182

8 Smith, W. L. (2008) Nutritionally essential fatty acids and biologically indispensable cyclooxygenases. Trends Biochem. Sci. **33**, 27–37

9 Gilroy, D. W. and Colville-Nash, P. R. (2000) New insights into the role of COX2 in inflammation. J. Mol. Med. **78**, 121–129

10 Chandrasekharan, N. V., Dai, H., Roos, K. L. T., Evanson, N. K., Tomsik, J., Elton, T. S. and Simmons, D. L. (2002) COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression. Proc. Natl. Acad. Sci. U.S.A. **99**, 13926–13931

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11 Ghosh, S., May, M. J. and Kopp, E. B. (1998) NF-κB and rel proteins: evolutionarily conserved mediators of immune responses. Annu. Rev. Immunol. **16**, 225–260
12 Hayden, M. S., West, A. P. and Ghosh, S. (2006) NF-κB and the immune response. Oncogene **25**, 6758–6780
13 Li, Q. T. and Verma, I. M. (2002) NF-κB regulation in the immune system. Nat. Rev. Immunol. **2**, 725–734
14 Saccani, S., Pantano, S. and Natoli, G. (2003) Modulation of NF-κB activity by exchange of dimers. Mol. Cell **11**, 1563–1574
15 Oeckinghaus, A. and Ghosh, S. (2009) The NF-κB family of transcription factors and its regulation. Cold Spring Harb. Perspect. Biol. **1**, a000034
16 Hayden, M. S. and Ghosh, S. (2004) Signaling to NF-κB. Genes Dev. **18**, 2195–2224
17 Chacon, M. R., Ceperuelo-Mallafre, V., Maymo-Masip, E., Mateo-Sanz, J. M., Arola, L., Guitierrez, C., Fernandez-Real, J. M., Ardevol, A., Simon, I. and Vendrell, J. (2009) Grape-seed procyanidins modulate inflammation on human differentiated adipocytes *in vitro*. Cytokine **47**, 137–142
18 Murphy, K. J., Chronopoulos, A. K., Singh, I., Francis, M. A., Moriarty, H., Pike, M. J., Turner, A. H., Mann, N. J. and Sinclair, A. J. (2003) Dietary flavanols and procyanidin oligomers from cocoa (*Theobroma cacao*) inhibit platelet function. Am. J. Clin. Nutr. **77**, 1466–1473
19 Terra, X., Montagut, G., Bustos, M., Llopiz, N., Ardevol, A., Blade, C., Fernandez-Larrea, J., Pujadas, G., Salvado, J., Arola, L. and Blay, M. (2009) Grape-seed procyanidins prevent low-grade inflammation by modulating cytokine expression in rats fed a high-fat diet. J. Nutr. Biochem. **20**, 210–218
20 Yilmaz, Y. and Toledo, R. T. (2004) Health aspects of functional grape seed constituents. Trends Food Sci. Tech. **15**, 422–433
21 Calder, P. C. (2005) Polyunsaturated fatty acids and inflammation. Biochem. Soc. Trans. **33**, 423–427
22 Simopoulos, A. P. (2002) Omega-3 fatty acids in inflammation and autoimmune diseases. J. Am. Coll. Nutr. **21**, 495–505
23 Grimminger, F., Walmrath, D., Seeger, W. and Lasch, H. G. (1993) Antiinflammatory effects of omega-3-lipinfusion in clinical and experimental studies. Med. Welt **44**, 207–216
24 Horrocks, L. A. and Yeo, Y. K. (1999) Health benefits of docosahexaenoic acid (DHA). Pharmacol. Res. **40**, 211–225
25 Weldon, S. M., Mullen, A. C., Loscher, C. E., Hurley, L. A. and Roche, H. M. (2007) Docosahexaenoic acid induces an anti-inflammatory profile in lipopolysaccharide-stimulated human THP-1 macrophages more effectively than eicosapentaenoic acid. J. Nutr. Biochem. **18**, 250–258
26 Schuehly, W., Huefner, A., Pferschy-Wenzig, E. M., Prettner, E., Adams, M., Bodensieck, A., Kunert, O., Oluwemimo, A., Haslinger, E. and Bauer, R. (2009) Design and synthesis of ten biphenyl-neolignan derivatives and their *in vitro* inhibitory potency against cyclooxygenase-1/2 activity and 5-lipoxygenase-mediated LTB4-formation. Bioorg. Med. Chem. **17**, 4459–4465
27 Zhang, W. Y., Liu, H. Q., Xie, K. Q., Yin, L. L., Li, Y., Kwik-Uribe, C. L. and Zhu, X. Z. (2006) Procyanidin dimer B2 epicatechin-(4β-8)-epicatechin suppresses the expression of cyclooxygenase-2 in endotoxin-treated monocytic cells. Biochem. Biophys. Res. Commun. **345**, 508–515
28 Serra, A., Macia, A., Romero, M. P., Salvado, M. J., Bustos, M., Fernandez-Larrea, J. and Motilva, M. J. (2009) Determination of procyanidins and their metabolites in plasma samples by improved liquid chromatography-tandem mass spectrometry. J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. **877**, 1169–1176
29 Serra, A., Macia, A., Romero, M. P., Valls, J., Blade, C., Arola, L. and Motilva, M. J. (2010) Bioavailability of procyanidin dimers and trimers and matrix food effects in *in vitro* and *in vivo* models. Br. J. Nutr. **103**, 944–952

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30 Marsen, T. A., Pollok, M., Oette, K. and Baldamus, C. A. (1992) Pharmacokinetics of omega-3-fatty-acids during ingestion of fish oil preparations. Prostaglandins Leukotrienes Essent. Fatty Acids **46**, 191–196
31 Williamson, G. and Manach, C. (2005) Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. Am. J. Clin. Nutr. **81**, 243S–255S
32 Rusca, A., Di Stefano, A. F. D., Doig, M. V., Scarsi, C. and Perucca, E. (2009) Relative bioavailability and pharmacokinetics of two oral formulations of docosahexaenoic acid/eicosapentaenoic acid after multiple-dose administration in healthy volunteers. Eur. J. Clin. Pharmacol. **65**, 503–510
33 Saw, C. L. L., Huang, Y. and Kong, A. N. (2010) Synergistic anti-inflammatory effects of low doses of curcumin in combination with polyunsaturated fatty acids: docosahexaenoic acid or eicosapentaenoic acid. Biochem. Pharmacol. **79**, 421–430
34 Baba, S., Osakabe, N., Natsume, M. and Terao, J. (2002) Absorption and urinary excretion of procyanidin B2 epicatechin-(4β-8)-epicatechin in rats. Free Radical Biol. Med. **33**, 142–148
35 Akira, S., Takeda, K. and Kaisho, T. (2001) Toll-like receptors: critical proteins linking innate and acquired immunity. Nat. Immunol. **2**, 675–680
36 Heinrich, P. C., Castell, J. V. and Andus, T. (1990) Interleukin-6 and the acute phase response. Biochem. J. **265**, 621–636
37 Kishimoto, T. (1989) The biology of interleukin-6. Blood **74**, 1–10
38 Vansnick, J. (1990) Interleukin-6: an overview. Annu. Rev. Immunol. **8**, 253–278
39 Tang, G. L., Minemoto, Y., Dibling, B., Purcell, N. H., Li, Z. W., Karin, M. and Lin, A. (2001) Inhibition of JNK activation through NF-κB target genes. Nature **414**, 313–317
40 Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T. and Tada, K. (1980) Establishment and characterization of a human acute monocytic leukemia-cell line (THP-1). Int. J. Cancer **26**, 171–176
41 Daigneault, M., Preston, J. A., Marriott, H. M., Whyte, M. K. B. and Dockrell, D. H. (2010) The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. PLoS ONE **5**, e8668
42 Smith, C. J., Morrow, J. D., Roberts, L. J. and Marnett, L. J. (1993) Differentiation of monocytoid THP-1 cells with phorbol ester induces expression of prostaglandin endoperoxide synthase-1 (COX-1). Biochem. Biophys. Res. Commun. **192**, 787–793
43 Smith, C. J., Morrow, J. D., Roberts, L. J. and Marnett, L. J. (1997) Induction of prostaglandin endoperoxide synthase-1 (COX-1) in a human promonocytic cell line by treatment with the differentiating agent TPA. In Eicosanoids and Other Bioactive Lipids in Cancer, Inflammation, and Radiation Injury 2, Pts A and B (Honn, K. V., Nigam, S. and Marnett, L. J., eds), pp. 99–106, Springer
44 Kang, Y. J., Wingerd, B. A., Arakawa, T. and Smith, W. L. (2006) Cyclooxygenase-2 gene transcription in a macrophage model of inflammation. J. Immunol. **177**, 8111–8122
45 Beinke, S., Belich, M. P. and Ley, S. C. (2002) The death domain of NF-κB1 p105 is essential for signal-induced p105 proteolysis. J. Biol. Chem. **277**, 24162–24168
46 Moorthy, A. K. and Ghosh, G. (2003) p105 center dot IκBγ and prototypical IκBs use a similar mechanism to bind but a different mechanism to regulate the subcellular localization of NF-κB. J. Biol. Chem. **278**, 556–566
47 Beinke, S. and Ley, S. C. (2004) Functions of NF-κB1 and NF-κB2 in immune cell biology. Biochem. J. **382**, 393–409
48 Guan, H. C., Hou, S. H. and Ricciardi, R. P. (2005) DNA binding of repressor nuclear factor-κB p50/p50 depends on phosphorylation of Ser337 by the protein kinase A catalytic subunit. J. Biol. Chem. **280**, 9957–9962
49 Tong, X., Yin, L., Washington, R., Rosenberg, D. W. and Giardina, C. (2004) The p50-p50 NF-κB complex as a stimulus-specific repressor of gene activation. Mol. Cell. Biochem. **265**, 171–183

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CHAPTER 3

Procyanidin B_2 inhibits inflammasome-mediated IL-1β production in lipopolysaccharide-stimulated human macrophages

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ABSTRACT

Macrophage stimulation with bacterial lipopolysaccharide (LPS) triggers inflammasome activation, resulting in proinflammatory interleukin (IL)-1β cytokine maturation and secretion. IL-1β underlies the pathologies of many diseases, including obesity-related disorders, such as type 2 diabetes or chronic inflammation. Thus, the modulation of the inflammatory response through bioactive food compounds present in the diet, such as procyanidins, is a powerful tool to promote homeostasis.

In this study, we provide the first evidence that procyanidin B_2 inhibits inflammasome activation and IL-1β secretion during LPS-induced acute inflammation in human macrophages. The procyanidin B_2-mediated inhibition of inflammasome activation includes the inactivation of the nuclear factor (NF)-κB signal pathway, the first stage required for the transcription of inflammasome precursors, through the inhibition of the nuclear translocation and DNA binding of the NF-κB-p65 subunit, resulting in the transcriptional repression of target genes, such as the cyclooxygenase 2 (COX_2), the inducible nitric oxide synthase (iNOS) and the production of IL-6 and nitric oxide (NO). Furthermore, procyanidin B_2 also decreases the cytoplasmic pools of NLRP3 and pro-IL-1β, limiting components of inflammasome activation and impeding NLRP3 inflammasome assembly and the activation of caspase-1, and finally the secretion of active IL-1β.

INTRODUCTION

Inflammation is a fundamental, acute response of the innate immune system to noxious stimuli [1]. The improper regulation of the inflammatory response underlies the pathogenesis of many chronic diseases including metabolic syndrome and its related pathologies, Alzheimer's disease and cancer [2].

Innate immune system effector cells, such as macrophages, trigger the inflammatory response through the identification of dangerous signals, such as the highly conserved pathogen-associated molecular patterns (PAMPs), and by sensing host-derived danger associated molecular patterns (DAMPs), which are involved in the disturbance of homeostasis [3,4]. This early sensing of dangerous signals involves pattern recognition receptors (PRRs) on the cell surface, such as membrane-bound Toll-like receptor (TLRs), and in the cytoplasm, such as NOD-like receptors (NLRs) [5].

Toll-like receptors function as PRRs in mammals and play an essential role in PAMP recognition; however, they also recognise endogenous DAMPs [6]. Therefore, the innate immune system senses gram-negative bacteria via Toll-like receptor 4 (TLR4) by identifying lipopolysaccharide (LPS), which is an integral component of the microbial outer membrane that triggers the inflammatory response. Then, LPS-induced TLR4 activation and signal transduction results in the activation and nuclear translocation of nuclear factor (NF)-κB. NF-κB plays a central role in immunity through its ability to modulate the transcription of multiple inflammatory and immune genes. Thus, once activated, it binds to recognition elements in the promoter regions of genes encoding inflammatory cytokines such as the interleukin (IL)-6, adhesion molecules, chemokines, growth factors and inducible enzymes such as cyclooxygenase 2 (COX_2) and inducible nitric oxide synthase (iNOS) [7,8].

Otherwise, the inflammasome is a cytosol-localised protein complex of the host innate immune system that consists of NOD-like receptor protein 3 (NLRP3), apoptosis-associated speck-like protein (ASC) and caspase-1, which is involved in the processing and secretion of interleukin (IL)-1β, a prominent inflammatory cytokine that is directly implicated in the pathogenesis of many disorders or indirectly implicated through the generation of other inflammatory mediators via IL-1 receptor signalling. NLRP3 inflammasome is unique because it can be activated in response to microorganism stimuli such as PAMPs and in response to endogenous danger signals, or DAMPs, e.g., during metabolic dysregulation.

The secretion of IL-1β is a result of a two-step process. First, a cytosolic pool of the immature form of the cytokine (pro-IL-1β) and NLRP3 inflammasome, which is mediated by the recognition of pro-inflammatory stimuli through toll-like receptors (TLRs), is produced and leads to the activation and nuclear translocation of NF-κB. Second, another stimulus or signal induces NLRP3 inflammasome assembly, promoting the proteolytically processed of pro-IL-1β to its active form (IL-1β) by caspase-1. IL-1β activation and secretion is then regulated at the transcriptional and post-transcriptional levels, and this mechanism is considered preventive against the unnecessary and harmfully production of IL-1β. Thus, IL-

1β is only secreted in conditions where pattern recognition-mediated activation of TLRs regulates the cytosolic pool of pro-IL-1β, whereas the inflammasome, through sensing cytosolic danger signals, modulates the proteolytic processing and subsequent secretion of the mature form of IL-1β.

The procyanidin B_2 is a phenolic compound composed by two molecules of the flavan-3-ol (-)-epicatechin and is mainly found in cocoa, apples or grapes and in beverages such as wine. Procyanidin B_2 is considered as a bioactive food component because of its implication in health promotion as well as in the restoration and maintenance of homeostasis [9]. One of these beneficial effects are its anti-inflammatory properties [10–13].

It has been described that the molecular basis underlying the inflammatory activity of LPS are based on TLR4 ligand-mediated activation of innate and adaptive immunity [14], but also in non-dependent TLR4 signal transduction [15]. Moreover, LPS induces IL-1β secretion, either by NLRP3 inflammasome induction [16,17] or by activating the caspase-11-governed inflammasome [18]. Furthermore and taking into account the role of procyanidin B_2 as an anti-inflammatory molecule as well as a promoter of the homeostasis, the objective of this study was to elucidate whether procyanidin B_2 modulates both the NF-κB signal transduction pathway and inflammasome-mediated IL-1β secretion in LPS-stimulated macrophages.

MATERIALS AND METHODS

Chemicals

The procyanidin B_2 dimer (epicatechin-(4β→8)-epicatechin) was acquired from ExtraSynthese (Lyon, France). RPMI 1640 culture medium was purchased from Gibco (Barcelona, Spain). Cell culture reagents were provided by BioWhittaker (Verviers, Belgium). The differentiating agent PMA (phorbol 12-myristate 13-acetate) was purchased from InvivoGen (San Diego, USA), and LPS (*Escherichia coli* 0111:B4) was purchased from Sigma-Aldrich (Steinheim, Germany).

Cell culture and stimulation

THP-1 human monocytes were obtained from the European Collection of Animal Cell Cultures (ECACC no. 88081201) and cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin, 25 mM HEPES and 10% foetal bovine serum at 37°C in a humidified incubator with 5% CO_2. THP-1 monocytes were differentiated to macrophages using fresh medium containing 0.5 µg/ml PMA for 24 h. After differentiation, the medium containing PMA was removed, and adherent THP-1 macrophages were incubated in RPMI medium for 24 h.

To determine the role of procyanidin B_2 in the modulation of LPS-induced inflammasome activation, THP-1 macrophages were primed with 1 µg/ml of LPS with or without the supplementation of 10 µM of procyanidin B_2 for the indicated times. When the experimental

design required, 5 mM of ATP stimuli was added to assess NRLP3-mediated inflammasome activation.

Cytosolic protein extraction and Western blotting

THP-1 macrophages were primed with 1 µg/ml of LPS, with or without 10 µM of procyanidin B_2 supplementation, for 2 h before stimulation with 5 mM of ATP for 4 h. Cytosolic cell fractions were isolated, and protein expression was determined by western blotting according to a previously described protocol[12]. Briefly, protein was separated in a 10% SDS-PAGE gel, transferred to PVDF membrane and blocked prior to detection with anti-COX_2 (Bioworld), anti-iNOS (SantaCruz), anti-p65 (Santa Cruz, USA), anti-NLRP3 or anti-IL-1β (Abcam). Semi-quantitative protein quantifications were performed using the ImageJ software (National Institutes of Health, Maryland, USA).

Nitric oxide (NO) production in primary monocyte-derived macrophages

Rat peripheral blood mononuclear cells (PBMCs) were isolated from healthy Wistar adult rats (Charles River Laboratories, Barcelona, Spain) by density-gradient centrifugation using HISTOPAQUE-1083 (Sigma-Aldrich, Steinheim, Germany). Once isolated, the monocytes were purified by adherence to plastic in serum-free RPMI 1640-supplemented medium. Briefly, 2×10^6 cells/ml were seeded into 12-well plates, and, after 2 h, non-adherent cells were removed by several washes with warm PBS. Freshly isolated monocytes were stimulated with 1 µg/ml of LPS and with or without 10 µM of B_2 procyanidin for 19 h. The nitrite concentration in the culture medium was determined as a measure of nitric oxide (NO) production according to the Griess reaction. Standard procedure was performed using the Griess reagent comprised of 1% (w/v) sulphanilamide, 12.5 mM naphthylenediamide and 6.5 M HCl. The secretion of nitrite was normalised to the protein content, which was determined following the Bradford method.

Caspase-1 activation assay

Caspase-1 activation was assessed at a range of ATP concentrations (0-10 mM) for 1 h in macrophages primed with 1 µg/ml of LPS. Furthermore, the procyanidin B_2 effects on LPS-mediated inflammasome activation was determined by treating THP-1 macrophages for 30 min with LPS (1 µg/ml) with or without adding 10 µM of dimeric procyanidin previous to its stimulation for 1 h with 5 mM of ATP. Caspase-1 activation was assessed by adding FAM-YVAD-fmk, a cell-permeable fluorescent probe able to bind covalently to the active form of caspase-1, to the cell culture. The percentage of activated caspase-1 was determined by fluorescence following the manufacturer’s instructions (Immunochemistry Technologies). Images were acquired using a Nikon Eclipse TE2000-E microscope.

IL-1β and IL-6 secretion

Secretion of the mature form of the IL-1β in THP-1 supernatants and the IL-6 cytokine released by primary murine macrophages were analysed in culture media samples using the

corresponding enzyme-linked immunosorbent assays (ELISA) following the manufacturer's instructions (BioLegend).

Electrophoretic mobility shift assay (EMSA) analysis of NF-κB transcriptional activation

Nuclear protein extracts of THP-1 macrophages were analysed to determine NF-κB transcriptional activation as previously described [19]. Biotin-labelled NF-κB oligonucleotides: forward 5'-AGTTGAGGGGACTTTCCCAGGC-3' and the complementary strand 5'-GCCTGGGAAAGTCCCCTCAACT-3', were used for the EMSA. The reaction mixture was loaded onto a 4% native polyacrylamide gel in 0.5% Tris-borate-EDTA and transferred onto nylon membranes (Bio-Rad, Hercules, CA, USA). After UV-crosslinking, the interaction between the biotin-labelled NF-κB oligonucleotides and NF-κB-p65 subunit was detected using the non-radioactive LightShift Chemiluminescent EMSA kit (Thermo Scientific, Rockforf, IL, USA).

Statistical analysis

Results are expressed as the mean ± S.E.M. The modulatory effects induced by LPS or procyanidin B_2 were evaluated using ANOVA. Tukey's test was used as a post-hoc test to make pair-wise comparisons. Significant differences were considered when the p values were < 0.05. Statistical analyses were performed using the SPSS 20.0 software.

RESULTS

Procyanidin B_2 inhibits the NF-κB transcriptional activation

To examine the effects of procyanidin B_2 in LPS-induced TLR4 signal transduction, the activation of the NF-κB pathway was evaluated (Figure 1). We reported that procyanidin B_2 inhibited not only the nuclear translocation of the NF-κB-p65 subunit (Figure 1 A and 1D) but also inhibits binding to the NF-κB DNA consensus sequence (Figure 1 B). Furthermore, this procyanidin B_2-mediated inactivation of the NF-κB pathway was also supported by the diminution in the protein expression of NF-κB target genes, including COX_2 (Figure 1 A and 1C) and iNOS (Figure 1 A and 1D).

Moreover, as expected, procyanidin B_2 also decreased the secreted concentration of IL-6, whose gene expression is induced by LPS and regulated through NF-κB (Figure 1G), and the iNOS-mediated generation of nitric oxide (NO) (Figure 1F) in LPS-stimulated rat primary macrophages.

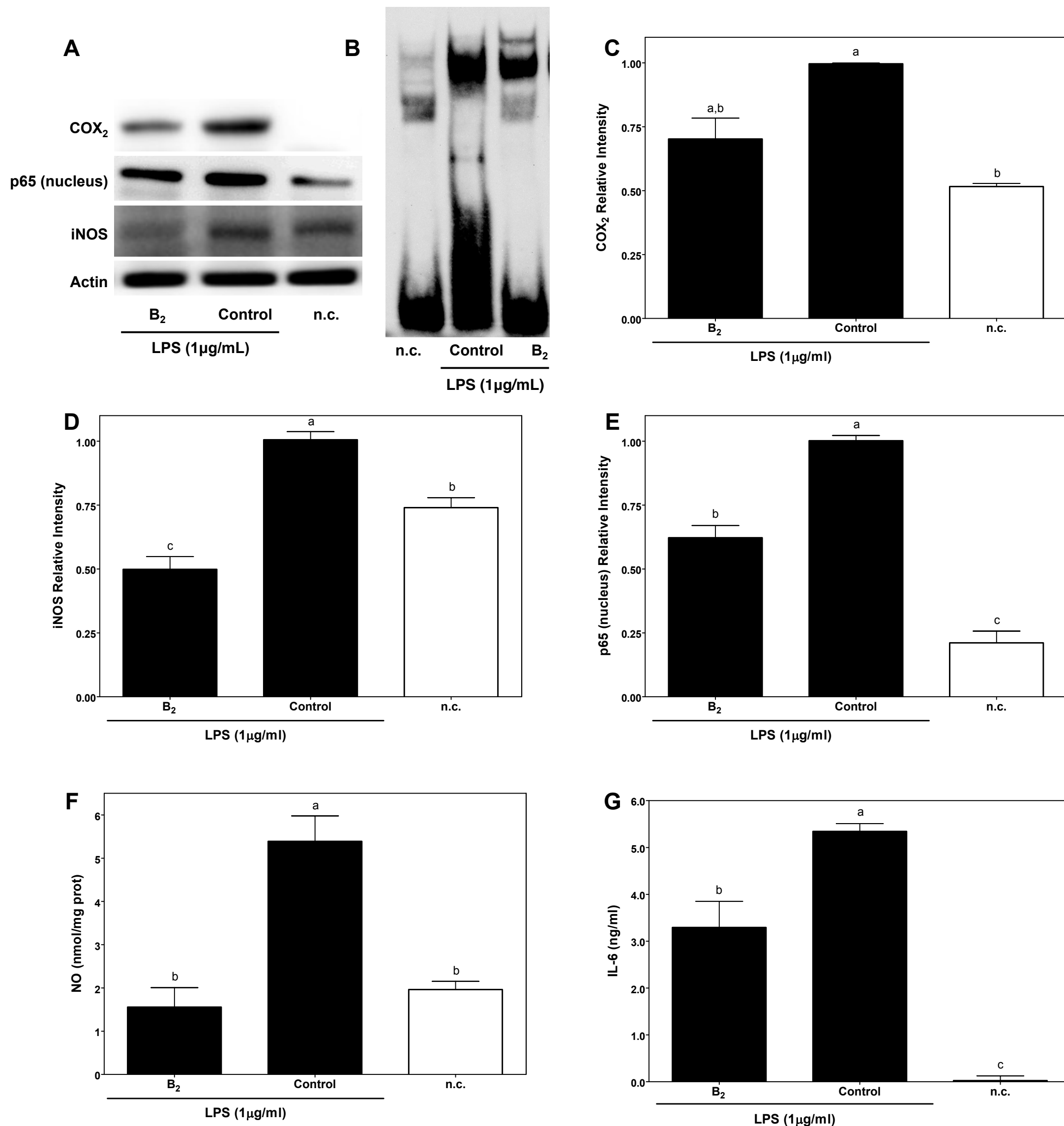

Figure 1. B_2 procyanidin inhibits LPS-induced NF-κB transcriptional activation. Dimeric procyanidin suppresses the LPS-induced activation of NF-κB by decreasing the transcriptional activation and the protein expression of target genes. THP-1 macrophages were stimulated by incubating them with 1 µg/ml LPS with or without 10 µM of B_2 dimer or with only vehicle (n.c.) for 2 h and then activating the inflammasome using 5 mM of ATP for 4 h. (A) Representative western blot analysis of COX_2, nuclear p65, iNOS and actin protein expression, (B) EMSA analysis of NF-κB transcriptional activation. (C, D and C) semi-quantification of the B_2–modulated COX_2, nuclear p65 and iNOS protein expression. (F and G) Secretion of nitric oxide and IL-6, respectively, in LPS-stimulated rat primary macrophages. The results represent the mean ± S.E.M. Bars sharing the same letters are not significantly different; otherwise, bars with different letters are significantly different (one-way ANOVA, Tukey's test, $P < 0.05$).

Procyanidin B2 decreases the inflammasome-priming signal

The effects of procyanidin B_2 as a modulator of the LPS-induced inflammasome priming signal was assessed after determining the cytoplasmic pools of NLRP3 and pro-IL-1β, the downstream effectors of this signal and required precursors for inflammasome activation. Thus, the protein expression of both, NLRP3 and pro-IL-1β, was significantly decreased by the procyanidin B2 in the cytoplasm of LPS-primed macrophages (Figure 2A-C).

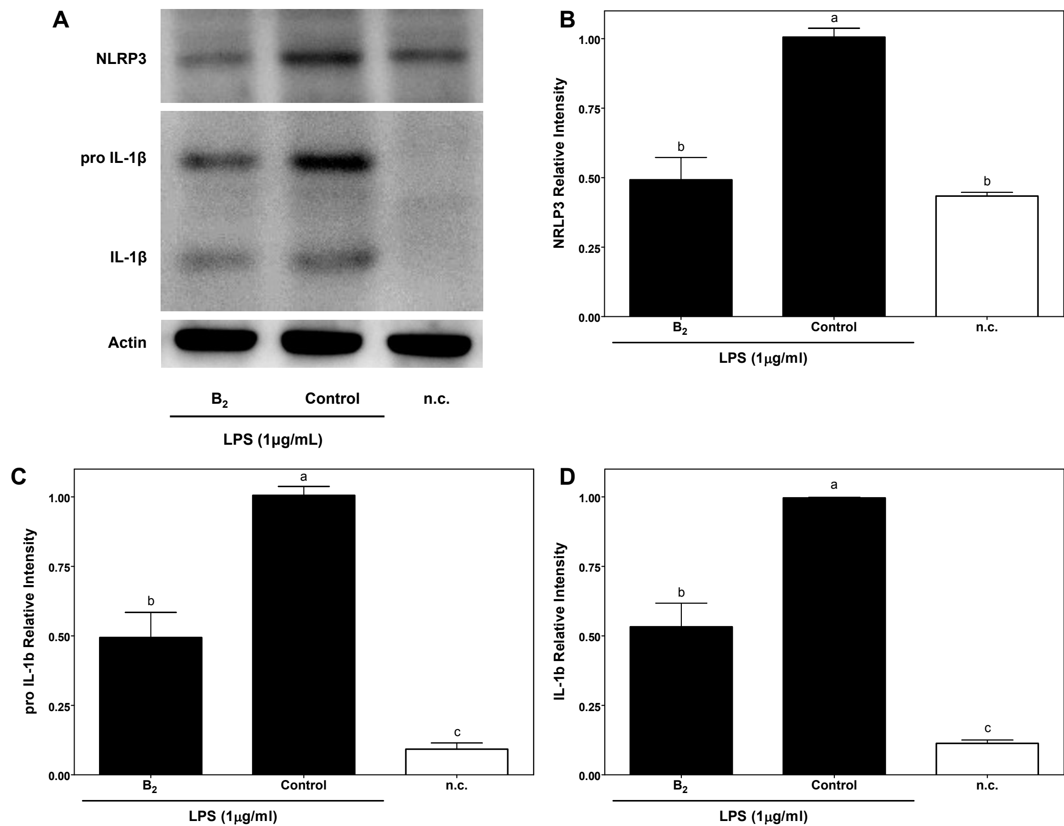

Figure 2. NLRP3 and IL-1β protein expression are inhibited by B_2 dimeric procyanidin. THP-1 macrophages were stimulated by incubating with 1 µg/ml LPS with or without 10 µM of B_2 dimer for 2 h, and, then, the inflammasome was activated with 5 mM of ATP for 4 h. The condition referred to as n.c. refers to THP-1 treated only with vehicle for 6 h. (A) Representative western blot analysis of NLRP3 and IL-1β protein expression after LPS-primed and ATP-induced inflammasome activation. Protein semi-quantifications are shown for NLRP3 (B), pro-IL-1β (C) and IL-1β (D). The results represent the mean ± S.E.M. Bars with different letters indicate significant differences (one-way ANOVA, Tukey's test, $P < 0.05$).

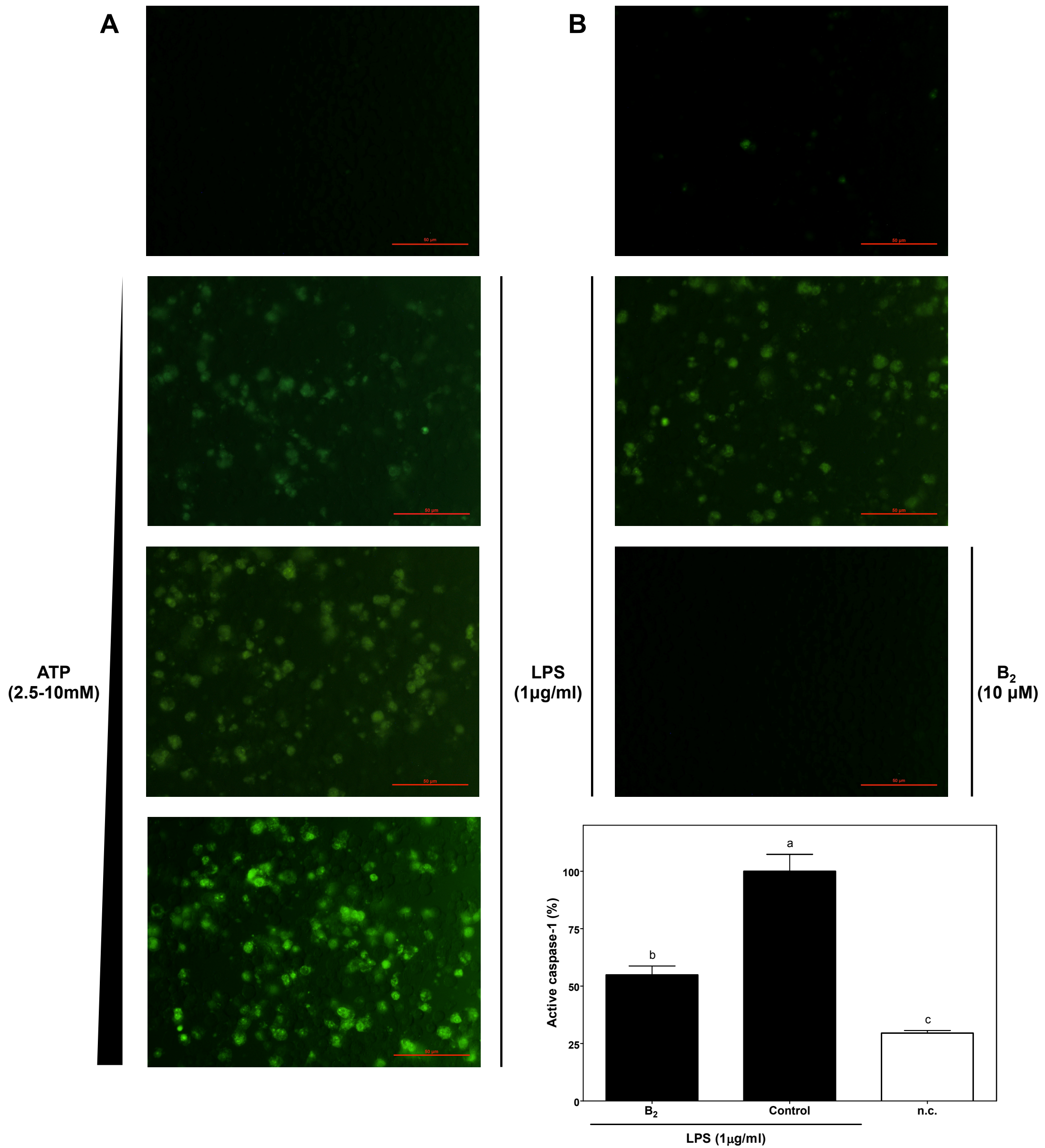

Figure 3. Inhibition of caspase-1 activation on LPS-primed macrophages by B_2 procyanidin. (A) THP-1 macrophages were primed with 1 µg/ml of LPS for 30 min, and caspase-1 was activated with different concentrations, from 0 to 10 mM, of ATP for 1 h. (B) Effects of co-incubation of 1 µg/ml LPS with or without 10 µM of B_2 for 30 min after caspase-1 was activated in THP-1 macrophages that were activated with 5 mM ATP. The condition referred to as n.c. refers to THP-1 cells treated only with vehicle for 1.5 h. The results represent the mean ± S.E.M. Bars with different letters are significantly different (one-way ANOVA, Tukey's test, $P < 0.05$).

Procyanidin B_2 inhibits inflammasome activation and IL-1β production

To explore the role of procyanidin B_2 in NLRP3 inflammasome activation, its effects on caspase-1 activation and the production and extracellular release of the mature form of IL-1β were determined.

First, we reported the increase of active caspase-1 in LPS-stimulated THP-1 macrophages by ATP in a dose-dependent manner (Figure 3A). Thus, the supplementation of LPS-primed macrophages with procyanidin B_2 significantly inhibited ATP-mediated inflammasome activation, as reflected by a significant decrease in the amount of activated caspase-1 detected (Figure 3B and 3C).

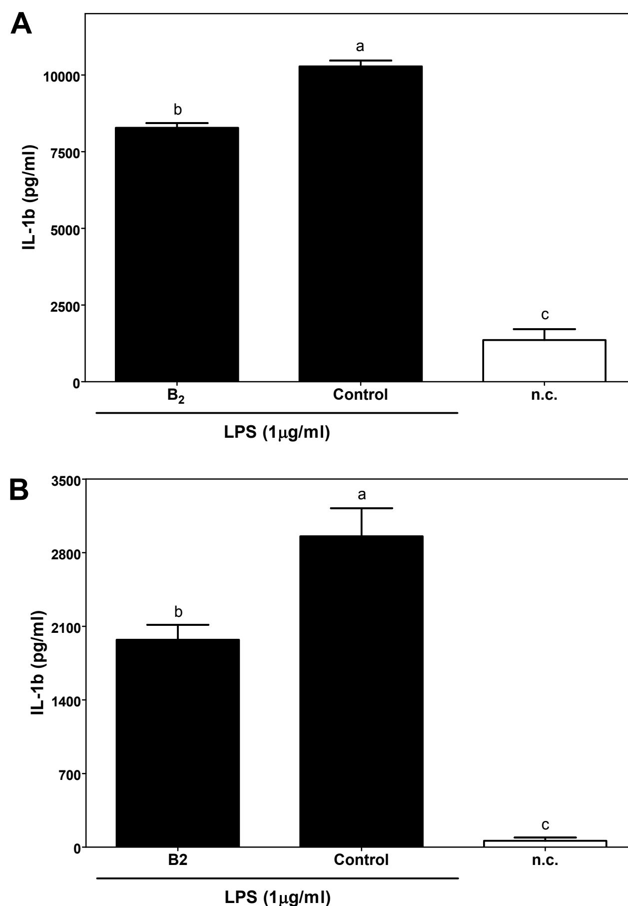

Figure 4. IL-1β secretion is inhibited by B_2 procyanidin in LPS-primed human macrophages. (A) Modulation of IL-1β secretion by 10 µM of B_2 in 1µg/ml LPS-primed macrophages for 2 h previous to 5 mM of ATP-mediated activation for 4 h. (B) Release of IL-1β in 1 µg/ml LPS-primed macrophages for 6 h with or without 10 µM B_2 without any other exogenous inflammasome-activating stimuli. The results are represented as the mean ± S.E.M. Different letters indicate significant differences (one-way ANOVA, Tukey's test, $P < 0.05$).

Moreover, we demonstrated the inhibition of inflammasome activation by the procyanidin B_2, after determining the cleavage and extracellular release of the mature form of IL-1β, as the primary function of inflammasome activation. Procyanidin B_2 significantly inhibited caspase-1-mediated IL-1β production, reflected as decreased amounts of intracellular mature IL-1β (Figure 2D) and a minor extracellular concentration of IL-1β secreted from LPS-primed and ATP-activated macrophages (Figure 4A). Moreover, procyanidin B_2 also significantly inhibited the secretion of IL-1β when THP-1 macrophages were only primed with LPS without the stimulation of any other NLRP3 inflammasome-activating stimuli (Figure 4B).

DISCUSSION

Acute inflammation, an early defence mechanism orchestrated through the innate immune system, is triggered via sensing dangerous signals, e.g., PAMPs and DAMPs, by pattern recognition receptors, such as TLRs and NLRs [20]. Inflammation, and the improper regulation of this mechanism underlies a wide range of physiological and pathological processes [2]. Moreover, the endotoxin LPS, is a pathogen-associated molecule of Gram-negative bacteria that, after recognition by PPRs, triggers a massive inflammatory response.

Procyanidins, featured bioactive components with beneficial properties, have been proposed as a powerful tool to promote the healthy state and homeostasis through the diet. The procyanidin B_2 is one of the forms found in plasma after procyanidin-rich food consumption [21], and this compound has been highlighted as one of the most immunologically active molecules within the procyanidins. Therefore, understanding how bioactive molecules contribute to a healthy lifestyle through an anti-inflammatory profile is an important objective of research.

Unlike other cytokines, two sequential stages regulate the production of IL-1β. Firstly, IL-1β is synthesised as an immature form, pro-IL-1β, and as a NF-κB target gene, the transcription of this cytokine is mediated through NF-κB pathway activation. Secondly, IL-1β maturation and secretion depends on caspase-1-mediated proteolytic cleavage. Pro-caspase-1, the inactive form of caspase-1, is activated through a multiprotein complex, the NLRP3 inflammasome, whose assembly depends on the amount of cytoplasmic NLRP3, an NF-κB target gene.

Given the pivotal role of inflammasome in the maintenance of homeostasis and the previously described anti-inflammatory effects of procyanidin B_2, particularly those involving the regulation of the NF-κB pathway, the modulation of inflammasome activation might be a potential mechanism underlying the anti-inflammatory effects promoted by the intake of procyanidin B_2. Thereby, in this study we have elucidated that procyanidin B_2 represses the NF-κB signal transduction pathway and inflammasome-mediated IL-1β secretion in LPS-stimulated macrophages.

Considering the sequential regulation of inflammasome activation, the NF-κB signal transduction pathway is considered an early event essential for inflammasome activation

and subsequent IL-1β secretion. Thus, the role of procyanidin B_2 in the down-regulation of NF-κB pathway activation and signal transduction was confirmed through the inhibition of the nuclear translocation of NF-κB-p65 subunit and binding to the consensus DNA sequence, both hallmarks of the LPS-induced NF-κB transcription complex activation. Furthermore, this inhibition in the NF-κB pathway was also reflected by a decrease in the transcription of the NF-κB target genes, determined as the protein expression of COX_2 and iNOS, two key enzymes for the regulation of inflammation, and the secretion of the IL-6, a pro-inflammatory biomarker. The B_2-promoted anti-inflammatory effects might reflect the COX_2-mediated down-regulation of the eicosanoids pathway, as previously observed for the procyanidin B_2-mediated inhibition of prostaglandin E_2 secretion [12]. Moreover, the procyanidin B_2 significantly inhibited not only the LPS-induced iNOS protein expression but also the secretion of NO, an important intracellular and intercellular signalling molecule that underlies the regulation of diverse physiological and pathophysiological mechanisms, supporting the B_2-mediated NO pathway-repressing effects previously observed for endotoxin-stimulated murine macrophages [11].

We also determined the B_2-mediated modulation of inflammasome activation by determining the protein expression of NLRP3 and proIL-1β, two limiting components for the NLRP3 assembly, caspase-1 activation and IL-1β maturation and secretion, the transcription of which are also regulated through the NF-κB pathway. We reported for the first time, that procyanidin B_2 significantly down-regulates the expression of NLRP3 and pro-IL-1β in LPS-primed and ATP-activated human macrophages. Notably, procyanidin B_2 also inhibited the activation of caspase-1, determined using a fluorochrome that exclusively binds to the intracellular active form of caspase-1.

Moreover, the modulation of IL-1β secretion, the main goal of inflammasome activation, in LPS-primed macrophages through B_2 procyanidin was analysed in two different conditions. Thus, procyanidin B_2 inhibited the LPS-induced release of IL-1β independently of inflammasome-triggered signals. Thus, we determined that procyanidin B_2 inhibited IL-1β secretion during LPS-primed and ATP-mediated NLRP3 inflammasome activation, and procyanidin B_2 also inhibited IL-1β secretion in LPS-primed and non-ATP inflammasome-activated macrophages. Thus, these results showed that although LPS-priming in the absence of any additional inflammasome-activating stimuli is sufficient for the promotion of IL-1β synthesis, supporting a role for LPS in boosting inflammasome activation independently of the NLRP3 inflammasome sensor induction as previously described [16], the activation of LPS-primed macrophages with ATP, an NLRP3 agonist, as the second signal, leads to the maturation and secretion of higher amounts of IL-1β.

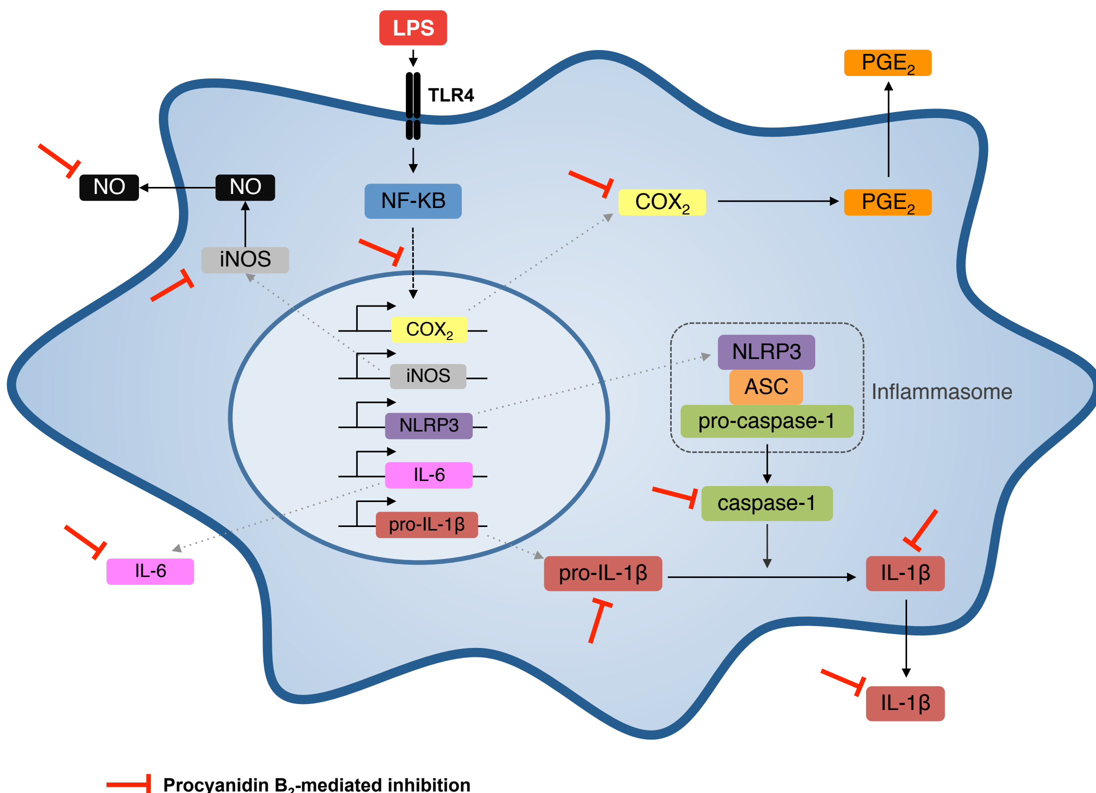

Figure 5. The proposed mechanism for the inhibition of inflammasome-mediated IL-1β secretion in LPS-stimulated macrophages by B_2 dimeric procyanidin. B_2 dimeric procyanidin inhibits the LPS-induced acute inflammatory response in macrophages through the inhibition of inflammasome activation at different levels, including the inhibition of NF-κB transcriptional activation, and the decrease of target genes protein expression, including the decrease of cytoplasmic pools of NLRP3 and pro-IL-1β, as well as the inhibition of caspase-1 activation and the production and release of IL-1β.

Therefore, the macrophage-mediated immune response to LPS is not focused exclusively on the cellular transcriptional response via TLR4-mediated NF-kB activation, but rather extends to the direct or indirect activation of a wide array of proinflammatory mechanisms, including autophagy, phagocytosis and oxidative bursts, to remove the triggering stimuli and promote the resolution of inflammation and the restoration of homeostasis [17,22]. Some authors also consider the redox-perturbing properties primarily exerted through reactive oxygen species (ROS) production, resulting from the LPS-induced oxidative burst response as a common feature of NLRP3 inflammasome activators [23,24]. In contrast, procyanidins and other plant polyphenols have been categorised as in vivo antioxidants. Furthermore, intact dimeric procyanidins can be transported inside cells and interact directly with signalling proteins and modulate its activity [25]. Thus, the inhibitory effects of B_2 procyanidins on IL-1β secretion might be associated with intrinsic antioxidant activity, and the potential repression of LPS-induced ROS production would be an added value for the strong anti-inflammatory effects of B_2 procyanidin described in this study.

In summary, procyanidin B_2 is a strong inhibitor of NF-κB signal pathway activation via the modulation of NF-κB transcriptional activation at different levels, including its nuclear translocation, DNA binding and transcriptional regulation of NF-κB-target genes. Moreover, we showed that procyanidin B_2 is also an inhibitor of inflammasome activation. Procyanidin B_2 decreases the cytoplasmic pool of NLRP3 and pro-IL-1β, inhibiting inflammasome assembly, the subsequent activation of caspase-1 and the maturation of IL-1β; thereby decreasing the levels of extracellular IL-1β secreted in LPS-stimulated human macrophages. Thus, these data provide new insights into the mechanisms underlying the anti-inflammatory effects described for procyanidin B_2, highlighting a new research direction concerning procyanidins as promising bioactive food compounds in terms of the prevention or resolution of disorders or diseases associated with the inappropriate activation of the inflammasome and IL-1β secretion.

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The authors declare no competing financial interest.

REFERENCES

1 Newton, K. and Dixit, V. M. (2012) Signaling in innate immunity and inflammation. Cold Spring Harb. Perspect. Biol., Cold Spring Harbor Lab **4**.

2 Tabas, I. and Glass, C. K. (2013) Anti-inflammatory therapy in chronic disease: challenges and opportunities. Science (80-.)., American Association for the Advancement of Science **339**, 166–172.

3 Nathan, C. F., Murray, H. W. and Cohn, Z. A. (1980) The macrophage as an effector cell. N. Engl. J. Med. **303**, 622–626.

4 Janeway Jr, C. A. and Medzhitov, R. (2002) Innate immune recognition. Sci. Signal., AAAS **20**, 197.

5 Schroder, K., Zhou, R. and Tschopp, J. (2010) The NLRP3 inflammasome: a sensor for metabolic danger? Science (80-.)., American Association for the Advancement of Science **327**, 296–300.

6 Akira, S., Takeda, K. and Kaisho, T. (2001) Toll-like receptors: critical proteins linking innate and acquired immunity. Nat. Immunol., Nature Publishing Group **2**, 675–680.

7 Barnes, P. J. (1997) Nuclear factor-κB. Int. J. Biochem. Cell Biol., Elsevier **29**, 867–870.

8 Tak, P. P. and Firestein, G. S. (2001) NF-κB: a key role in inflammatory diseases. J. Clin. Invest., Am Soc Clin Investig **107**, 7–11.

9 Osakabe, N. (2013) Flavan 3-ols improve metabolic syndrome risk factors: evidence and mechanisms. J. Clin. Biochem. Nutr., The Society for Free Radical Research Japan **52**, 186.

10 Pallarès, V., Calay, D., Cedó, L., Castell-Auví, A., Raes, M., Pinent, M., Ardévol, A., Arola, L. and Blay, M. (2012) Additive, antagonistic, and synergistic effects of procyanidins and

polyunsaturated fatty acids over inflammation in RAW 264.7 macrophages activated by lipopolysaccharide. Nutrition, Elsevier **28**, 447–457.

11 Terra, X., Valls, J., Vitrac, X., Mérrillon, J.-M., Arola, L., Ardèvol, A., Bladé, C., Fernández-Larrea, J., Pujadas, G. and Salvadó, J. (2007) Grape-seed procyanidins act as antiinflammatory agents in endotoxin-stimulated RAW 264.7 macrophages by inhibiting NFkB signaling pathway. J. Agric. Food Chem., ACS Publications **55**, 4357–4365.

12 Martinez-Micaelo, N., Gonzalez-Abuin, N., Terra, X., Richart, C., Ardevol, A., Pinent, M. and Blay, M. (2012) Omega-3 docosahexaenoic acid and procyanidins inhibit cyclo-oxygenase activity and attenuate NF-kappaB activation through a p105/p50 regulatory mechanism in macrophage inflammation. Biochem. J., Portland Press Ltd. **441**, 653–663.

13 Martinez-Micaelo, N., González-Abuín, N., Ardèvol, A., Pinent, M. and Blay, M. T. (2012) Procyanidins and inflammation: Molecular targets and health implications. BioFactors, Wiley Online Library **38**, 257–265.

14 Beutler, B. (2000) Tlr4: central component of the sole mammalian LPS sensor. Curr. Opin. Immunol., Elsevier **12**, 20–26.

15 Kayagaki, N., Wong, M. T., Stowe, I. B., Ramani, S. R., Gonzalez, L. C., Akashi-Takamura, S., Miyake, K., Zhang, J., Lee, W. P. and Muszyński, A. (2013) Noncanonical inflammasome activation by intracellular LPS independent of TLR4. Science (80-.)., American Association for the Advancement of Science **341**, 1246–1249.

16 Schroder, K., Sagulenko, V., Zamoshnikova, A., Richards, A. A., Cridland, J. A., Irvine, K. M., Stacey, K. J. and Sweet, M. J. (2012) Acute lipopolysaccharide priming boosts inflammasome activation independently of inflammasome sensor induction. Immunobiology, Elsevier.

17 Kagan, J. C. (2013) Sensing Endotoxins from Within. Science (80-.)., American Association for the Advancement of Science **341**, 1184–1185.

18 Hagar, J. A., Powell, D. A., Aachoui, Y., Ernst, R. K. and Miao, E. A. (2013) Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock. Science (80-.)., American Association for the Advancement of Science **341**, 1250–1253.

19 Deree, J., Martins, J. O., Melbostad, H., Loomis, W. H. and Coimbra, R. (2008) Insights into the regulation of TNF-alpha production in human mononuclear cells: the effects of non-specific phosphodiesterase inhibition. Clinics (Sao Paulo). **63**, 321–8.

20 Medzhitov, R. (2008) Origin and physiological roles of inflammation. Nature, Nature Publishing Group **454**, 428–435.

21 Serra, A., Macià, A., Romero, M.-P., Valls, J., Bladé, C., Arola, L. and Motilva, M.-J. (2010) Bioavailability of procyanidin dimers and trimers and matrix food effects in in vitro and in vivo models. Br. J. Nutr., Cambridge Univ Press **103**, 944.

22 Ortega-Gómez, A., Perretti, M. and Soehnlein, O. (2013) Resolution of inflammation: an integrated view. EMBO Mol. Med., Wiley Online Library.

23 Tschopp, J. and Schroder, K. (2010) NLRP3 inflammasome activation: The convergence of multiple signalling pathways on ROS production? Nat. Rev. Immunol., Nature Publishing Group **10**, 210–215.

24 Rubartelli, A., Gattorno, M., Netea, M. G. and Dinarello, C. A. (2011) Interplay between redox status and inflammasome activation. Trends Immunol., Elsevier **32**, 559–566.

25 Fraga, C. G. and Oteiza, P. I. (2011) Dietary flavonoids: role of (–)-epicatechin and related procyanidins in cell signaling. Free Radic. Biol. Med., Elsevier **51**, 813–823.

CHAPTER 4

Dietary fatty acid composition is sensed by the NLRP3 inflammasome in human macrophages

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Submitted

ABSTRACT

The Nod-like receptor protein 3 (NLRP3) inflammasome is considered a pivotal host platform responsible for sensing of exogenous and endogenous danger signals, including those generated as a result of metabolic dysregulation and for the subsequent, IL-1β-mediated orchestration of inflammatory and innate immunity responses. In this way, although the molecular link between diet-induced obesity and inflammasome activation is still unclear, free fatty acids have been proposed as one triggering event.

We report that dietary fatty acid composition is sensed by the NLRP3 inflammasome in human macrophages. For this purpose, we have analysed three roles of fatty acid supplementation: as a priming signal for ATP-activated macrophages, in determining where the administration of dietary fatty acids interferes with LPS-mediated inflammasome activation and by inducing inflammasome activation *per se*. In this manner, saturated (SFAs) or polyunsaturated (PUFAs) fatty acids exerted different modulatory effects on inflammasome activation, whereas SFAs stimulated the secretion of the IL-1β cytokine, and PUFAs were mainly inhibitors.

INTRODUCTION

The incidence of obesity worldwide has increased dramatically in recent years. Obesity is associated with a cluster of several chronic metabolic and inflammatory alterations known as metabolic syndrome, that includes impaired insulin sensitivity, type 2 diabetes, dyslipidaemia and atherosclerosis [1,2].

Immunity and metabolism are considered fundamental systems for survival. In fact, cell homeostasis is linked to the integration and continuous crosstalk between the immune system and metabolism [3]. In this way, an immune response is influenced by the nutritional status. Obesity is associated with chronic post-absorptive and postprandial lipid overload that results in hyperlipidaemia and lipid accumulation in adipose or non-adipose metabolic tissues. Hypertrophy and hyperplasia of adipose tissue as well as ectopic fat accumulation underlie the activation of the inflammatory response and promote the recruitment of immune cells, which are exposed to environments with high concentrations of fatty acids [4–6].

Tissues and blood fatty acids are mainly derived from the diet and have been used as dietary intake biomarkers [7]. Moreover, elevated levels of fatty acids are usually one of the hallmarks of obesity-related pathologies [8]. Fatty acids present in the diet can be divided into two major classes depending on their chemical structure: saturated (SFAs) or unsaturated fatty acids (UFAs). Palmitate (16:0) and stearate (18:0) are the most important saturated fatty acids in the blood, representing 90% of the total SFAs [7]. Moreover, UFAs can be divided into monounsaturated and polyunsaturated fatty acids (MUFAs and PUFAs, respectively), and PUFAs are further divided into two categories, namely, omega-6 and omega-3, based on the location of the first double bond from the methyl end of the fatty acid molecule. Arachidonic acid (C20:4 n-6) and docosahexaenoic acid (DHA, C22:6 n-3) are both considered essential fatty acids because the omega-6/omega-3 ratio is directly related to the pathogenesis of many diseases, including those related to the dysregulation of inflammatory or immune response [9].

On the other hand, innate immunity provides a first line of host defence that is responsible for recognising external pathogenic microorganisms by targeting highly conserved molecular patterns (also known as pathogen-associated molecular patterns, PAMPs) and for sensing host-derived danger signals (also known as danger associated molecular patterns, DAMPs). The recognition of PAMPs and DAMPs is primarily mediated through the expression of an array of germline-encoded, pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) and Nod-like receptors (NLRs) [10,11]. Sensing of PAMPs and DAMPs by PRRs on innate immune cells, such as macrophages, results in the activation of different intracellular signalling pathways that lead to the activation of the inflammatory response [12,13].

TLRs, a family of transmembrane proteins, play a major role in immune signal recognition and transduction that promotes inflammation and innate immune response activation and triggers the adaptive immune response [10]. Then, signal transduction from TLR4 induces

the activation of nuclear factor κB (NF-κB), which is a key transcription factor involved in regulation of the immune and inflammatory responses. In this way, cyclooxygenase 2 (PGH_2 or COX_2), a NF-κB regulated gene, catalyses the rate-limiting step of prostaglandin and thromboxane biosynthesis, and its protein overexpression is considered a hallmark of inflammation.

NLRs form central molecular platforms that are mostly expressed in the cytosol and organise signalling complexes such as inflammasomes [14]. Structurally, inflammasomes are large, multiprotein complexes consisting of Nod-like receptor protein 3 (NLRP3), apoptosis-associated speck-like protein (ASC) and caspase-1 that recognise a diverse set of unrelated stimuli including PAMPs, DAMPs and even completely inorganic molecules such as silica, asbestos or alum. Activation of NLRP3, which is the best-characterised NLR family member, promotes inflammasome assembly, which triggers an inflammatory cascade that leads to mediation of the ASC protein adaptor, the recruitment and autocatalytic activation of caspase-1 and inducing the subsequent proteolytic activation of the proinflammatory cytokines interleukin 1β (IL-1β) and IL-18[14]. IL-1β is a key proinflammatory mediator that underlies the pathogenesis of metabolic disorders, including the obesity-associated inflammatory response and mediation of the development of insulin resistance [15]. Therefore, inflammasome activation must be tightly regulated at the transcriptional and post-transcriptional levels to avoid potentially harmful inflammation [16].

Therefore, the activation of the inflammasome and IL-1β secretion requires two signals. A first signal is responsible for priming the cell to induce the transcription of the immature form of IL-1β, pro-IL-1β and NLRP3, which are limiting factors required for inflammasome activation, via the TLR4-mediated NF-κB activation pathway. The second signal, the activation signal, promotes conformational changes that induce NRLP3 inflammasome assembly and drives caspase-1 activation and, therefore, IL-1β maturation and secretion [16].

Otherwise, the NRLP3 inflammasome might be activated by host-derived DAMP signals, which are abundant in metabolic stress. Therefore, inflammasome activation through diet-associated sensing signals can be considered a strong link between inflammation and metabolic disturbances [13,14,17]. Indeed, it has been described that obesity-induced inflammasome activation in key metabolic tissues promote chronic inflammation and contribute to the development of type 1 and type 2 diabetes [18].

Consequently, obesity is caused by an energy imbalance and total fat intake; and different compositions of dietary fat (in terms of individual fatty acids) may cause different effects on the onset of metabolic complications. In the present study, we analysed the inflammasome-mediated inflammatory effects of dietary fatty acids that were dependent upon its structure-based chemical properties. For this reason, the two most important SFAs in blood, palmitate and stearate, and two PUFAs, DHA and arachidonic acid, were tested as physiological inducers of NLRP3 inflammasome activation. The aim of this study was to determine

whether the fatty acid composition of the diet differentially affected NLRP3 inflammasome priming and the activation of macrophages in a fatty acid-rich environment.

MATERIAL AND METHODS

Chemicals

The dietary fatty acids sodium palmitate and cis-4-,7,10,13,16,19-docosahexaenoic acid were obtained from Sigma-Aldrich (Steinheim, Germany), arachidonic acid was provided by Cayman Chemical (Michigan, USA), and stearic acid was purchased in Panreac (Barcelona, Spain). The culture medium RPMI 1640 was purchased from Gibco (Barcelona, Spain). Cell culture reagents were provided by BioWhittaker (Verviers, Belgium), except for the differentiating agent PMA (phorbol 12-myristate 13-acetate), which was purchased from InvivoGen (San Diego, USA), and LPS (*Escherichia coli* 0111:B4) and adenosine 5'-triphosphate (ATP), which were obtained from Sigma-Aldrich (Steinheim, Germany).

Cell culture and stimulation

Human THP-1 monocytes that were purchased from the European Collection of Animal Cell Cultures (ECACC no. 88081201) were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin, 25 mM HEPES and 10% foetal bovine serum at 37°C in a humidified incubator with 5% CO_2. They were differentiated into macrophages by cultivation in fresh medium containing 0.5 µg/ml PMA for 24 h. After differentiation, the medium containing PMA was removed, and adherent THP-1 macrophages were incubated in supplemented medium for 24 h.

To explore the role of individual fatty acids on the modulation of inflammasome priming and activation, three different roles were tested (Figure 1). First, the priming capacity of each individual fatty was determined, and, for this purpose, THP-1 macrophages were primed with 250 µM of BSA-conjugated fatty acids for the indicated times, and the NLRP3 inflammasome was activated using 5 mM of ATP. Second, the interfering of dietary fatty acids with LPS-induced inflammasome priming and its subsequent activation were analysed. Therefore, THP-1 cells were primed with 1 µg/ml of LPS for the indicated times before supplementation with 250 µM of each BSA-conjugated fatty acid. Third, the capacity of each fatty acid to activate the inflammasome *per se* was determined in terms of IL-1β secretion. In this case, THP-1 macrophages were supplemented with 250 µM of BSA-conjugated fatty acids without any other priming or activating external stimuli. Dietary fatty acids were conjugated to fatty acid-free BSA, which was obtained from Sigma-Aldrich (Steinheim, Germany) at a 4:1 molar ratio.

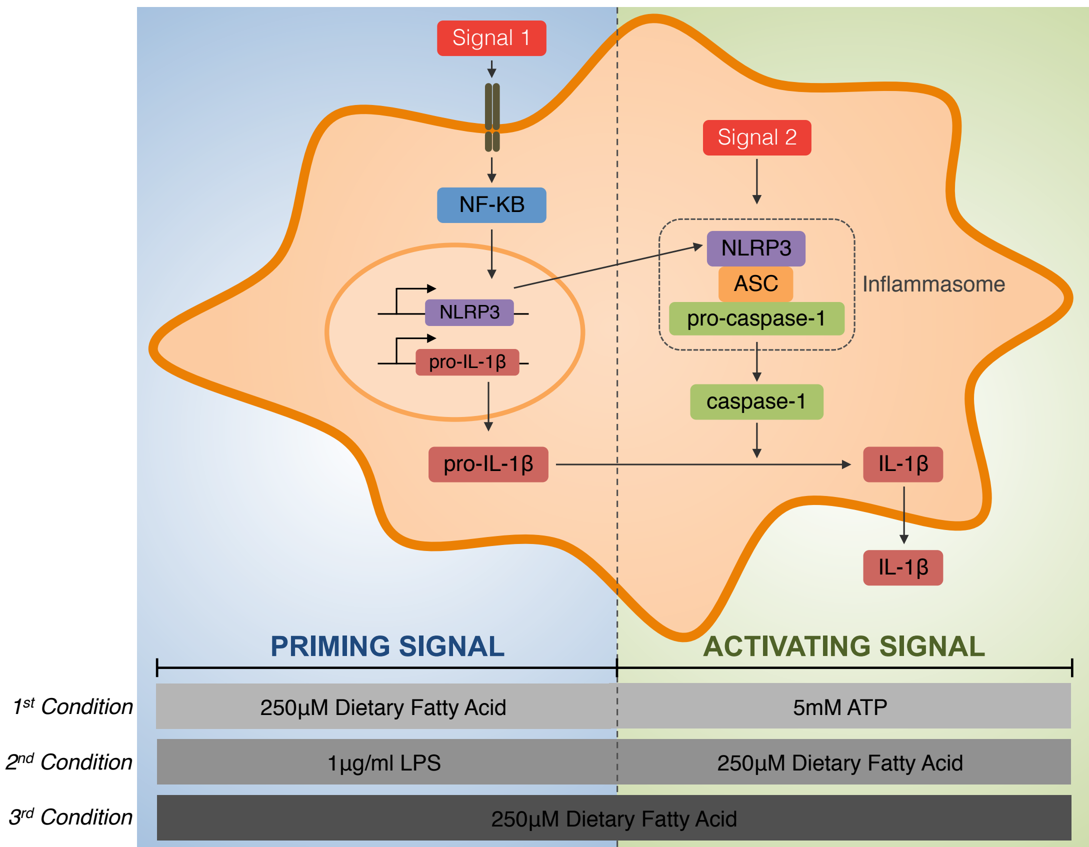

Figure 1. Experimental design. Two steps are required for the processing and release of the mature form of IL-1β. Primarily, a pro-inflammatory stimulus (signal 1) is triggered via the TLR4 membrane receptor, the activation NF-κB and the transcription of immature pro-IL-1β and NLRP3, which are two crucial proteins for NLRP3 inflammasome activation. In a second step, an activation signal promotes NLRP3 assembly, which induces the ASC-mediated recruitment and autocatalytic activation of caspase-1 into a large cytosolic protein named the inflammasome. Activated caspase-1 processes the immature form of IL-1β into its biologically active cytokine. Therefore, three different conditions have been used to evaluate the roles of individual dietary fatty acids as modulators of inflammasome priming and activation. In the first condition, the effects of dietary fatty acids as priming stimuli were determined; therefore, macrophages were primed with fatty acids before ATP-mediated inflammasome activation. In the second condition, the capacity of dietary fatty acids to interfere with LPS-induced inflammasome activation was analysed. For this purpose, LPS-primed macrophages were supplemented with the studied fatty acids. In the third condition, the capacity of each dietary fatty acid to induce inflammasome activation *per se* was studied.

Protein extraction and Western blotting

After 2 h of priming and 4 h of activation with the indicated compounds (either fatty acids, LPS or ATP), cytosolic fractions were extracted, and protein expression was determined by western blotting following a previously described procedure [19]. Briefly, protein was separated in a 10% SDS-PAGE gel, transferred to PVDF membrane and blocked prior to detection with anti-COX_2 (Bioworld, Barcelona, Spain), anti-NLRP3 or anti-IL-1β (Abcam,

Cambridge, UK). Semi-quantification of protein levels was performed using the ImageJ software (National Institutes of Health, Maryland, USA).

Caspase-1 activation assay

THP-1 macrophages were primed for 30 min with either 250 µM of the corresponding fatty acids or LPS (1 µg/ml), followed by activation for 1 h with the corresponding stimuli according to Figure 1 (5 mM of ATP or 250 µM of fatty acids) before the addition of FAM-YVAD-fmk, which is a fluorescent, cell-permeable probe that covalently binds only to the active form of caspase-1. Active caspase-1 was determined by fluorescence according to the manufacturer's instructions (Immunochemistry Technologies). The fluorescent intensity was determined at $\lambda ex=490$ and $\lambda em=520$ using the FLx800 Multi-Detection Microplate Reader (Biotek, Winooski, USA).

IL-1β secretion

Mature IL-1β was analysed in culture media using an enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's protocol (BioLegend). Levels of secreted IL-1β were normalised according to the protein content and measured using the Bradford assay.

Statistical analysis

Results are expressed as the mean ± S.E.M. The effects of fatty acids were assessed using ANOVA. Tukey's test was used to make pair-wise comparisons. Differences were considered significant when the p values were <0.05. Calculations were performed using the SPSS 20.0 software.

RESULTS

Fatty acids modulate the activation of NF-κB

To determine whether the fatty acid composition of a diet might have a crucial role in macrophage inflammasome activation, modulation of NF-κB signalling pathway was first analysed.

Given the potential role of NF-κB in relaying proinflammatory signals, COX_2 protein expression was determined to test whether fatty acid structure (saturated or polyunsaturated) could be involved in activation upstream of the NF-κB signalling pathway. The effects of dietary fatty acids on NF-κB and subsequent COX_2 protein expression were determined for two different conditions (Figure 2 and 3, respectively). First, we determined the role of dietary fatty acids as a priming signal, and, for this purpose, macrophages were primed for 2 h with each of the studied fatty acids before their activation with ATP for 4 h. In this first condition (Figure 2A and B), only the use of palmitate as a priming signal potentially increased COX_2 protein expression in THP-1 macrophages. On the other hand, the use of stearic acid or unsaturated fatty acids as priming signals for inflammasome activation did not produce any significant effect regarding COX_2 protein expression.

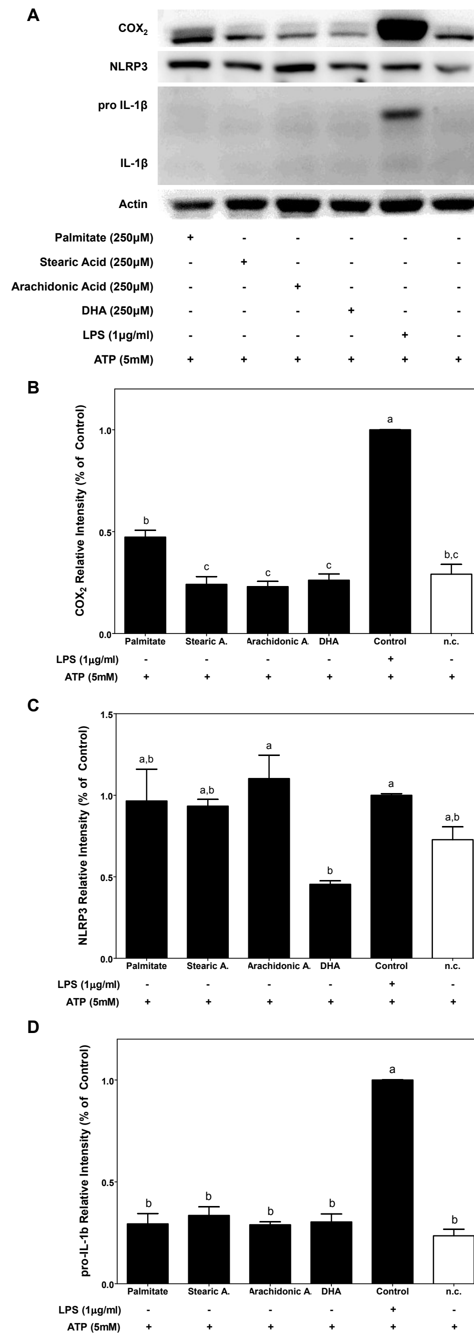

Figure 2. Modulation of NF-κB activation in dietary fatty acid-primed macrophages. Saturated palmitate and stearic acids as well as unsaturated n-6 arachidonic acids and n-3 DHA are modulators of regulated NF-κB protein expression. (A) Representative western blot analysis and semi-quantification of COX_2 (B), NLRP3 (C) and IL-1β (D) proteins expression after priming macrophages with the indicated fatty acids for 2 h and 4 h of ATP-inflammasome activation. The results represent the mean ± S.E.M. Bars sharing the same letters are not significantly different; otherwise, bars with different letters are significantly different ($P < 0.05$).

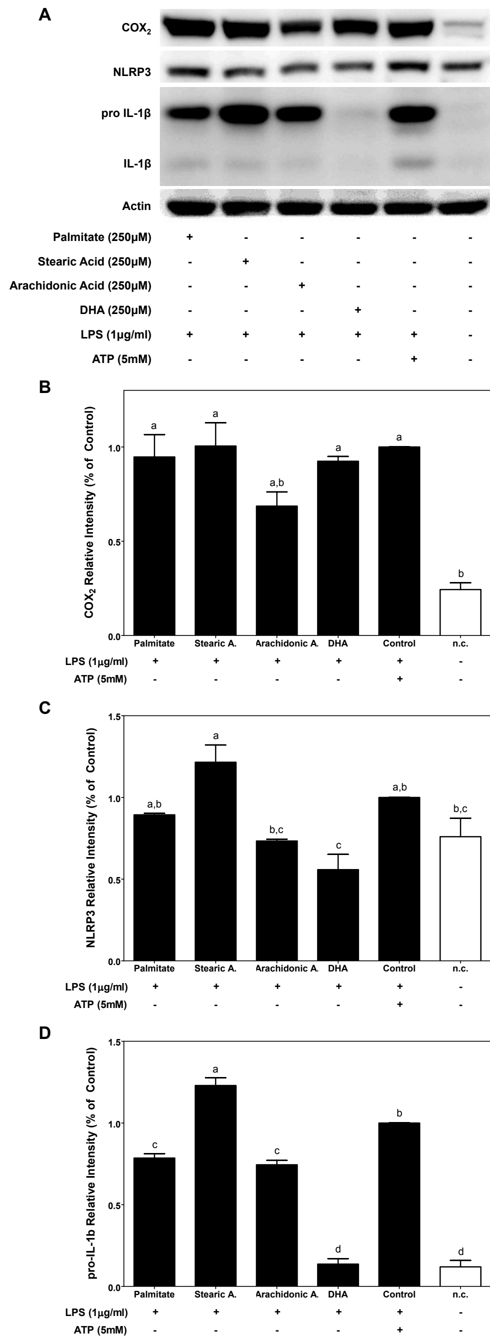

Figure 3. Effects of fatty acids on NF-κB activation in LPS-primed macrophages. Saturated palmitate and stearic acids as well as unsaturated n-6 arachidonic acids and n-3 DHA can modulate the expression of NF-κB-regulated proteins. (A) Representative western blot analysis of COX_2, NLRP3 and IL-1β protein expression after priming for 2 h with 1 µg/ml of LPS prior to its supplementation with the indicated fatty acids for 4 h. Protein semi-quantifications are shown for COX_2 (B), NLRP3 (C) and IL-1β (D). The results are represented by the mean ± S.E.M. Bars with different letters are significantly different ($P < 0.05$).

Second, the effects of dietary fatty acids on COX_2 protein expression induced by LPS-primed macrophages were determined. THP-1 macrophages were primed for 2 h with LPS prior to supplementation with the corresponding fatty acid for 4 h (Figure 3A). In this situation, COX_2 protein expression that was induced after priming macrophages with LPS was decreased by supplementation with n-6 arachidonic acid (Figure 3B).

Saturated and unsaturated fatty acids modulate NLRP3 inflammasome priming

Among the NF-κB up-regulated proinflammatory mediators, the increase in NLRP3 and IL-1β transcription, which is known as the priming step, is a critical checkpoint that is required for inflammasome activation. In this way, to study the capacities of dietary fatty acids in modulating the NLRP3 and IL-1β cytosolic pools, their protein expressions were determined.

In this way, while supplementation of macrophages with 250 µM of each saturated fatty acid (palmitate and stearic acid) before ATP activation were not effective in increasing the cytosolic pool of NLRP3, the n-3 DHA unsaturated fatty acids significantly decreased NLRP3 protein expression in macrophages (Figure 2A and C). On the other hand, in conditions where fatty acids were used as priming signals, a significant increase in the cytosolic expression of the immature or mature forms IL-1β were observed (Figure 2A and D).

When macrophages were primed with LPS before fatty acid supplementation, the cytosolic pool of NLRP3 proteins was increased after supplementation with stearic acid, and it was decreased by n-6 arachidonic acid and, more importantly, by n-3 DHA (Figure 3A and C).

Moreover, the expression of immature IL-1β that was induced by priming macrophages with LPS was significantly blocked by dietary fatty acids. In this way, while stearic acid induced an increase in the cytoplasmic expression of pro-IL-1β, palmitate and n-6 and n-3 unsaturated fatty acids significantly decreased its expression. It should be noted that the dramatic inhibition of pro-IL-1β protein expression that was promoted by DHA supplementation lowered the protein amount to levels similar to those of the untreated control (Figure 3A and D).

Fatty acids are modulators of caspase-1 activation

To analyse the role of dietary fatty acid on inflammasome activation, the percentage of activated caspase-1, an essential step in controlling IL-1β maturation after NLRP3 assembly, was determined.

For the first condition that was analysed, fatty acids were used as a priming stimulus for caspase-1 (Figure 4A). Fluorometric determinations identified n-6 arachidonic acid as a direct caspase-1 activator.

Although the supplementation of macrophages with palmitate, n-6 and n-3 fatty acids slightly increased the amount of active caspase-1 when macrophages were primed with LPS, only stearic acid induced a significant increase in caspase-1 activation, which enhanced the activating effects that were induced by LPS (Figure 4B).

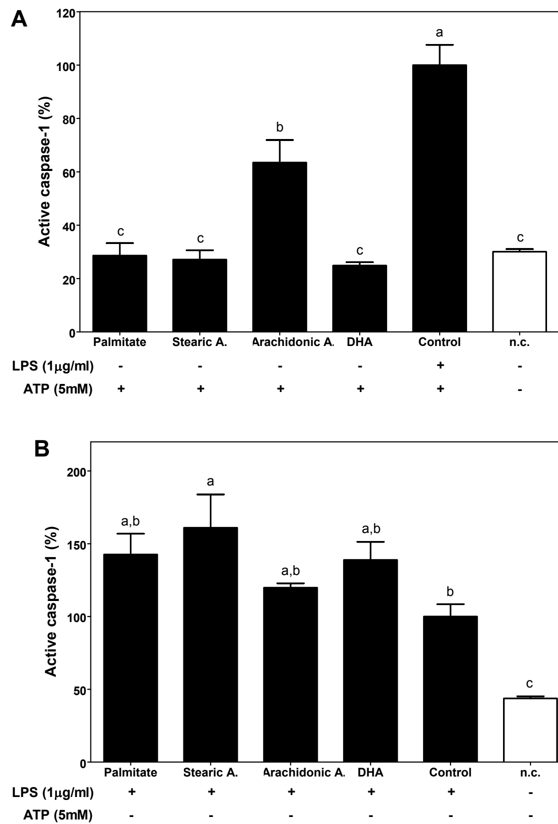

Figure 4. Dietary fatty acids are modulators of caspase-1 activation. (A) THP-1 macrophages were primed with 250 µM of the indicated fatty acids for 30 min, and caspase-1 was activated with 5mM of ATP for 1 hour. (B) After 30 min of 1 µg/ml LPS priming, 250 µM of the indicated dietary acids were administered for 1 hour. The results are represented by the mean ± S.E.M. Bars with different letters are significantly different ($P < 0.05$).

IL-1β secretion is induced by fatty acid administration

To determine whether dietary fatty acids modulated inflammasome activation, the secretion of IL-1β was assessed (Figure 5). The activity of fatty acids on inflammasome activation and the resulting IL-1β secretion was evaluated using the three different conditions, as detailed in Figure 1.

First, fatty acids were used as priming signals before inflammasome activation with ATP (Figure 5A). Priming macrophages with either of the saturated fatty acids, palmitate or stearic, or with n-6 arachidonic acid significantly enhanced the secretion of IL-1β.

However, DHA was ineffective as a priming stimulus and in the subsequent induction of IL-1β secretion.

Secondly, in LPS stimulated macrophages prior to supplementation with dietary fatty acids (second condition described in Figure 1), LPS-induced IL-1β secretion was slightly blocked by treating cells with palmitate and n-3 DHA and was significantly decreased by n-6 arachidonic acid (Figure 5B).

Finally, the effect of dietary fatty acids in directly activating IL-1β secretion without any other activating or stimulating signal was measured (Figure 5C). In this third condition, only macrophages stimulated by the saturated fatty acid palmitate were sufficient to induce a significant increase in IL-1β secretion.

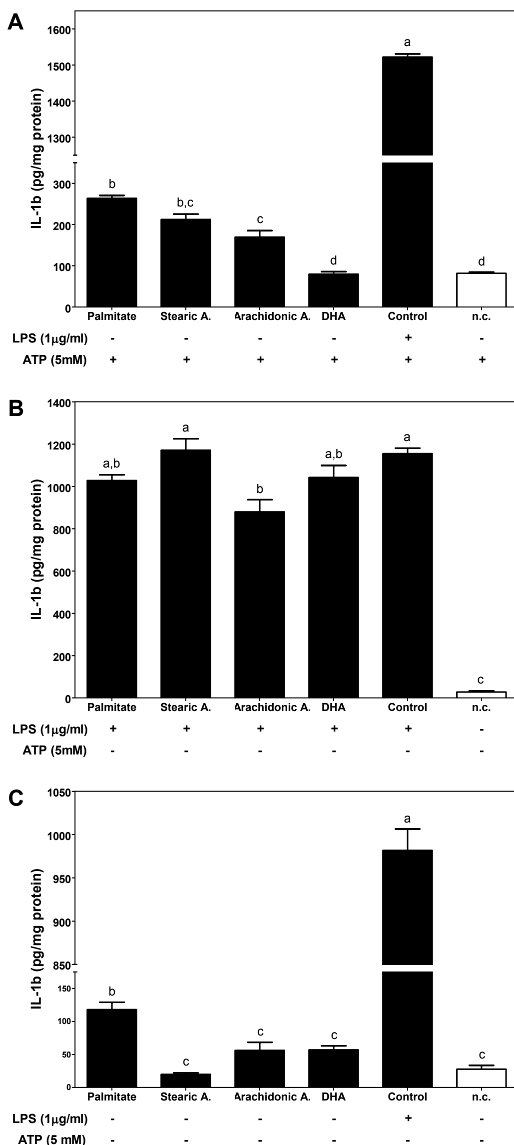

Figure 5. Modulation of IL-1β secretion by dietary fatty acids. (A) Modulation of IL-1β secretion in fatty acid-primed macrophages for 2 h previous to ATP-mediated activation. (B) Release of IL-1β in macrophages primed with LPS (1 µg/ml) for 2 h and supplemented for 4 h with the indicated fatty acids. (C) IL-1β production induced by administration of the indicated fatty acids for 6 h. The results are represented by the mean ± S.E.M. Bars with different letters are significantly different ($P < 0.05$).

DISCUSSION

Obesity is associated with a continuous increase in post-absorptive and postprandial fatty acid concentrations, which underlies the development of obesity-related metabolic complications. In this way, in chronic high-fat intake conditions, tissues and plasma-free fatty acid levels are commonly elevated [7,8].

Dietary fat has a dual role in human physiology. Besides its functions as a source of energy or cell membrane structural components, dietary fatty acids can also act as signalling molecules at different levels[20], including nuclear receptor ligands [21,22], or as modulators of the immune system activation. In this sense, the capacity of dietary fatty acids to interact with TLR4 has been previously described [23]. For this reason, in addition to the total dietary fat intake during the onset and progression of metabolic disturbances, the different composition of fat, in terms of the degree of fatty acid saturation, plays a key role [6,24]. In fasting conditions, the levels of free fatty acids in human blood are approximately of 700 µM; however, this concentration can be widely modified after the intake of a fatty meal [25]. Therefore, blood-circulating cells, such as monocytes or tissue-infiltrated macrophages, would be constantly exposed to relatively high concentrations of free fatty acids [26].

Moreover, dietary fatty acid composition can be sensed by the innate immune response, thus, in a diet-induced obesity context, the hyperlipidaemia-promoted excess of fat accumulation in metabolic tissues triggers the recruitment and activation of immune cells. In this way, inflammasomes are intracellular, innate immune sensors[16]. Subsequently, danger signals resulting from metabolic disturbance can be sensed by NLRP3, a NOD-like cytosolic pattern recognition receptor that is expressed mostly in cells involved in the innate immune response, resulting in caspase-1-mediated IL-1β maturation and secretion. This metabolic-triggered inflammasome activation has been linked to the development and progression of many metabolic disorders, including, among others, atherosclerosis and type 2 diabetes [6,7,27].

In this manner, based on the assumption that dietary fatty acids might influence the immune system as well the function of various cellular components, the aim of this study was to determine whether the chemical properties that are linked to the degree of fatty acid saturation might differentially affect inflammasome activation and IL-1β secretion. For this purpose, the capacity of four different fatty acids that are present in diets, two SFAs (palmitate and stearic acid) and two PUFAs (n-6 arachidonic acid and n-3 DHA) as modulators of NLRP3 inflammasome activation was determined by supplementing human macrophages with physiological concentrations of fatty acids under the three different conditions (Figure 1). First, the role of fatty acids as a priming signal before ATP-induced NLRP3 inflammasome activation was studied. Second, the capacity of fatty acids to interfere with IL-1β secretion in LPS-primed macrophages was determined. Third, the inflammasome-activating properties of dietary fatty acids *per se*, that is, without the administration of any other priming or activating exogenous signal were analysed.

In this way, in the first proposed condition, the effects of dietary fatty acids acting as priming signals in inflammasome activation were evaluated. We demonstrated that both SFAs that were analysed, palmitate and stearic acid, as well as the n-6 PUFA acted as priming signals of inflammasome activation, but n-3 DHA had no effect. In this manner, the supplementation of macrophages with SFAs or n-6 PUFA before ATP-mediated inflammasome activation significantly increased IL-1β secretion, while n-3 DHA supplementation showed no stimulating effect on IL-1β secretion. Regarding, the inflammasome activation checkpoints that are regulated by these fatty acids, palmitate was the only fatty acid that was able to enhance COX_2 protein expression in macrophages, supporting the role of palmitate as a TLR4 agonist and as an activator of NF-κB. Then, the modulation of the cytosolic pool of NLRP3 was only significantly modulated by DHA, which induced a decrease in its protein expression. Otherwise, caspase-1 activation levels were only significantly enhanced when n-6 arachidonic was used as a priming stimulus.

On the other hand, although it has been controversial for years, it has currently been described that acute priming of LPS is solely required to boost the inflammasome and activate caspase-1 and the resulting secretion of mature IL-1β [28–30]. Thus, by using the second experimental condition, the capacities of dietary fatty acids to interfere with LPS-induced inflammasome activation were determined. Of the dietary fatty acids that were tested, the supplementation of LPS-primed macrophages with stearic acid potentiated the proinflammatory effects induced by LPS, which enhanced the cytosolic pool of the two limiting components of inflammasome activation, NLRP3 and pro-IL-1β, and caspase-1 activation.

Surprisingly, arachidonic acid interfered inversely with LPS-induced NF-κB activation. We showed that supplementation of LPS-primed macrophages with arachidonic acid decreased COX_2, NLRP3 and pro-IL-1β protein abundance in the cytoplasm of LPS-primed macrophages and decreased IL-1β secretion. These results indicate that supplementation of macrophages with n-6 arachidonic acid might modulate the efficiency of LPS to induce NF-κB activation. Although, arachidonic acid is a precursor of the cyclooxygenase-mediated synthesis of eicosanoids, it is also stored in the cell membrane. It has been described that the modification of cell membrane fatty acid composition influences membrane fluidity, which, in turn, would modify membrane receptor function as well as signal transduction mechanisms[31]. Thus, an arachidonic acid-rich diet could modify the cell membrane composition, which could affect its fluidity and, then, signal transduction, as was observed for LPS-primed macrophages.

Interestingly, n-3 DHA inhibited LPS-mediated inflammasome activation. This inhibition was primary mediated by the significant repression of NLRP3 and pro-IL-1β protein expression, which, in turn, translated to a slight decrease in the secretion of mature IL-1β, confirming that DHA modulation of inflammasome activation is mainly through the inhibition of LPS-induced NF-κB activation.

Finally, in the third condition, the abilities of dietary fatty acids to modulate *per se* the priming and activation of the inflammasome and IL-1β were analysed. We demonstrated that only palmitate was able, *per se* without any additional stimulus (either priming or activating), to significantly induce the secretion of IL-1β. These results are in accordance with the description of palmitate as a ligand for TLR4, which promotes the activation of NF-κB and the transcription of its proinflammatory target genes [23]. Moreover, it has been described previously that the stimulation of macrophages with palmitate can induce the production of reactive oxygen species (ROS) [32]. Therefore, palmitate may induce the activation of the inflammasome and the secretion of IL-1β in macrophages by directly acting as a priming signal through its TLR4-agonist activity and by sensing NLRP3, which mediates the production of ROS, similar to that described previously for LPS [33].

In summary, we can conclude that dietary fatty acid composition can be sensed by NLRP3 inflammasome activation and that the chemical structure of these fatty acids, regarding the degree of saturation, is critical for its regulating effects. Whereas saturated fatty acids, such as palmitate and stearic acid, show mainly a proinflammatory profile by promoting inflammasome activation and IL-1β secretion, polyunsaturated fatty acids are inhibitors of inflammasome activation by mainly interfering with LPS-TLR4 signal transduction, NF-κB and the inflammasome activation step. With these results, we have provided new insights regarding the molecular-sensing mechanisms involved in signal transduction by high levels of fatty acids to trigger intracellular inflammatory signalling, which is a mechanism that underlies the development of diet-induced chronic inflammation and metabolic disturbances. In this manner, the diet and, more accurately, the modification of fatty acid composition can be a powerful tool for regulating the inflammatory and innate immunity responses as well as for the promotion of homeostasis.

ACKNOWLEDGEMENTS

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REFERENCES

1 Speakman, J. R. (2004) Obesity: the integrated roles of environment and genetics. J. Nutr., Am Soc Nutrition **134**, 2090S–2105S.

2 Miranda, P. J., DeFronzo, R. A., Califf, R. M. and Guyton, J. R. (2005) Metabolic syndrome: definition, pathophysiology, and mechanisms. Am. Heart J., St. Louis, CV Mosby Co. **149**, 33–45.

3 Hotamisligil, G. S. (2006) Inflammation and metabolic disorders. Nature, Nature Publishing Group **444**, 860–867.

4 Iyer, A., Fairlie, D. P., Prins, J. B., Hammock, B. D. and Brown, L. (2010) Inflammatory lipid mediators in adipocyte function and obesity. Nat. Rev. Endocrinol., Nature Publishing Group **6**, 71–82.

5 Lumeng, C. N. and Saltiel, A. R. (2011) Inflammatory links between obesity and metabolic disease. J. Clin. Invest., American Society for Clinical Investigation **121**, 2111.

6 Schaffer, J. E. (2003) Lipotoxicity: when tissues overeat. Curr. Opin. Lipidol., LWW **14**, 281–287.

7 Hodson, L., Skeaff, C. M. and Fielding, B. A. (2008) Fatty acid composition of adipose tissue and blood in humans and its use as a biomarker of dietary intake. Prog. Lipid Res., Elsevier **47**, 348–380.

8 Fielding, B. A., Callow, J., Owen, R. M., Samra, J. S., Matthews, D. R. and Frayn, K. N. (1996) Postprandial lipemia: the origin of an early peak studied by specific dietary fatty acid intake during sequential meals. Am. J. Clin. Nutr., Am Soc Nutrition **63**, 36–41.

9 Simopoulos, A. P. (2002) The importance of the ratio of omega-6/omega-3 essential fatty acids. Biomed. Pharmacother., Elsevier **56**, 365–379.

10 Barton, G. M. and Kagan, J. C. (2009) A cell biological view of Toll-like receptor function: regulation through compartmentalization. Nat. Rev. Immunol., Nature Publishing Group **9**, 535–542.

11 Zhang, G. and Ghosh, S. (2001) Toll-like receptor–mediated NF-κB activation: a phylogenetically conserved paradigm in innate immunity. J. Clin. Invest., Am Soc Clin Investig **107**, 13–19.

12 Janeway, C. A. and Medzhitov, R. (2002) Innate immune recognition. Annu. Rev. Immunol. **20**, 197–216.

13 Schroder, K. and Tschopp, J. (2010) The inflammasomes. Cell, Elsevier **140**, 821–832.

14 Martinon, F., Mayor, A. and Tschopp, J. (2009) The inflammasomes: guardians of the body. Annu. Rev. Immunol., Annual Reviews **27**, 229–265.

15 Wen, H., Ting, J. P. Y. and O'Neill, L. A. J. (2012) A role for the NLRP3 inflammasome in metabolic diseases [mdash] did Warburg miss inflammation [quest]. Nat. Immunol., Nature Publishing Group **13**, 352–357.

16 Strowig, T., Henao-Mejia, J., Elinav, E. and Flavell, R. (2012) Inflammasomes in health and disease. Nature, Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved. **481**, 278–86.

17 De Nardo, D. and Latz, E. (2011) NLRP3 inflammasomes link inflammation and metabolic disease. Trends Immunol., Elsevier **32**, 373–379.

18 Stienstra, R., Tack, C. J., Kanneganti, T.-D., Joosten, L. A. B. and Netea, M. G. (2012) The inflammasome puts obesity in the danger zone. Cell Metab., Elsevier **15**, 10–18.

19 Martinez-Micaelo, N., Gonzalez-Abuin, N., Terra, X., Richart, C., Ardevol, A., Pinent, M. and Blay, M. (2012) Omega-3 docosahexaenoic acid and procyanidins inhibit cyclo-oxygenase activity and attenuate NF-kappaB activation through a p105/p50 regulatory mechanism in macrophage inflammation. Biochem. J., Portland Press Ltd. **441**, 653–663.

20 Duplus, E., Glorian, M. and Forest, C. (2000) Fatty acid regulation of gene transcription. J. Biol. Chem., ASBMB **275**, 30749–30752.

21 Kliewer, S. A., Sundseth, S. S., Jones, S. A., Brown, P. J., Wisely, G. B., Koble, C. S., Devchand, P., Wahli, W., Willson, T. M. and Lenhard, J. M. (1997) Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors α and γ. Proc. Natl. Acad. Sci., National Acad Sciences **94**, 4318–4323.

22 Michalik, L., Desvergne, B. and Wahli, W. (2004) Peroxisome-proliferator-activated receptors and cancers: complex stories. Nat. Rev. Cancer, Nature Publishing Group **4**, 61–70.

23 Shi, H., Kokoeva, M. V, Inouye, K., Tzameli, I., Yin, H. and Flier, J. S. (2006) TLR4 links innate immunity and fatty acid–induced insulin resistance. J. Clin. Invest., Am Soc Clin Investig **116**, 3015–3025.

24 Bray, G. A., Lovejoy, J. C., Smith, S. R., DeLany, J. P., Lefevre, M., Hwang, D., Ryan, D. H. and York, D. A. (2002) The influence of different fats and fatty acids on obesity, insulin resistance and inflammation. J. Nutr., Am Soc Nutrition **132**, 2488–2491.

25 Jump, D. B. (2004) Fatty acid regulation of gene transcription. Crit. Rev. Clin. Lab. Sci., Informa UK Ltd UK **41**, 41–78.

26 Shapiro, H., Lutaty, A. and Ariel, A. (2011) Macrophages, Meta-Inflammation, and Immuno-Metabolism. ScientificWorldJournal., Hindawi Publishing Corporation **11**, 2509.

27 Schroder, K., Zhou, R. and Tschopp, J. (2010) The NLRP3 inflammasome: a sensor for metabolic danger? Science (80-.)., American Association for the Advancement of Science **327**, 296–300.

28 Netea, M. G., Nold-Petry, C. A., Nold, M. F., Joosten, L. A. B., Opitz, B., van der Meer, J. H. M., van de Veerdonk, F. L., Ferwerda, G., Heinhuis, B. and Devesa, I. (2009) Differential requirement for the activation of the inflammasome for processing and release of IL-1β in monocytes and macrophages. Blood, American Society of Hematology **113**, 2324–2335.

29 Schroder, K., Sagulenko, V., Zamoshnikova, A., Richards, A. A., Cridland, J. A., Irvine, K. M., Stacey, K. J. and Sweet, M. J. (2012) Acute lipopolysaccharide priming boosts inflammasome activation independently of inflammasome sensor induction. Immunobiology, Elsevier.

30 Kagan, J. C. (2013) Sensing Endotoxins from Within. Science (80-.)., American Association for the Advancement of Science **341**, 1184–1185.

31 Calder, P. C., Bond, J. A., Harvey, D. J., Gordon, S. and Newsholme, E. A. (1990) Uptake and incorporation of saturated and unsaturated fatty acids into macrophage lipids and their effect upon macrophage adhesion and phagocytosis. Biochem.J **269**, 807–814.

32 Shrestha, C., Ito, T., Kawahara, K., Shrestha, B., Yamakuchi, M., Hashiguchi, T. and Maruyama, I. (2013) Saturated fatty acid palmitate induces extracellular release of histone H3: A possible mechanistic basis for high-fat diet-induced inflammation and thrombosis. Biochem. Biophys. Res. Commun., Elsevier.

33 Tschopp, J. and Schroder, K. (2010) NLRP3 inflammasome activation: The convergence of multiple signalling pathways on ROS production? Nat. Rev. Immunol., Nature Publishing Group **10**, 210–215.

CHAPTER 5

The role of the peripheral lymphoid organs, thymus and spleen, in the diet-induced adiposity-triggered immune response

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Submitted

ABSTRACT

Objective: To determine whether diet-induced obesity affects inflammatory and immune responses and characterise the effect of chronic overfeeding on the regulation of lymphoid organs functionality.

Design and Methods: A Cafeteria diet or a standard diet was administered to Wistar rats for 10 weeks. We studied the obesity-driven local inflammatory response in adipose tissue and the responsiveness of the thymus and the spleen to diet by gene expression analysis, as well as we determined the circulating levels of systemic inflammation biomarkers.

Results: Our results support a pivotal role for adipose tissue as the central site for overfeeding-triggered low-grade chronic inflammation, resulting in the up-regulation of proinflammatory genes (*F4/80*, *IL-6* and *TNF-α*) as well as the down-regulation of the anti-inflammatory (*IL-10*). However, the functionality of lymphoid organs in response to obesity-derived noxious stimuli was weakened as a consequence of diet-induced obesity, reflected by the down-regulated expression of the *F4/80*, *IL-6* and *IL-10* gene expression in the thymus and the repression of *F4/80*, *IL-6*, *Crp* and *IL-10* gene expression in the spleen.

Conclusion: Diet-induced obesity compromises the complex crosstalk between the metabolism and the immune system. Diet-induced obesity induces low-grade inflammation, mainly triggered by the adipose tissue, and impairs the immune system affecting thymus and spleen function and gene expression.

INTRODUCTION

Obesity, resulting from an imbalance between energy intake and expenditure, has been associated with several metabolic alterations, including insulin resistance, impaired glucose tolerance, type 2 diabetes mellitus, dyslipidaemia, atherosclerosis, hypertension and the expression of pro-inflammatory biomarkers, a cluster of pathologies widely known as Metabolic Syndrome [1–3].

Indeed, there is complex and continuous crosstalk between metabolic and immune systems because both systems are considered among the most fundamental requirements for survival, and the regulation of metabolism and the immune response are integrated, whereby the proper functioning of each is dependent on the other [4]. Thus, the organs and tissues involved in the regulation of the metabolism, aside from providing the necessary nutrients to trigger and sustain the immune response, contain resident populations of immune cells, suggesting that the immune system is poised to respond to nutrient-derived signals. Furthermore, obesity-associated metabolic dysfunction might result in the malfunctioning of the immune system; thus, regulation of the immune system is indispensable for the maintenance of metabolic homeostasis [5].

Under chronic overfeeding conditions, there is a continuous imbalance between energy intake and expenditure, resulting in an excessive accumulation of triglycerides in the adipocytes, endangering the functionality of these cells and triggering a local low-grade inflammatory response [6]. Consequently, excessive levels of proinflammatory cytokines, such as tumour necrosis factor-α (TNF-α) or interleukin 6 (IL-6), are continuously produced in adipose tissue, promoting the recruitment of immune cells and the activation of a low-grade inflammatory response. Although several chemotactic factors have been implicated in the recruitment of monocytes to adipose tissue, monocyte chemotactic protein-1 (MCP-1) might play a dominant role [7,8]. Thus, obesity-induced inflammation in adipose tissue underlies the systemic activation of the inflammatory response, leading to the widespread activation of the innate immune system and systemic metabolic dysfunction, an event associated with the development of several obesity-related disorders. The systemic effects of inflammation comprise among others, a global reaction known as the acute-phase response, characterised by pronounced changes in the concentration of circulating acute-phase proteins, such as the C-reactive protein (CRP), complement component C3 (C3) and orosomucoid [9,10]. Moreover, adipose tissue is also considered as a key endocrine organ, mediating the release of multiple bioactive substances known as adipose-derived secreted factors or adipokines, which also play pivotal roles in the proper integration of metabolic and immune systems. The adipokines leptin and adiponectin are both involved in the control of energy expenditure, lipid and carbohydrate metabolism and the regulation of the immune response.

Thus, inflammation is a coordinated physiological response of the innate immune system to harmful stimuli, whether physical, chemical or biological, to restore the system to the homeostasis. However, in an obesity context, a continuous surplus of energy intake

promotes the chronic low-grade activation of the innate immune system, impairing not only the resolution of inflammation but also inducing the activation of the adaptive immunity, reflecting the nature of the triggering stimulus.

The immune system, comprising a complex network of organs, tissues and cells that integrates the innate and adaptive immunity, might play a pivotal role in the development of metabolic diseases [4]. Moreover, whereas immune cells and secreted mediators are involved in obesity-induced adipose tissue inflammation, suggesting a prominent role for the cellular components of both the innate and the adaptive arms of the immune system in obesity-induced immune activation, little is known about the role and regulation of the lymphoid organs in the development of obesity and obesity-related disorders. Thus, both the thymus, the primary lymphoid organ involved in bone marrow-derived T cell maturation, and the spleen, a secondary lymphoid organ considered as the immunologic filter of the blood, play pivotal roles in host immune function [11].

The activation and deregulation of the immune system in response to diet-induced chronic lipid overload leads to the impairment of both metabolic and immune homeostasis. However, the mechanisms underlying the continuous activation of inflammation and the responsiveness of lymphoid organs to obesity remain unclear. The aim of this study was to examine the diet-induced activation of immune system and to determine the role of the thymus and spleen, as key central and peripheral lymphoid organs, in the obesity-induced improper deregulation of the immune system.

MATERIALS AND METHODS

Animals and experimental design

Male Wistar rats weighing 150 g were obtained from Charles River Laboratories (Barcelona, Spain). The animals were housed individually in a 12 h light-dark cycle. After adaptation, the animals were randomly distributed into two groups (n=7), fed for 10 weeks with either standard (STD) chow or a cafeteria (CAF) diet. A CAF diet comprised bacon, cookies, muffins, pate, cheese, carrots, milk and sugar added to the standard chow, generating a hypercaloric diet comprising 11.5% protein, 34.5% carbohydrates and 37.8% fat. The animals were fasted for 3 h and sacrificed through abdominal aortic exsanguination. Subsequently, blood samples were collected from the abdominal aorta, and heparinised plasma was obtained through centrifugation. Adipose fat depots (mesenteric, perirenal and epididymal), were excised, weighed, immediately frozen in liquid nitrogen and stored at -80ºC until further analysis. The thymus and spleen were rapidly removed and weighed, and either thymocytes or splenocytes were immediately isolated. All procedures were approved through the Animal Ethics Committee of Rovira i Virgili University.

Measurement of circulating biochemical parameters

After sacrifice, blood samples were collected, and heparinised plasma was obtained through centrifugation. Circulating levels of leptin and adiponectin were determined using specific EIAs according to the manufacturer's instructions (Biosource International Inc., CA, USA). The plasmatic concentrations of orosomucoid and complement factor C3 were determined using ELISA kits according to the manufacturer's instructions (GenWay, San Diego, USA). The ELISA kit for MCP-1 quantification was purchased from Abcam (Cambridge, USA).

Thymocyte and splenocyte isolation.

Thymocyte and splenocyte cell suspensions were obtained from freshly isolated lymphoid tissue through mechanical disruption and fractionation using a 70-µm nylon cell strainer. The isolated cells were treated with ACK lysis buffer (SIGMA, Barcelona, Spain) to eliminate erythrocytes. Trypan blue staining was performed to determine the number of live cells.

Gene expression analysis using real-time RT-PCR

Total RNA from mesenteric fat depots and isolated thymocytes and splenocytes was obtained using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Barcelona, Spain) and purified using RNeasy Mini Kit spin columns (Qiagen, Valencia, CA, USA). cDNA was generated using the reverse transcription reagent kit (Applied Biosystems, Madrid, Spain). The mRNA expression of *Tnf-α*, *IL-6*, *IL-10*, *F4/80*, *Crp* and cyclophilin peptidylprolyl isomerase A (*Ppia*), as reference gene, was determined through RT-PCR amplification using SsoFast EvaGreen SuperMix (Bio-Rad). The forward and reverse primers used for the different genes are described in Table 1. However, the expression of *Bax*, *Bcl-2* and *Actb,* as a reference gene, was determined through RT-PCR amplification using TaqMan Mix (Applied Biosystems, Madrid, Spain) and gene-specific TaqMan probes: Rn99999125_m1 for *Bcl-2*, Rn01480160_g1 for *Bax* and Rn00667869_m1 for *Actb*. The values for the relative gene expression were normalised to the Ppia mRNA levels according to the $2^{-\Delta\Delta Ct}$ method.

Analysis of DNA fragmentation

The formation of DNA fragmentation ladders was analysed using agarose gel electrophoresis according to a previously described method [12]. Briefly, the extracted thymocytes or splenocytes were suspended in 1 ml of hypotonic lysis buffer (0.2% Triton X-100, 1 mM EDTA and 10 mM Tris/HCl, pH 7.5) and incubated for 20 min at 4ºC. The lysed cells were centrifuged for 5 min at 14,000 g at 4ºC, and the supernatants were collected and incubated with 200 µg/ml of RNase A for 1 h at 37ºC. After digesting with 200 µg/ml of proteinase K for 30 min at 50ºC, the DNA was precipitated overnight at -20ºC in 50% isopropanol containing 0.5 M NaCl. The precipitated DNA was pelleted through centrifugation for 15 min at 14,000 g, air-dried and suspended in buffer containing 1 mM of EDTA and 10 mM Tris/HCl, pH 7.5. Equal amounts of isolated DNA (10 µg) were subjected to electrophoresis on a 2% agarose gel. Subsequently, the bands were stained with ethidium bromide and photographed.

Table 1. Rat-specific primer sequences.

Gene		Primer sequence
Tnf-α	Forward	5' CCTCACACTCAGATCATCTTCTC 3'
	Reverse	5' TTGGTGGTTTGCTACGACGTG 3'
IL-6	Forward	5' CTCTCCGCAAGAGACTTCC 3'
	Reverse	5' GCCATTGCACAACTCTTTTCTC 3'
IL-10	Forward	5' GCAGGACTTTAAGGGTTACTTGG 3'
	Reverse	5' GGAGAAATCGATGACAGCGT 3'
F4/80	Forward	5' ATGCATAATCGCTGCTGGCTGAA 3'
	Reverse	5' GAGGGCAGAGTTGATCGTGATGATC 3'
Crp	Forward	5' GGCTTTTGGTCATGAAGACATG 3'
	Reverse	5' TCTTGGTAGCGTAAGAGAAGA 3'
Ucp2	Forward	5' CCTCTGGAAAGGGACCTC 3'
	Reverse	5' GAGGTCGTCTGTCATGAGG 3'
Ppia	Forward	5' CTTCGAGCTGTTTGCAGACAA 3'
	Reverse	5' AAGTCACCACCCTGGCACATG 3'

Statistical analysis

The results are expressed as the mean ± S.E.M. The effects of the CAF diet were assessed using Student's t-test. Differences were considered significant when the p values were <0.05. The calculations were performed using SPSS 20.0 software.

RESULTS

Cafeteria diet promotes adiposity-induced inflammatory responses

To determine the modulatory effects of the CAF diet on the functionality of primary and secondary lymphoid organs, we assessed the diet-induced effects on the activation of inflammatory and immune responses.

Thereby, feeding rats a CAF diet based on high-energy foods resulted in an increase in body weight, with a consequent increase in different fat depots (Table 2). Furthermore, this diet-induced increase in adiposity enhanced macrophage recruitment in adipose tissue, reflected as an increase in *F4/80* gene expression, a macrophage-selective marker, and the activation of the local inflammatory response, defined by the significant transcription of pro-inflammatory genes, including *IL-6* and *Tnf-α*, but not anti-inflammatory genes, such as *IL-10* (Figure 1).

Table 2. Cafeteria diet-induced body weight and adiposity increase. *P<0.05 vs. STD diet-fed rats.

	Standard diet	Cafeteria diet
Body weight gain (%)	34.92 ± 0.67	45.40 ± 0.83*
Tissue weight		
Mesenteric fat (g)	6.25 ± 0.64	14.00 ± 1.52*
Epididymal fat (g)	10.02 ± 0.70	21.54 ± 2.44*
Perirenal fat (g)	8.97 ± 0.41	20.17 ± 2.02*
Adiposity Index (mg/g bw)	55.51 ± 1.29	105.86 ± 5.38*

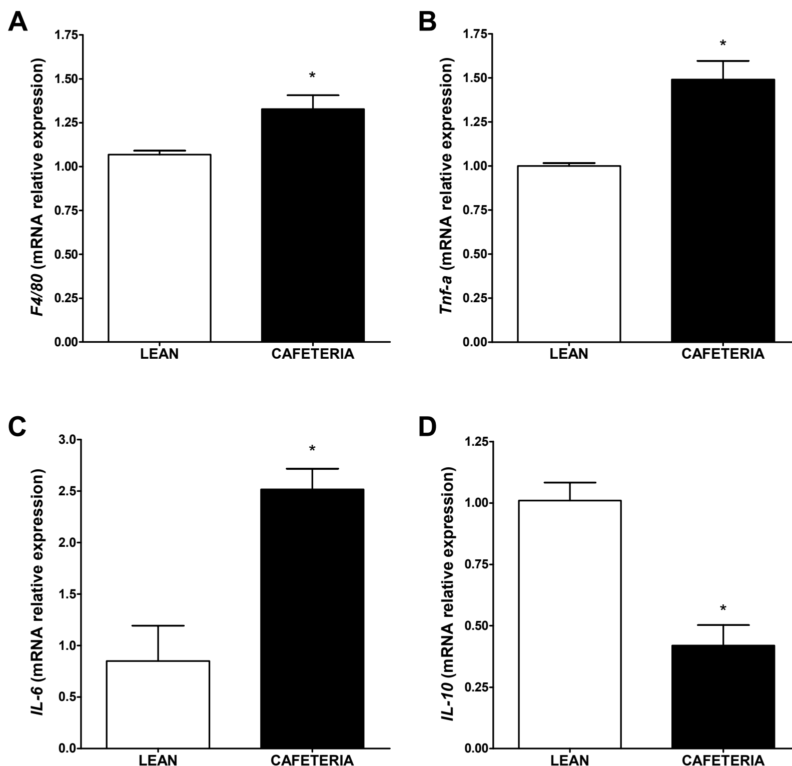

Figure 1. The cafeteria diet induces the modulation of inflammatory biomarkers mRNA expression in the adipose tissue. Gene expression was measured using RT-PCR. (A) Diet effect on *F4/80* macrophage selective marker. (B), (C) and (D) Obesity-induced modulation of *Tnf-α*, *IL-6* and *IL-10* gene expression. Data represent the mean ± SEM. *P<0.05 vs. STD diet-fed rats.

Moreover, these local diet-induced modulatory effects also affected the levels of fat-derived plasma biomarkers (Figure 2). Indeed, the CAF diet induced a significant increase in the levels of circulating leptin, and although no differences were observed in the adiponectin concentration, the leptin/adiponectin ratio was higher in CAF-fed rats. In addition, MCP-1, orosomucoid and C3 plasma levels were significantly increased after the rats were fed a CAF diet.

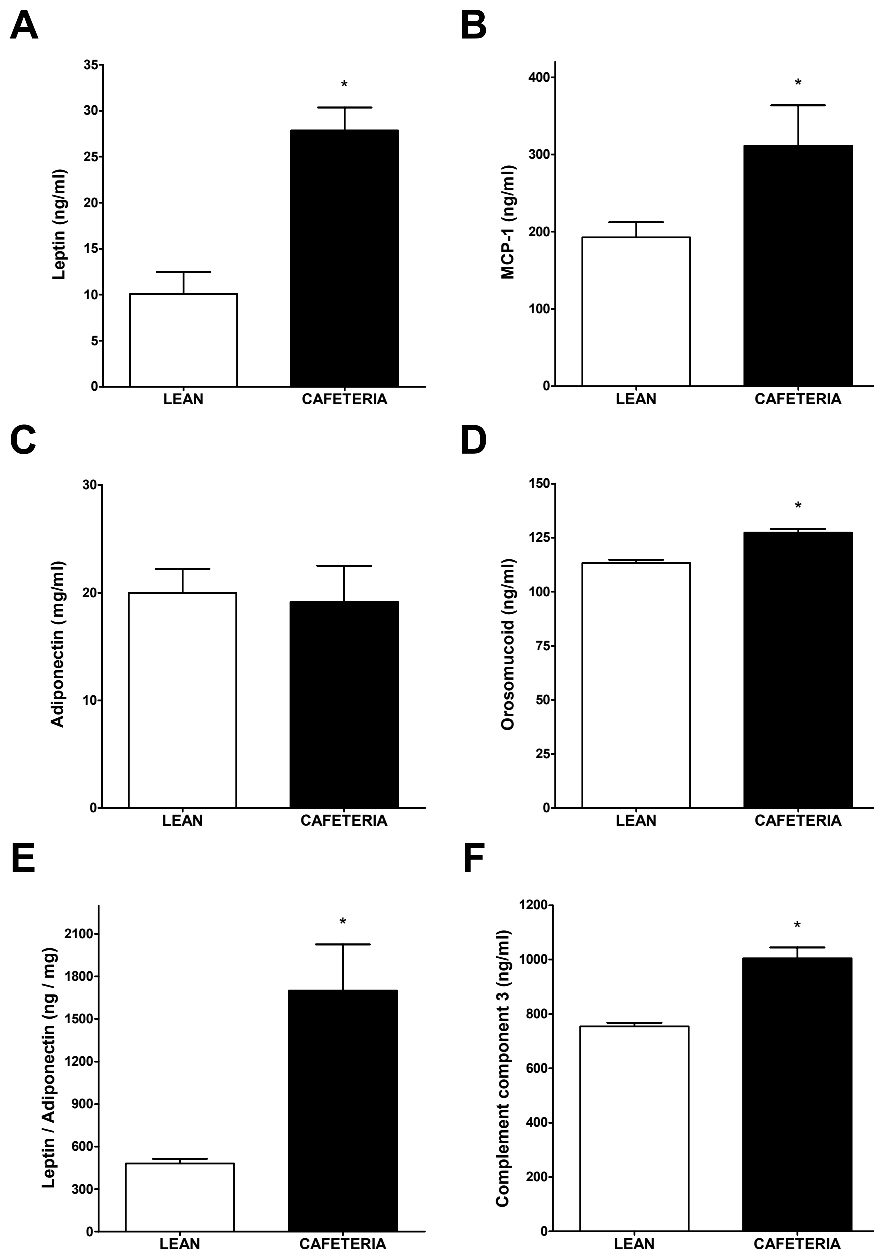

Figure 2. Circulating concentration of biomarkers involved in the systemic responsiveness to diet-induced obesity. Levels of leptin (A), MCP-1 (B), adiponectin (C), orosomucoid (D), the ration between leptin and adiponectin (E) and complement component 3 (F) were measured in plasma of STD and CAF fed rats. Data represent the mean ± SEM. *P<0.05 between the rats fed with STD and CAF diets.

Diet-induced obesity influences the features of the thymus

The modulatory effects of the CAF diet on the functionality of thymus, the primary lymphoid organ involved in the maturation of T cells, were determined based on the regulation of thymic weight and size and the expression of genes involved in the modulation of the inflammatory response (Figure 3). Although no significant diet-induced effects were observed in terms of the thymus weight, the visual appearance of this organ was completely different, suggesting that the CAF diet promoted the accumulation of ectopic fat in the thymus. Furthermore, the CAF diet decreased the presence of macrophages, reflected by the down-regulated expression of the macrophage-specific gene *F4/80*, whereas no significant diet-induced differences were observed for *Tnf-α* transcription, and the CAF diet promoted the down-regulation of both *IL-6* and *IL-10* gene expression.

Moreover, DNA fragmentation in the thymus was also analysed as a marker of diet-induced thymic atrophy. However, the rats fed either STD or CAF diets showed similar DNA ladder integrity patterns. Furthermore, whereas slight but not significant CAF diet-induced effects on the expression of the pro-apoptotic *Bax* gene, the CAF diet down-regulated the transcription of *Bcl-2*, an anti-apoptotic gene.

The spleen functionality is modulated by cafeteria diet

The diet-induced modulation of the immune system was also assessed in terms of spleen regulation and functionality to determine the regulation of this secondary lymphoid organ responsible for integrating innate and adaptive immune responses (Figure 4). The CAF diet induced a significant decrease in spleen weight, although no visual differences were observed regarding the morphology or composition of the spleen cells.

Moreover, the expression of the genes involved in the regulation of the immune response in splenocytes was significantly affected in response to the CAF diet. Indeed, the CAF diet induced the significant repression of macrophage marker *F4/80*, accompanied by a significant decrease in *IL-6*, *Crp* and *IL-10* gene expression; however, *Ucp-2* was significantly overexpressed in the spleens of CAF-fed rats. In addition, although apoptotic DNA fragmentation was clearly visualised, mediating DNA laddering, no diet-induced effect was observed in the modulation of DNA integrity.

DISCUSSION

The immune system is a complex network of tissues and organs comprising molecular, cellular and humoral components that sense perturbations in normal physiology and trigger responses to restore homeostasis. Immune system components that sense obesity-induced metabolic stress resulting from the continuous metabolic surplus and the integration of the functionality of both metabolism and the immune system contribute to the proper regulation of the immune response as a pivotal event associated with the progression of obesity and obesity-related pathologies.

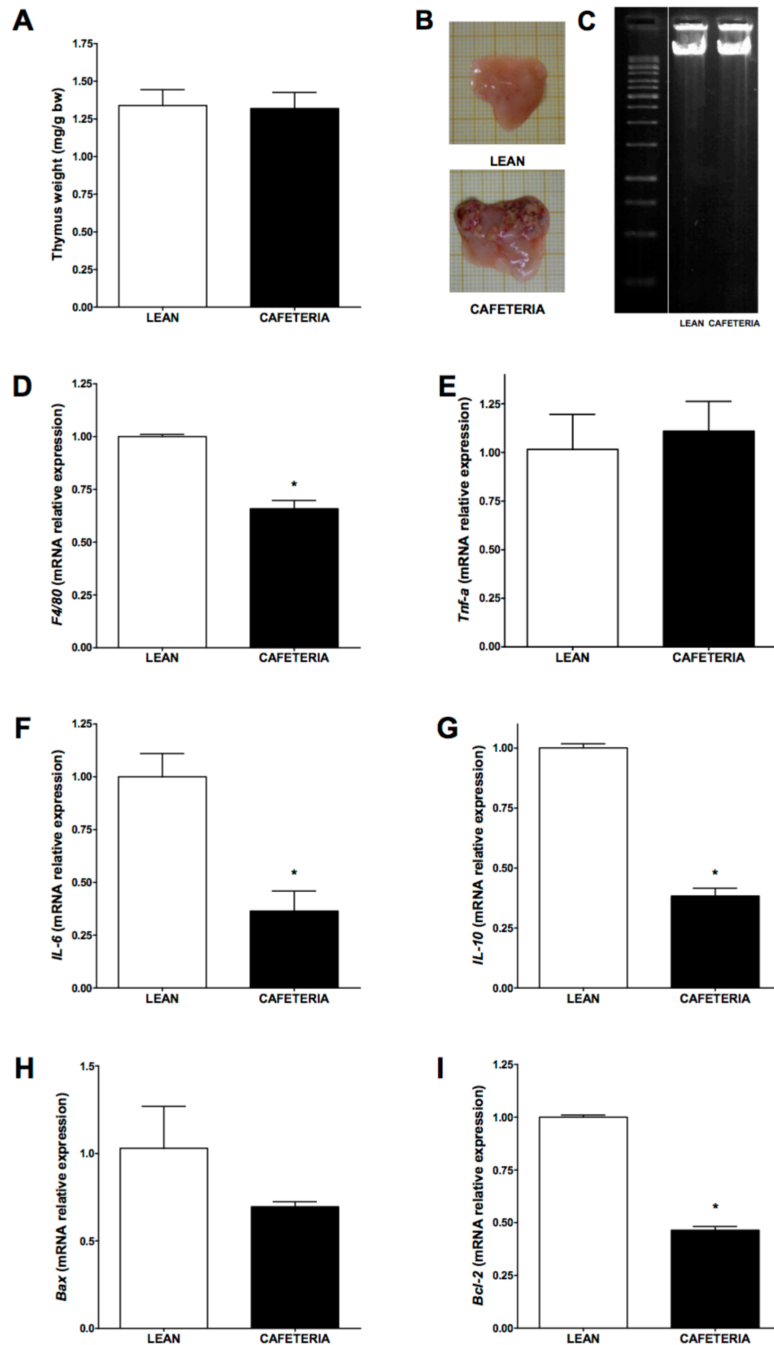

Figure 3. Cafeteria diet modulates thymus functionality. (A) Weight of thymus from standard and cafeteria fed rats. (B) Macroscopic pictures of the thymus from rats fed with standard and also cafeteria diet. Each small square represents 1 mm. (C) Gel electrophoresis of DNA fragmentation ladders. DNA was isolated from the thymocytes of rats under both fed conditions. (D) mRNA gene expression of the selective macrophage marker *F4/80*. (E), (F) and (G) modulation of *Tnf-α*, *IL-6* and *IL-10* the gene transcription. (H) and (I) Effects of diet on the modulation of the expression of apoptotic *Bax* and *Bcl-2* genes. Data represent the mean ± SEM. *P<0.05 vs. STD diet-fed rats.

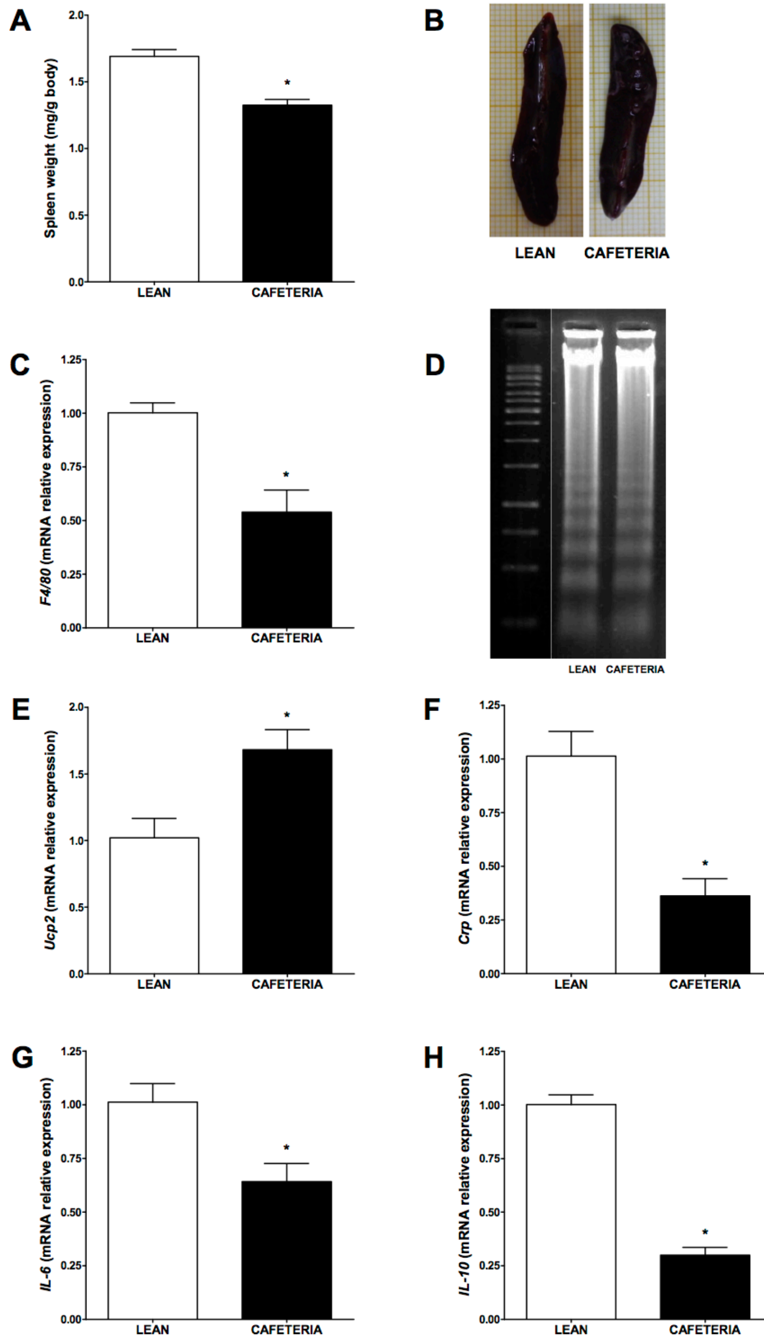

Figure 4. Spleen functionality is impaired by diet-induced obesity. (A) Weight of the spleens obtained from standard and cafeteria-fed rats. (B) Macroscopic pictures of the spleens from rats fed with standard and cafeteria diet. Each square represents 1 mm. (C) Diet modulation of *F4/80* mRNA expression. (D) Electrophoretic analysis of thymocytes DNA fragmentation. (E), (F) and (G) modulation of *Ucp-2*, *CRP*, *IL-6* and *IL-10* gene transcription. Data represent the mean ± SEM. **P<0.05 between the rats fed with STD and CAF diets.

Thus, the purpose of this study was to determine the influence of diet-induced obesity as both a triggering event for innate immunity and a chronic stimulus for the adaptive immune response through an analysis of local inflammatory responses in adipose tissue, systemic inflammation, and the responsiveness of the two major primary and secondary lymphoid organs, the thymus and the spleen, respectively.

The cafeteria diet, a robust model for human metabolic syndrome, was used to investigate the obesogenic and inflammatory implications of diet-induced obesity [13]. Using this model, the animals were provided free access to a STD diet and concurrently offered highly palatable and energy-dense food. Therefore, the hedonistic overfeeding induced through a CAF diet in Wistar rats was reflected by an increase in body weight resulting primarily from the dramatic gain of adiposity. This expansion of adipose tissue activated the inflammatory response as a consequence of the up-regulated transcription of *IL-6* and *Tnf-α*, key genes involved in the diet-induced activation of local inflammation, and the down-regulated gene expression of *IL-10*. CAF diet-induced inhibitory modulation was also associated with the activation of the inflammatory response. These results suggest that adipose tissue not only acts as storage depot for excess calorie intake, but is also involved in the obesity-induced inflammatory response [14]. Furthermore, diet-induced adiposity increase was accompanied by macrophage infiltration into adipose tissue, reflected as an increase in circulating MCP-1 levels and expression of the macrophage-selective *F4/80* gene, consistent with previous results associating an increase in adipose tissue-infiltrating macrophages with an increase in IL-6 and TNF-α production [15,16] and highlighting a pivotal role for macrophages in the activation of innate immunity [17].

Although adipose tissue is the central location for obesity-triggered local inflammation, mediated through adipocytes and innate and adaptive immune cells, the dysregulation of metabolism and immunity also affects systemic immune response activation. Furthermore, the specificity of the obesity-induced immune response lies in the periodicity of the triggering stimulus; that is, as consequence of a chronic surplus of energy, metabolic stress is continuously induced, impeding not only the resolution of inflammation and the deactivation of innate immunity but also affecting the activation of adaptive immunity [7,18].

However, diet-induced obesity also modulates the circulating pattern of immune regulation components, including orosomucoid and C3, whose plasma concentrations were increased in rats after feeding with a CAF diet. Orosomucoid and C3 are acute phase response proteins with key roles in the regulation of innate immune system, and these proteins are secreted by adipose tissue and at other metabolically active sites, such as the liver, in response to stressful conditions, such as metabolic perturbations. High circulating levels of both immunomodulators have been associated with the development of obesity and obesity-related pathologies [19,20].

Moreover, considering the roles of adipose tissue as an endocrine and immunologically active organ [1,21–23], increased adiposity lead to increased plasma leptin levels, a

hallmark for diet-induced leptin resistance [24], and decreased circulating adiponectin levels. Both of these molecules are the most abundant adipokines secreted by adipose tissue, thus confirming the close and inverse relationship between leptin and adiponectin plasma concentrations and the proportion of fat storage [22]. Therefore, in addition to being essential for the modulation of appetite and energy expenditure, leptin is considered as one of the most important mediators linking nutritional status, metabolism and adipose tissue with the immune response [25–27]. The pivotal roles of leptin are evidenced through the dysregulation of leptin signalling in response to metabolic perturbations that not only impair the proper regulation of metabolism but also achieve appropriate immune functionality. Thus, high circulating levels of leptin as a consequence of diet increase adiposity, and the attenuation of leptin signal transduction through different converging molecular mechanisms prevents adequate sensing through its cognate receptor in the hypothalamus. In addition, leptin receptor expression is not only limited to the central nervous system, but this protein is also expressed in immune cells, thereby implicating leptin in the cell-mediated immune response and highlighting that the attenuation of leptin signalling might also affect both innate and adaptive immune responses. Indeed, genetically obese mice with impaired leptin signalling, reflecting the inhibition of either leptin (ob/ob) or leptin receptor (db/db) transcription, exhibit impaired immune function, resulting in inability of the immune system to appropriately respond to pathogenic stimuli [28,29]. Moreover, the immunomodulatory effects of leptin are not exclusively associated with cell-mediated immunity regulation, but leptin deficiency is also genetically associated with lymphoid organ atrophy [26].

Thus, taken together, these results suggest that the effectiveness of the immune system, as a homeostatic promoter, is based on the highly sensitive recognition of harmful stimulus, and immuno-tolerance and weakened immune responses result from chronic immune activation in a diet-induced obesity context, yielding extremely deleterious consequences to the host. Therefore, to obtain deeper insight into obesity-associated immunocompetence, we also examined peripheral immune responsiveness to diet-induced obesity, including the regulation of thymus and spleen functionality, which are essential for the appropriate response of the host immune system to harmful exogenous and endogenous stimuli.

The thymus, the primary lymphoid organ responsible for *de novo* T cell maturation and output, is a vital organ for the homeostatic maintenance of the peripheral immune system. Thus, although we have not observed differences regarding the weight, the appearance of the thymus was strongly affected by diet, suggesting that a CAF diet could promote thymus atrophy through the induction of ectopic lipogenesis [30,31].

Furthermore, the CAF diet induced the down-regulation of *F4/80,* the selective macrophage marker, which is abundantly expressed in thymic cells [32]. In the thymus, macrophages bind and phagocytose thymic lymphocytes, and this interaction is crucial for T cell maturation. However, no diet-induced changes were observed for the expression of *TNF-α* in thymocytes, although this pleiotropic cytokine has been described as an immunostimulator of T cells proliferation, whose expression is increased in the developing thymus [33].

Notably, the expression of *IL-6* and *IL-10* was down-regulated in thymocytes in response to a CAF diet. The down-regulation of *IL-6* gene expression could be associated with a decrease in the proliferation and *de novo* generation of mature T cells, leading to the impairment of thymus-mediated immune functions. *IL-10*, in turn, also regulates T cell maturation, and the dysregulation of *IL-10* expression leads to a severe T cell immunodeficiency [34]. The diet-induced decrease in the expression of both cytokines, with opposite inflammatory functions, might reflect a decrease in the macrophage subset.

However, some authors have hypothesised the existence of immune-metabolic interactions in the thymus. Indeed, the thymus is a plastic tissue that undergoes massive architectural changes with age, and thymocytes are replaced with adipocytes, through a process known as thymic involution [35]. Thus, one of the mechanisms associated with thymic atrophy-induced immunodeprivation is the increase in apoptosis leading to increased DNA fragmentation. Several studies have reported that apoptosis plays a critical role in T cell development and apoptotic thymocytes are cleared by thymic macrophages [36]. Therefore, we analysed the degradation of DNA in the thymocytes obtained from rats fed either STD or CAF diets; however, no DNA ladders or differences regarding the type of diet were observed. Consistently, the gene expression of *Bax* and *Bcl-2*, apoptotic proteins, in thymocytes was determined. Notably, only the transcription of the anti-apoptotic gene *Bcl-2* was significantly down-regulated in response to CAF diet consumption, which might reflect the obesity-induced atrophy of thymus functionality [37]. Thus, the CAF diet inhibited the immune-stimulatory capacity of the thymus through a decrease in the thymic macrophage population.

The spleen, the largest lymphoid organ in the body, plays an important role not only as blood filter but also in the regulation of the immune-metabolic-endocrine network [38], considering that obesity significantly modifies the pattern of circulating metabolic and immune metabolites, suggesting that the spleen is crucial for immune response regulation. Furthermore, there is also an important association between the liver and spleen for the regulation of metabolism and immunity [39], and the splenic role might be more important in the response to homeostatic perturbations.

These results showed a decrease in the weight of the spleens obtained from CAF diet-fed rats, likely reflecting a reduction in the number of splenocytes. Indeed, the spleen is the storage site for several cell types, including erythrocytes, monocytes and macrophages, and serves as a key resource for rapid macrophage deployment and immunity regulation. Thus, the CAF diet induced a decrease in splenic macrophages, reflected by the down-regulation of *F4/80* gene expression. Moreover, uncoupling protein 2 (UCP2), a mitochondrial protein robustly expressed in the spleen (Lamas et al., 2004), is involved in the regulation of metabolism and in immune function, controlling macrophage activation through the modulation of mitochondrial reactive species (ROS) production, showing a direct association between increased *Ucp2* mRNA levels in the spleens of obese rats with poor oxidative burst activities [40]. Therefore, we demonstrated that diet-induced obesity not only reduces the

number of macrophages present in the spleen, limiting its capacity to counteract inflammatory stimuli but also that the obesity-induced weakened immune response might reflect limited oxidation capacity resulting from the up-regulation of splenic *Ucp2* gene expression. In addition, diet-induced obesity also regulates the mRNA expression of *IL-6* and *Crp*, systemic biomarkers of inflammation, whose expression in splenocytes is suppressed as result of CAF diet, consistent with the results of previous studies [41], likely inhibiting proper immune reactivity against obesity-related systemic biomarkers. We also showed that diet-induced obesity resulted in the inability of the spleen to synthesise *IL-10*. Thus, activated B cells primarily contribute to splenic *IL-10* production, playing a regulatory role in the suppression of harmful immune responses. It has been hypothesised that the obesity-induced down-regulation of splenic *IL-10* expression results in chronic inflammation in adipose tissue, the liver and hypothalamus [11,42].

Furthermore, whereas no differences were observed regarding diet-induced apoptotic effects, a clear DNA degradation pattern was observed for the splenocytes obtained from both the STD and CAF-fed rats, demonstrated by the corresponding oligonucleosomal fragments from 300 to 50 kb in length [43], corresponding to apoptotic splenocytes. This diet-independent apoptosis hallmark in the splenocytes of rats from both groups might reflect the involvement of the spleen in the maintenance of peripheral tolerance via the clearance of circulating apoptotic cells [39].

In summary, we demonstrated that the obesity-triggered low-grade inflammatory response occurs primarily in adipose tissue, rather than lymphoid organs, and the macrophages are crucial cells, orchestrating not only local inflammatory responses but also inhibiting appropriate responses to harmful obesity-derived stimuli in primary and secondary lymphoid organs. Remarkably, diet-induced prolonged metabolic overload modulates functionality in the thymus and the spleen, reflecting a weakened sensibility of the immune response, promoting immune tolerance and affecting not only the regulation of metabolic and immune systems but also the restoration and maintenance of the homeostatic state in the entire body.

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The authors declare no competing financial interest.

REFERENCES

1 Galic, S., Oakhill, J. S. and Steinberg, G. R. (2010) Adipose tissue as an endocrine organ. Mol. Cell. Endocrinol., Elsevier **316**, 129–139.

2 Miranda, P. J., DeFronzo, R. A., Califf, R. M. and Guyton, J. R. (2005) Metabolic syndrome: definition, pathophysiology, and mechanisms. Am. Heart J., St. Louis, CV Mosby Co. **149**, 33–45.

3 Speakman, J. R. (2004) Obesity: the integrated roles of environment and genetics. J. Nutr., Am Soc Nutrition **134**, 2090S–2105S.

4 Hotamisligil, G. S. (2006) Inflammation and metabolic disorders. Nature, Nature Publishing Group **444**, 860–867.

5 Lumeng, C. N. and Saltiel, A. R. (2011) Inflammatory links between obesity and metabolic disease. J. Clin. Invest., American Society for Clinical Investigation **121**, 2111.

6 Iyer, A., Fairlie, D. P., Prins, J. B., Hammock, B. D. and Brown, L. (2010) Inflammatory lipid mediators in adipocyte function and obesity. Nat. Rev. Endocrinol., Nature Publishing Group **6**, 71–82.

7 Chawla, A., Nguyen, K. D. and Goh, Y. P. S. (2011) Macrophage-mediated inflammation in metabolic disease. Nat. Rev. Immunol., Nature Publishing Group.

8 Kanda, H., Tateya, S., Tamori, Y., Kotani, K., Hiasa, K., Kitazawa, R., Kitazawa, S., Miyachi, H., Maeda, S. and Egashira, K. (2006) MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. J. Clin. Invest., Am Soc Clin Investig **116**, 1494.

9 Baumann, H. and Gauldie, J. (1994) The acute phase response. Immunol. Today **15**, 74.

10 Epstein, F. H., Gabay, C. and Kushner, I. (1999) Acute-phase proteins and other systemic responses to inflammation. N. Engl. J. Med., Mass Medical Soc **340**, 448–454.

11 Gotoh, K., Inoue, M., Masaki, T., Chiba, S., Shimasaki, T., Ando, H., Fujiwara, K., Katsuragi, I., Kakuma, T. and Seike, M. (2012) A Novel Anti-inflammatory Role for Spleen-Derived Interleukin-10 in Obesity-Induced Inflammation in White Adipose Tissue and Liver. Diabetes, Am Diabetes Assoc **61**, 1994–2003.

12 Saeki, K., Kobayashi, N., Inazawa, Y., Zhang, H., Nishitoh, H., Ichijo, H., Saeki, K., Isemura, M. and Yuo, A. (2002) Oxidation-triggered c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein (MAP) kinase pathways for apoptosis in human leukaemic cells stimulated by epigallocatechin-3-gallate (EGCG): a distinct pathway from those of chemically induced and recep. Biochem. J. **368**, 705–20.

13 Sampey, B. P., Vanhoose, A. M., Winfield, H. M., Freemerman, A. J., Muehlbauer, M. J., Fueger, P. T., Newgard, C. B. and Makowski, L. (2011) Cafeteria Diet Is a Robust Model of Human Metabolic Syndrome With Liver and Adipose Inflammation: Comparison to High-Fat Diet. Obesity, Wiley Online Library **19**, 1109–1117.

14 Osborn, O. and Olefsky, J. M. (2012) The cellular and signaling networks linking the immune system and metabolism in disease. Nat. Med., Nature Publishing Group **18**, 363–374.

15 Weisberg, S. P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R. L. and Ferrante, A. W. (2003) Obesity is associated with macrophage accumulation in adipose tissue. J. Clin. Invest., Am Soc Clin Investig **112**, 1796–1808.

16 Lumeng, C. N., Deyoung, S. M., Bodzin, J. L. and Saltiel, A. R. (2007) Increased inflammatory properties of adipose tissue macrophages recruited during diet-induced obesity. Diabetes **56**, 16–23.

17 Lumeng, C. N. (2013) Innate immune activation in obesity. Mol. Aspects Med. **34**, 12–29.

18 Duncan, B. B. and Schmidt, M. I. (2001) Chronic activation of the innate immune system may underlie the metabolic syndrome. Sao Paulo Med. J. **119**, 122–7.

19 Lee, Y. S., Choi, J. W., Hwang, I., Lee, J. W., Lee, J. H., Kim, A. Y., Huh, J. Y., Koh, Y. J., Koh, G. Y., Son, H. J., et al. (2010) Adipocytokine orosomucoid integrates inflammatory and metabolic signals to preserve energy homeostasis by resolving immoderate inflammation. J. Biol. Chem. **285**, 22174–85.

20 Engström, G., Hedblad, B., Eriksson, K.-F., Janzon, L. and Lindgärde, F. (2005) Complement C3 is a risk factor for the development of diabetes: a population-based cohort study. Diabetes **54**, 570–5.

21 Kershaw, E. E. and Flier, J. S. (2004) Adipose tissue as an endocrine organ. J. Clin. Endocrinol. Metab., Endocrine Soc **89**, 2548–2556.

22 Coelho, M., Oliveira, T. and Fernandes, R. (2013) Biochemistry of adipose tissue: an endocrine organ. Arch. Med. Sci. **9**, 191–200.

23 Kaminski, D. A. and Randall, T. D. (2010) Adaptive immunity and adipose tissue biology. Trends Immunol., Elsevier **31**, 384–390.

24 Zhang, Y. and Scarpace, P. J. (2006) The role of leptin in leptin resistance and obesity. Physiol. Behav. **88**, 249–56.

25 Lam, Q. L. K. and Lu, L. (2007) Role of leptin in immunity. Cell. Mol. Immunol. **4**, 1–13.

26 Matarese, G. (2000) Leptin and the immune system: how nutritional status influences the immune response. Eur. Cytokine Netw., John Libbey Eurotext; 1999 **11**, 7–14.

27 Loffreda, S., Yang, S. Q., Lin, H. Z., Karp, C. L., Brengman, M. L., Wang, D. J., Klein, A. S., Bulkley, G. B., Bao, C., Noble, P. W., et al. (1998) Leptin regulates proinflammatory immune responses. FASEB J. **12**, 57–65.

28 Amar, S., Zhou, Q., Shaik-Dasthagirisaheb, Y. and Leeman, S. (2007) Diet-induced obesity in mice causes changes in immune responses and bone loss manifested by bacterial challenge. Proc. Natl. Acad. Sci. U. S. A. **104**, 20466–71.

29 Ikejima, S., Sasaki, S., Sashinami, H., Mori, F., Ogawa, Y., Nakamura, T., Abe, Y., Wakabayashi, K., Suda, T. and Nakane, A. (2005) Impairment of host resistance to Listeria monocytogenes infection in liver of db/db and ob/ob mice. Diabetes **54**, 182–9.

30 Kanneganti, T.-D. and Dixit, V. D. (2012) Immunological complications of obesity. Nat. Immunol., Nature Publishing Group **13**, 707–712.

31 Yang, H., Youm, Y.-H., Vandanmagsar, B., Rood, J., Kumar, K. G., Butler, A. A. and Dixit, V. D. (2009) Obesity accelerates thymic aging. Blood **114**, 3803–12.

32 Wood, G. W. (1985) Macrophages in the thymus. Surv. Immunol. Res. **4**, 179–91.

33 Chatzidakis, I. and Mamalaki, C. (2010) T cells as sources and targets of TNF: implications for immunity and autoimmunity. Curr. Dir. Autoimmun. **11**, 105–18.

34 Rouleau, M., Cottrez, F., Bigler, M., Antonenko, S., Carballido, J. M., Zlotnik, A., Roncarolo, M. G. and Groux, H. (1999) IL-10 transgenic mice present a defect in T cell development reminiscent of SCID patients. J. Immunol., American Association of Immunologists **163**, 1420–7.

35 Dixit, V. D. (2010) Thymic fatness and approaches to enhance thymopoietic fitness in aging. Curr. Opin. Immunol. **22**, 521–8.

36 Surh, C. D. and Sprent, J. (1994) T-cell apoptosis detected in situ during positive and negative selection in the thymus. Nature **372**, 100–3.

37 Mandal, D., Bhattacharyya, A., Lahiry, L., Choudhuri, T., Sa, G. and Das, T. (2005) Failure in peripheral immuno-surveillance due to thymic atrophy: importance of thymocyte maturation and apoptosis in adult tumor-bearer. Life Sci. **77**, 2703–16.

38 Tarantino, G., Savastano, S., Capone, D. and Colao, A. (2011) Spleen: A new role for an old player? World J. Gastroenterol. **17**, 3776–84.

39 Tarantino, G., Scalera, A. and Finelli, C. (2013) Liver-spleen axis: intersection between immunity, infections and metabolism. World J. Gastroenterol. **19**, 3534–42.

40 Arsenijevic, D., Onuma, H., Pecqueur, C., Raimbault, S., Manning, B. S., Miroux, B., Couplan, E., Alves-Guerra, M. C., Goubern, M., Surwit, R., et al. (2000) Disruption of the uncoupling protein-2 gene in mice reveals a role in immunity and reactive oxygen species production. Nat. Genet. **26**, 435–9.

41 Lamas, O., Martínez, J. A. and Marti, A. (2004) Decreased splenic mRNA expression levels of TNF-α and IL-6 in diet-induced obese animals. J. Physiol. Biochem. **60**, 279–283.

42 Gotoh, K., Inoue, M., Masaki, T., Chiba, S., Shimasaki, T., Ando, H., Fujiwara, K., Katsuragi, I., Kakuma, T., Seike, M., et al. (2012) A novel anti-inflammatory role for spleen-derived interleukin-10 in obesity-induced hypothalamic inflammation. J. Neurochem. **120**, 752–64.

43 Oberhammer, F., Wilson, J. W., Dive, C., Morris, I. D., Hickman, J. A., Wakeling, A. E., Walker, P. R. and Sikorska, M. (1993) Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. EMBO J. **12**, 3679–84.

CHAPTER 6

Procyanidins and docosahexaenoic acid suppress cafeteria diet-triggered inflammation and boost immune system to counteract obesity-induced immunosuppression

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Submitted

ABSTRACT

Objective: Nutrition can be considered as a two-side coin: although an imbalance in the energy content is associated with the induction of obesity and obesity-related pathologies, a healthy state can be induced through the intake of food containing immunologically active compounds, such as grape seed procyanidin extract (GSPE) or docosahexaenoic acid (DHA). Thus, the aim of this study was to determine the immunomodulatory properties of these dietary components in cafeteria diet-fed rats.

Design: Five groups of rats were fed either a standard diet (STD) or a cafeteria diet (CAF) for 13 weeks and three of the CAF-diet fed groups were supplemented with either GSPE at 25 mg per kg of body weight, DHA at 500 mg per kg of body weight or the combination of GSPE and DHA during the last 3 weeks of the study.

Results: GSPE and DHA suppress diet induced-inflammation by promoting a phenotypic switch in the molecular and cell profile of mesenteric adipose tissue. Furthermore, the healthy properties of GSPE and DHA supplementation are not limited to the modulation of adipose tissue inflammation, but also boost immune system through the modulation of cell-mediated immunity, involving macrophages and T lymphocytes subsets, as well as stimulate the functionality of thymus and spleen to counteract the obesity-induced weakened immune responses. Moreover, the combination of the bioactivity of GSPE and DHA potentiates the immunomodulatory properties observed for each compound administered individually.

Conclusion: Diet supplementation with GSPE and DHA in obese rats induces a healthy state, not only promoting the resolution of adipose tissue-driven inflammation but also boosting the obesity-induced weakened immune response.

INTRODUCTION

Obesity is a complex and multifactorial disease primarily reflecting imbalanced energy metabolism, associated with a high incidence of metabolic disorders, including impaired glucose metabolism, dyslipidaemia, atherosclerosis, hypertension and the activation of inflammatory responses; altogether, these pathologies are known as Metabolic Syndrome [1,2].

The immune system is a coordinated physiological response comprising both innate and the adaptive immunity, associated with a complex network of molecules, cells and organs to detect perturbations, trigger adequate immune responses and restore the homeostatic state. Moreover, obesity has been associated with the impairment of the immune function, interfering with the proper regulation of the production of immune biomarkers, altering leukocyte counts and affecting the functionality of lymphoid organs [3,4].

Thus, the regulation of the metabolism and the immune system are highly integrated and dependent on each other, indeed this interface is a central homeostatic mechanism, the dysfunction of which leads to the improper functionality of both systems [5]. Therefore, obesity is characterised in part by lipid accumulation in adipose tissue, the major site for the storage of excess energy in the body, triggering the activation of a local inflammatory response, characterised by the infiltration of immune cells, the over production of pro-inflammatory cytokines, including tumour necrosis factor-α (TNF-α) and interleukin 6 (IL-6) and the reduced production of anti-inflammatory cytokines, such as interleukin 10 (IL-10) [6,7]. Thus, the perpetuation of this metabolic dysfunction, leads to chronic cellular stress, leading to an adipose-tissue driven systemic inflammatory response, considered as a pivotal event for the development of metabolic diseases [5]. Therefore, systemic inflammation is characterised by enhanced circulating concentrations of several chemotactic factors, including the monocyte chemotactic protein-1 (MCP-1) [8,9], and the activation of an overall acute-phase response, characterised by increased levels of acute-phase proteins, such as the C-reactive protein (CRP), the complement component C3 (C3) and orosomucoid [10,11]. Moreover, adipose tissue, as an endocrine organ, plays a pivotal role in the integration between energy balance and immune function through the synthesis and secretion of fat-derived neuroendocrine peptides, including leptin and adiponectin. Thus, due to a chronic positive energy balance, these neuroendocrine-immune interactions are heightened.

Leptin is a 16-kDa hormone synthesised in adipocytes proportionally to the amount of energy stored in adipose tissue to centrally regulate energy metabolism. However, the leptin receptor is not only expressed in the central nervous system but also in peripheral tissue and cells, including those involved in the immune system, demonstrating a role for leptin as a modulator of the immune response [12]. Thus, leptin is a potent immunomodulator, promoting lymphopoiesis and myelopoiesis, and leptin deficiency is associated with an atrophy of the thymus that primarily affects the size and cellularity of this organ [13]. In addition, leptin is also necessary for the induction and maintenance of the pro-inflammatory immune response, suggesting a link between nutritional status and the T cell profile.

Moreover, the direct interactions between increased obesity-induced leukocyte populations, including macrophages and lymphocytes, in adipose tissue, and the incremented adipocyte numbers within the lymphoid microenvironment, are considered as important adaptive responses to changes in energy balance and the pivotal cross-talk, whose impairment underlies the development of obesity-related pathologies [14,15]. Thus, cell-mediated immunity plays a pivotal role in the orchestration of the immune response to metabolic disturbances. In adults, the two major T cell lineages, $CD4^+$ helper and the $CD8^+$ cytotoxic T cells, are derived from bone marrow, traffic through the blood, and are subsequently imported into the thymus, where thymocytes receive the corresponding signals to mature into T lymphocytes [16]. Naïve T cells circulate in the bloodstream, as part of the peripheral blood mononuclear cells (PBMCs) and are activated in the peripheral lymphoid tissues, where the spleen is the largest secondary lymphoid organ and is essential for the host immune function [17]. Furthermore, a specialised subset of $CD4^+$ T cells, known as Tregs, plays a key role for the maintenance of immunological homeostasis. Tregs has immunosuppressive activity based on the exclusive presence of a master transcriptional regulator, the transcription factor forkhead box P3 (Foxp3) [18]. Thus, diet induced-obesity is associated with the infiltration of pro-inflammatory macrophages (M1), the infiltration of pro-inflammatory $CD4^+$ and $CD8^+$ T cells into adipose tissue, and the reduction of anti-inflammatory regulatory T cells (Tregs) [19].

However, the modulation of metabolic and immune systems by the diet reduces the impairment of homeostasis; nevertheless, the intake of foods containing biologically active molecules improves the health status and reduces pathologies associated to immunometabolic disorders. Thus, a variety of bioactive food components have been shown to modulate the inflammatory response, including procyanidins and docosahexaenoic acid (DHA). However, the implications of these foods in the regulation of the immune system as a result of a diet-induced obesity are not fully understood. Procyanidins are phenolic compounds widely distributed in cereals, vegetables and in fruits, such as grapes, berries, cocoa or apples, associated with beneficial effects on health, such as the promotion of anti-inflammatory activities [20,21]. Furthermore, polyunsaturated fatty acids (PUFAs) are important constituents of cells, mediators in metabolic and physiological processes, and important bioactive lipids. DHA, a ω-3 fatty acid, is present in certain aquatic organisms, such as fish (fish oil) or marine microalgae with known anti-inflammatory effects [22].

Diet-induced obesity has been associated with an adipose tissue-driven low-grade chronic inflammation and impairment in the immune response to both harmful exogenous and endogenous stimuli. Therefore, the modulation of the obesity-mediated interference of the innate and adaptive immune systems through the intake of food containing biologically active compounds could not only enhance immunity but also counteract metabolic disorders. Thus, the aim of this study was to determine the potentially beneficial effects of diet supplementation with biologically active components, including procyanidins and DHA, alone or in combination, in the context of diet-induced obesity. For this purpose, the immunomodulatory effects of the bioactive compounds were determined either for the

inflammatory response, including the adipose tissue-driven local and systemic inflammation, for cell-mediated immunity, or the modulation of lymphoid organ functionality, involving the thymus and spleen.

MATERIALS AND METHODS

Grape Seed Proanthocyanidin Extract (GSPE)

Grape seed procyanidin extract (GSPE) was obtained from Les Dérives Résiniques et Terpéniques (Dax, France). According to previous studies [23], procyanidin extract derived from grape seeds comprises catechin (58 μmol/g), epicatechin (52 μmol/g), epigallocatechin (5.50 μmol/g), epicatechin gallate (89 μmol/g), epigallocatechin gallate (1.40 μmol/g), dimeric procyanidins (250 μmol/g), trimeric procyanidins (15.68 μmol/g), tetrameric procyanidins (8.8 μmol/g), pentameric procyanidins (0.73 μmol/g), and hexameric procyanidins (0.38 μmol/g).

Oil rich in docosahexaenoic acid (DHA-OR)

DHA-OR, obtained from Market DHA^{TM}-S, was derived from the marine algae *Schizochitrium sp.*, a rich source of fatty acids, and this oil primarily comprises DHA.

Animals and experimental design

Male Wistar rats weighing 150 g were obtained from Charles River Laboratories (Barcelona, Spain). The animals were housed individually in a 12-h light/dark cycle. After the adaptation period, the rats were distributed in two groups, fed for 13 weeks with either standard (STD) chow or a cafeteria (CAF) diet. The CAF diet comprised bacon, cookies, muffins, pate, cheese, carrots, milk and sugar added to the standard chow, yielding an hypercaloric diet comprising 11.5% protein, 34.5% carbohydrates and 37.8% fat. After 10 weeks, the CAF-fed rats were 20 % overweight with respect to the STD diet-fed rats, and these rats were randomly distributed into four groups (n=7). In three of these groups, the CAF diet was supplemented for the last 3 weeks with GSPE (25 mg / kg of body weight), DHA-OR (500 mg DHA / kg of body weight) or both (25 mg / kg of body weight + 500 mg DHA/kg of body weight) as shown in Figure 1. At the end of 13 weeks, the animals were fasted for 3 h and sacrificed through abdominal aortic exsanguination. After sacrifice, the plasma was obtained through centrifugation of the blood samples. The adipose fat depots (mesenteric, perirenal and epididymal) were excised, weighed, immediately frozen in liquid nitrogen and stored at -80ºC until further analysis. The thymus and spleen were rapidly removed, weighed, and the thymocytes and splenocytes were immediately isolated. All procedures were approved through the Animal Ethics Committee of Rovira i Virgili University.

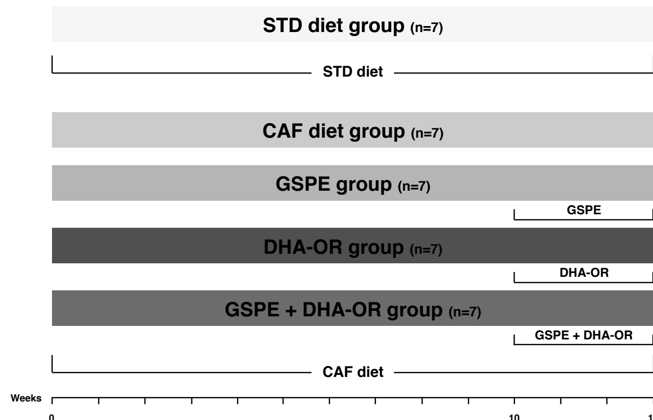

Figure 1. Experimental design. Rats were fed with either standard (STD) or cafeteria (CAF) diets for 10 weeks. Subsequently, 3 of the 4 CAF-diet fed groups were supplemented with GSPE (25 mg/kg bw), DHA-OR (500 mg/kg bw) or the combination of both (25 mg/kg bw of GSPE and 500 mg/kg bw of DHA-OR) for three weeks.

Isolation of thymocytes and splenocytes.

Thymocyte and splenocyte cell suspensions were obtained from freshly isolated lymphoid tissue through mechanical disruption and fractionation through a 70-µm nylon cell strainer. The isolated cells were treated with ACK lysis buffer (SIGMA, Barcelona, Spain) to eliminate erythrocytes. Trypan blue staining was performed to determine the number of live cells.

Peripheral blood mononuclear cells (PBMCs) isolation

Peripheral blood mononuclear cells (PBMCs) were isolated through density-gradient centrifugation using HISTOPAQUE-1083 according to the manufacturer's instructions (SIGMA, Madrid).

Flow cytometric T cell subset analysis

Freshly isolated splenocytes, thymocytes and PBMCs single cell suspensions were stained with a three-color reagent using the Rat T cell lymphocyte cocktail (BD Bioscience, San Diego, CA) according to the manufacturer's instructions. Major subsets of T lymphocytes were determined based on the expression of $CD3^+$, $CD4^+$ and $CD8^+$ surface markers using flow cytometry analysis (FACS ARIA III, BD Bioscience, San Diego, CA).

Measurement of biochemical parameters

Circulating leptin and adiponectin levels were quantified using specific EIAs according to the manufacturer's instructions (Biosource International Inc., CA, USA). Orosomucoid and Complement factor C3 plasma concentrations were determined using ELISAs kits according to the manufacturer's instructions (GenWay, San Diego, USA). The MCP-1 determination was based on the ELISA kit purchased in (Abcam, Cambridge, USA).

Gene expression analysis using real-time RT-PCR

Total RNA from the isolated thymocytes and splenocytes and the mesenteric fat depots was isolated using TRIzol reagent according o the manufacturer's instructions (Invitrogen, Barcelona, Spain) and purified using the RNeasy Mini Kit spin columns (Qiagen, Valencia, CA, USA). Complementary DNA was generated using the reverse transcription reagent kit (Applied Biosystems, Madrid, Spain) and subsequently subjected to RT-PCR amplification using the SsoFast EvaGreen SuperMix (Bio-Rad). The forward and reverse primers for *Tnf-α* were 5' CCTCACACTCAGATCATCTTCTC 3' and 5' TTGGTGGTTTGCTACGACGTG 3', respectively. For *IL-6*, 5' CTCTCCGCAAGAGACTTCC 3' and 5' GCCATTGCACAACTCTTTTCTC 3' were used for the forward and reverse primers, respectively. The forward and reverse primers for *IL-10* were 5' GCAGGACTTTAAGGGTTACTTGG 3' and 5' GGAGAAATCGATGACAGCGT 3', respectively. *F4/80* was detected using the 5' ATGCATAATCGCTGCTGGCTGAA 3' as the forward primer and 5' GAGGGCAGAGTTGATCGTGATGATC 3' as the reverse primer. iNOS gene expression was determined using 5′ GGATCTTCCCAGGCAACCA 3′ as forward and 5′ AATCCACAACTCGCTCCAAGATT 3′ reverse primers, respectively. For *Foxp3*, 5' AAGCCAGGCTGATCCCTCTC 3' and 5' TCCAAGTCTCGTGTGAAGGC 3', were used as forward and reverse primers, respectively, and the primers used for *Ppia* detection were 5' CTTCGAGCTGTTTGCAGACAA 3' as a forward primer and 5' AAGTCACCACCCTGGCACATG 3' as a reverse primer. Relative gene expressions were normalised according to the Ppia mRNA levels according to the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

The results are expressed as the mean ± S.E.M. The effects of different diet consumption were assessed using ANOVA. Tukey's test was used to make pair wise comparisons. Differences were considered significant when the p values were <0.05. The calculations were performed using SPSS 20.0 software.

RESULTS

GSPE and DHA-OR, individually or together, supress diet-induced adipose tissue inflammatory response

To determine the immunomodulatory effects of procyanidins and DHA supplementation in diet-induced obese rats, the effects of the type of diet on obesity-induced adipose tissue dysfunction were assessed.

Thus, the supplementation of CAF diet with bioactive compounds had no effect regarding the modulation of the CAF diet-induced increase in body weight (Figure 2A). Furthermore, whereas GSPE or DHA alone did not modulate the CAF diet-promoted increase in mesenteric fat depots, the mesenteric adipose tissue was significantly overexpanded in rats supplemented with the combination of GSPE and DHA-OR (Figure 2B).

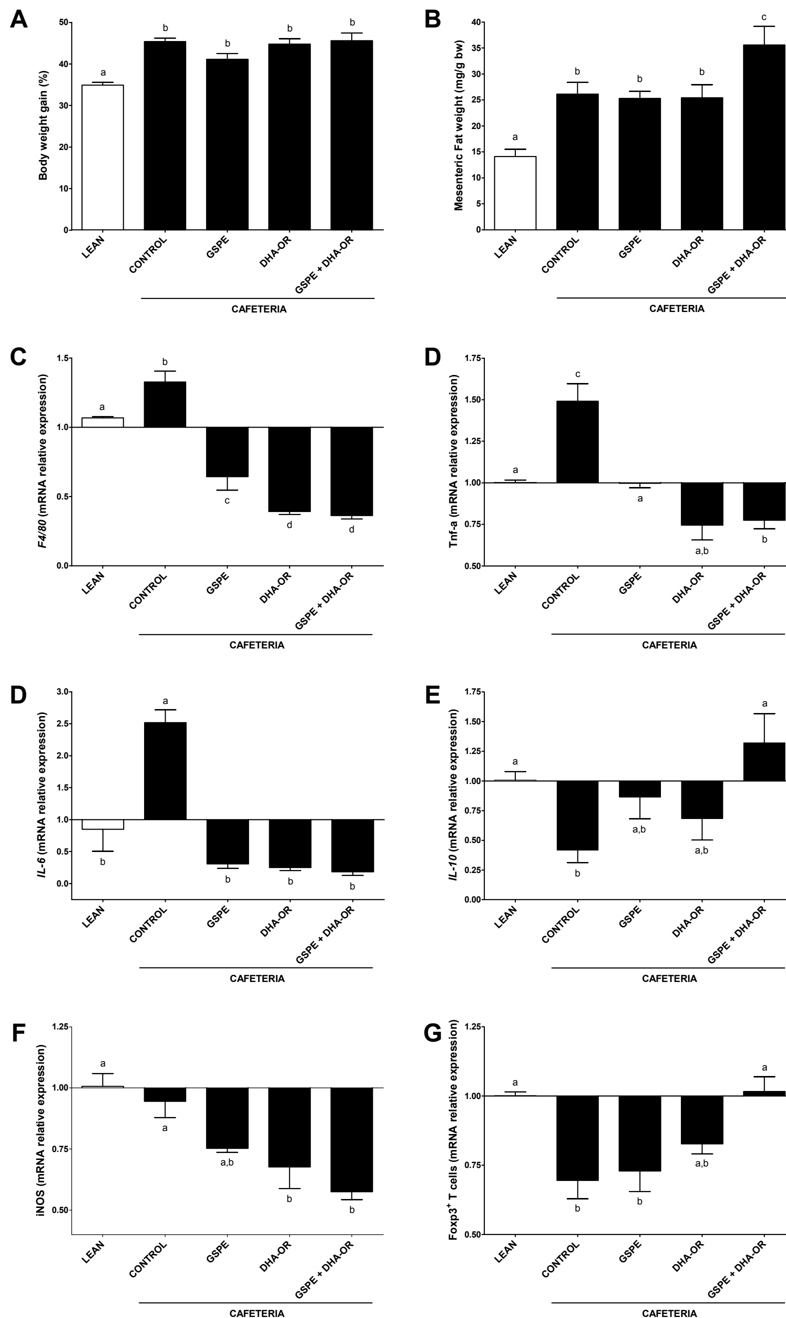

Figure 2. The supplementation of cafeteria diet with GSPE and/or DHA-OR suppresses CAF diet-induced adipose tissue-triggered local inflammatory response. (A) Body weight gain resulting from the 13 weeks of experiment. (B) Weight of the mesenteric adipose tissue. (C) Diet-induced modulatory effect on F4/80 macrophage selective marker. (D), (E), (F) and (G) Modulation of mRNA expression for the inflammatory biomarkers *Tnf-α*, *IL-6*, *IL-10* and *iNOS*. (H) Diet-induced regulation *of Foxp3^{+}* regulatory T lymphocytes. Gene expression was measured using RT-PCR. Data represent the mean ± SEM. Different letters denote significant differences between the different groups (P<0.05).

Nevertheless, the bioactive compounds dramatically counteracted the CAF diet-induced adipose tissue inflammation (Figure 2C-F). Thus, the presence of macrophages was significantly decreased, reflected through the down-regulation of *F4/80* gene expression, a macrophage-selective marker, and the CAF diet-induced transcription of pro-inflammatory genes, including *Tnf-α*, *IL-6* and *iNOS* were also inhibited. The transcription of the anti-inflammatory *IL-10* cytokine was up-regulated in response to the bioactive compounds. Remarkably, the modulation of *Tnf-α* and *IL-10* gene expression was significantly greater in animals whose diets were supplemented with the combination of GSPE and DHA-OR.

However, the combination of GSPE and DHA-OR also improved the immunomodulatory effects of both compounds alone in terms of Tregs cell recruitment to mesenteric adipose tissue, thereby counteracting the CAF diet-induced down-regulation of regulatory T cells, reflected through increased *Foxp3* gene expression in adipose tissue.

Obesity-induced systemic inflammatory response is mitigated through diet supplementation with GSPE and/or DHA-OR.

To determined the immunomodulatory effects of diet supplementation with bioactive compounds on the systemic activation of inflammation, the circulating levels of biomarkers involved in the regulation of immune and metabolic homeostasis were determined (Figure 3).

The circulating concentration of leptin was differentially modulated through the bioactives. Thus, whereas GSPE supplementation had no effect regarding the CAF-diet induced leptin overproduction, the supplementation with DHA-OR and most significantly, the combination of GSPE and DHA-OR increased leptin-circulating levels. Nevertheless, although DHA-OR and the combined bioactive compounds slightly increased adiponectin levels, only the supplementation of the CAF diet with GSPE produced a significant increase in the plasmatic adiponectin concentration.

Moreover, the supplementation of the CAF diet with any bioactive compound induced the restoration of the obesity-induced systemic inflammatory biomarkers to levels similar to those observed in STD-fed rats. Thus, circulating levels of MCP-1, orosomucoid and complement C3 were reduced through the supplementation of CAF diet with GSPE, DHA and the combination of both bioactive compounds.

CAF diet supplementation with GSPE and/or DHA-OR modulates thymus functionality

The capacity to modulate the adaptive response through diet supplementation with the bioactive compounds in CAF-diet induced obesity was determined based on the regulation of thymus, the largest primary lymphoid organ responsible of T cells maturation, in terms of morphology, the transcription of the genes involved in immune regulation and the modulation of T cell populations (Figure 4).

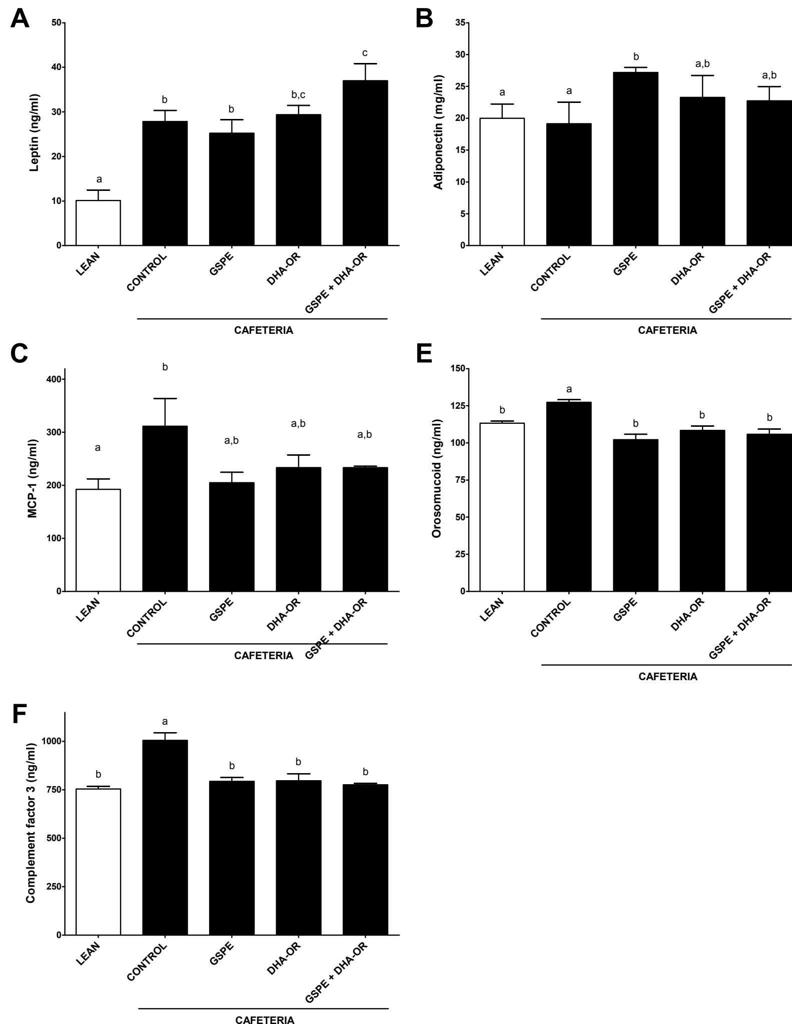

Figure 3. Effects of bioactive compounds, GSPE and/or DHA-OR in the concentration of circulating biomarkers involved in systemic responsiveness to diet-induced obesity. Levels of leptin (A), adiponectin (B), MCP-1 (C), orosomucoid (D) and complement component 3 (E) were measured in the plasma. Data represent the mean ± SEM. Identical letters indicate no differences in the indicated parameter between the different group diets (P<0.05).

Although the CAF diet had no effect in terms of thymus weight, the visual appearance of the thymus obtained from rats fed a cafeteria diet and independently supplemented or not with a bioactive compound was completely different from the thymus derived from STD-fed rats. Furthermore, the thymus weight was only slightly modulated after the supplementation of the CAF diet with GSPE and DHA-OR, and this increment of weight was significantly higher for the thymus from rats whose diets were supplemented with the combination of GSPE and DHA-OR. In addition, the number of thymocytes reflected the observed weight gain; that is, the more heavy the thymus, the higher the number of thymocytes obtained.

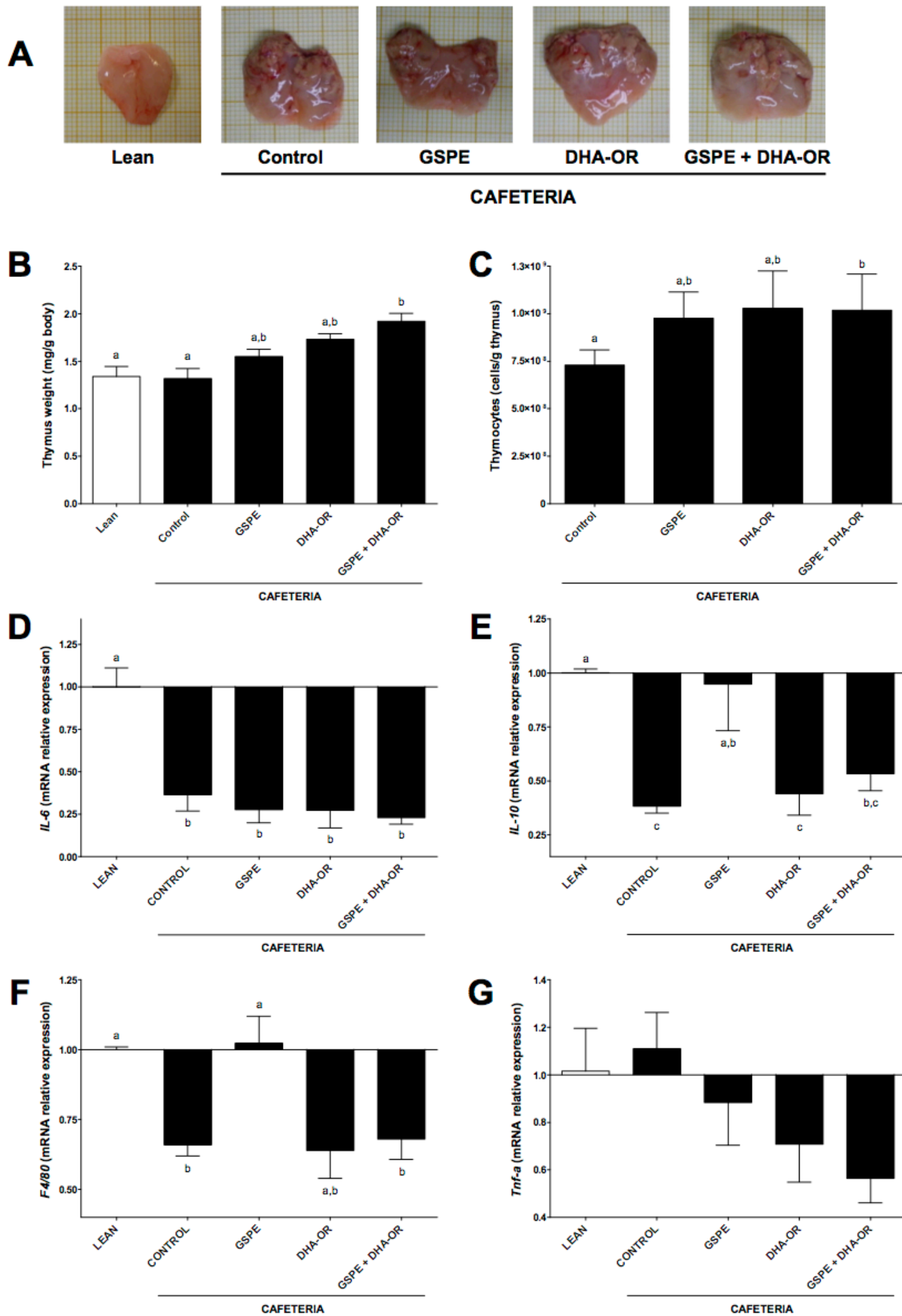

Figure 4. Cafeteria diet supplementation with GSPE, DHA-OR and the combined bioactive compounds modulates thymus functionality. (A) Macroscopic pictures of the thymus from rats fed with standard and also with cafeteria diet alone or supplemented with the indicated bioactives. Each small square represents 1 mm. (B) Weight of the thymus after the 13 weeks of the experiment. (C) Total number of thymocytes isolated. (D), (E), (F) and (G) mRNA gene expression of genes involved in the regulation of inflammation, including the cytokines *IL-6* and *IL-10* and the selective macrophage markers *F4/80 and Tnf-α*.

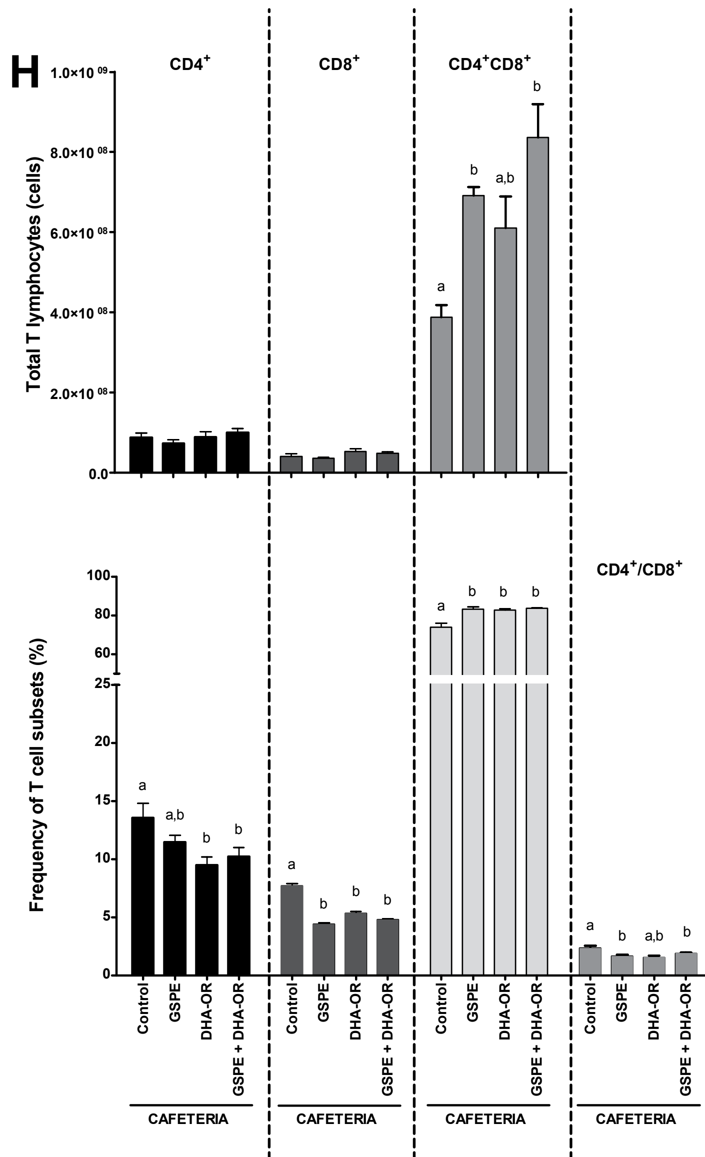

Figure 4. Cafeteria diet supplementation with GSPE, DHA-OR and the combined bioactive compounds modulates thymus functionality. ((H) Flow-cytometric phenotyping of thymic cells. The distribution and total T cell count for the major T lymphocytes subsets were determined based on the expression of $CD3^+$, $CD4^+$ and $CD8^+$ surface markers. Data represent the mean ± SEM. Different letters denote significant differences between the different groups for the determination of each parameter ($P<0.05$).

However, regarding the regulation of the expression of the genes involved in immune regulation on thymocytes, no bioactive-mediated modulatory effects were observed for *IL-6* and *Tnf-α* gene transcription, and the expression of *IL-10* was only modulated after supplementation with GSPE alone or in combination with DHA-OR. Furthermore, the CAF-diet induced a decrease in the macrophage count, and this effect was partially counteracted

through supplementation with DHA-OR and totally balanced in the thymus from GSPE-supplemented rats.

Moreover, the effect of GSPE and DHA-OR diet supplementation on the counts of lymphocyte subpopulations was determined. Thus, not only the T cell subsets frequency but also the total number of thymus-contained T cells was determined. Whereas the three bioactive compounds differentially modulated the frequency of $CD4^+$, $CD8^+$ and the ratio of $CD4^+/CD8^+$, the total count of these T cell subsets within the thymus were not modulated in response to diet supplementation with the bioactive compounds. Furthermore, both, the frequency and the total double positive T cells, i.e., $CD4^+CD8^+$, were significantly increased in the thymus of rats whose CAF diet was complemented with GSPE, DHA-OR or the combination of both compounds.

Spleen functionality is modulated through supplementation of the cafeteria diet with GSPE, DHA-OR and the combination of both bioactive components

The effect of enriching the CAF diet with biologically active compounds on spleen weight and the modulation of the expression of immune genes was determined. Furthermore, spleen immune function was evaluated through the analysis of the composition of lymphocyte population (Figure 5).

Thus, the bioactive compounds, particularly the GSPE and DHA-OR combination, counteracted CAF-diet induced spleen atrophy. Unlike the thymus, no bioactive effects were determined regarding the number of splenocytes.

Furthermore, regarding the immunosuppressed gene expression profile resulting from the CAF diet, characterised by the down-regulation of macrophages and inflammatory biomarkers, including, *IL-6*, *Tnf-α* and *IL-10*, only the supplementation of the CAF diet with GSPE, particularly in combination with DHA-OR, significantly counterbalanced the obesity-induced expression profile. Thus, the GSPE and DHA-OR combination counteracted the CAF diet-induced down-regulation of *IL-6* and *Tnf-α* gene expression in splenocytes, while only GSPE slightly modulated *IL-10* gene expression. Moreover, although the CAF diet-induced diminution of the macrophage count in splenocytes was partially neutralised through supplementation with the combination of GSPE and DHA-OR, only GSPE supplementation restored the expression of the selective macrophage marker *F4/80*, similar to the observations in the splenocytes obtained from the STD-fed rats. However, the bioactive-mediated regulation of splenic immune function primarily reflected the decrease in the ratio between the relative counts of $CD4^+$ and $CD8^+$ T cells and the increase in $CD8^+$ T cells.

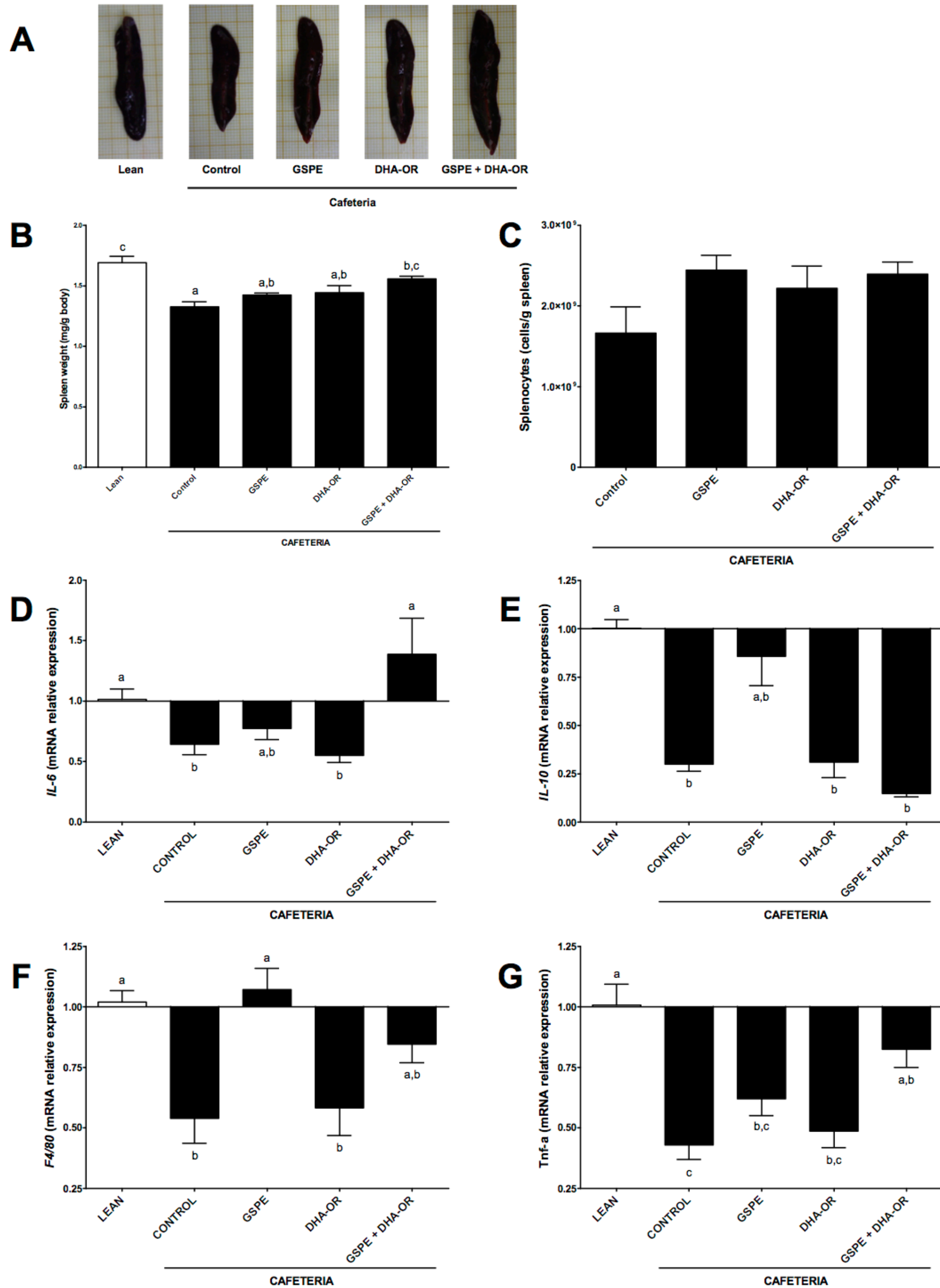

Figure 5. Spleen functionality is impaired by diet-induced obesity. (A) Macroscopic pictures of the spleens from rats fed standard and cafeteria diets alone or supplemented with the indicated bioactives. Each small square represents 1 mm. (B) Weight of the spleen after 13 weeks of the experiment. (C) Total number of splenocytes isolated. (D), (E), (F) and (G) mRNA expression of the genes involved in the regulation of inflammation, including *IL-6* and *IL-10*, and the selective macrophage marker *F4/80 and Tnf-α*.

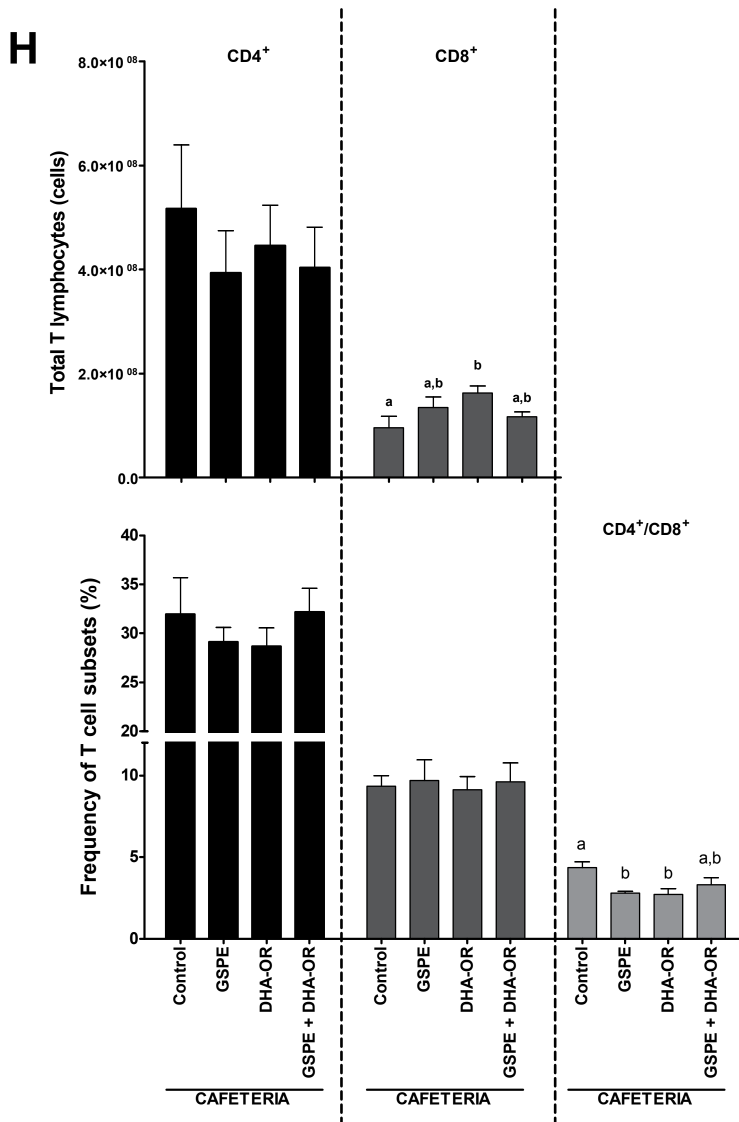

Figure 5. Spleen functionality is impaired by diet-induced obesity. (H) Flow-cytometric phenotyping of splenic cells. The distribution and total T cell number for the major T lymphocytes subsets were determined based on the expression of $CD3^+$, $CD4^+$ and $CD8^+$ surface markers. Data represent the mean ± SEM. Different letters denote significant differences between the different groups for the determination of each parameter ($P<0.05$).

The lymphocyte counts in PBMCs are influenced through supplementation of the CAF diet with GSPE, DHA-OR or the combination of both bioactive components

The effects of the supplementation of the CAF diet with biologically active molecules on the modulation of obesity-mediated T cell populations was determined based on the analysis of the major T lymphocyte counts in PBMCs.

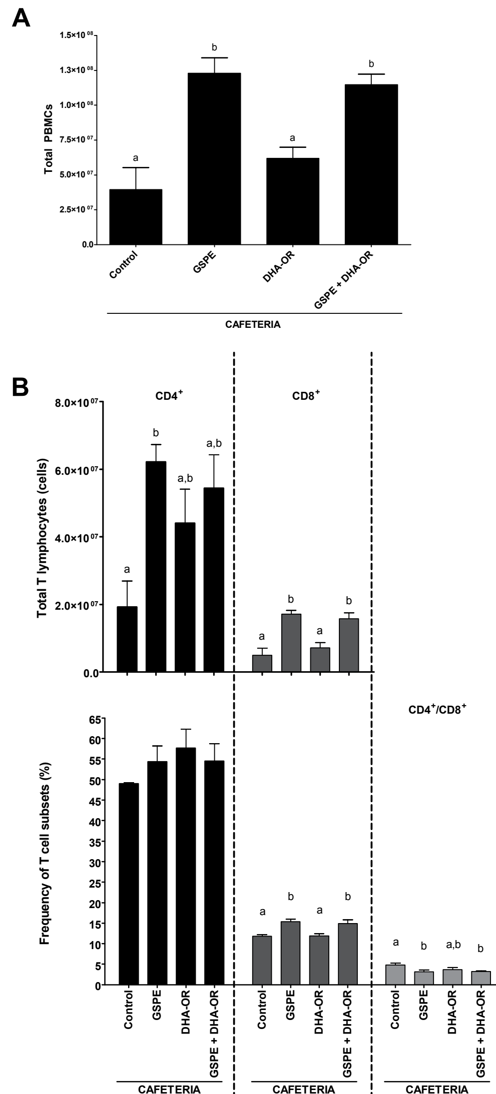

Figure 6. Supplementation of cafeteria diet with GSPE, DHA-OR and its combination modulate obesity-induced T lymphocyte subsets. (A) Weight (B) Flow-cytometric phenotyping of peripheral blood mononuclear cells. The distribution and the total T cell counts for the major T lymphocyte subsets were determined based on the expression of $CD3^+$, $CD4^+$ and $CD8^+$ surface markers. Data represent the mean ± SEM. Different letters denote significant differences between the different groups for the determination of each parameter (P<0.05).

Thus, supplementation with GSPE alone and in combination with DHA-OR significantly increased the total number of PBMCs circulating in the bloodstream compared with the counts obtained in rats fed CAF diets without supplementation. Furthermore, a significant increase in the frequency of the $CD8^+$ T cells and significant decrease in the ratio of the relative counts of $CD4^+$ and $CD8^+$ T lymphocytes was observed. In addition, an increase in the total counts of circulating $CD4^+$ lymphocytes, particularly circulating $CD8^+$ T cells, was detected.

DISCUSSION

Nutritional status plays a pivotal role in the regulation of overall immune function. In addition to metabolic changes, obesity has also been associated with chronic low-grade inflammation and a weakened immune response. Thus, an impairment of the reciprocal interactions between metabolism and the immune system underlies the pathogenesis of obesity and obesity-related diseases [15]. The cafeteria diet is considered a robust model for human metabolic syndrome, characterised by promoting obesity as a consequence of the hedonistic hyperphagia of highly palatable and energy-dense food [24].

Thus, these results suggest that after feeding with a cafeteria diet, rats become obese, and this pathological state is hallmarked by a dramatic increase in adiposity. Furthermore, diet-induced obesity triggers a local inflammatory response in adipose tissue characterised by the increased production of pro-inflammatory, not anti-inflammatory, biomarkers [7,25]. This pro-inflammatory state activates resident leukocytes and also triggers the recruitment and infiltration of circulating leukocytes, including macrophages, circulating monocytes, and lymphocytes, thereby resulting in the alteration of the stromal vascular fraction of adipose tissue [26]. An overall inflammatory response has also been observed, although obesity weakens lymphoid organs-mediated immune responses [3,27]. Thus, in a diet-induced obesity context, homeostasis is disrupted as a consequence of metabolic and immune dysfunctions resulting from continuous positive energy imbalance, promoting a pathological state.

Because nutrition plays a central role for pathology development in diet-induced obesity, we proposed the supplementation of diet with nutritional bioactive compounds, including GSPE and DHA-OR, as an intrinsic treatment for the amelioration of obesity-induced disturbances and the promotion of the healthy state. Thus, we determined the potential role of compounds contained in foods present constitutively in the Mediterranean diet, highlighted by metabolically and immunologically properties, as nutritional tools to supress the diet-induced inflammation and boost the immune system to restore homeostasis.

There is growing evidence that adipose tissue is a fundamental player in immuno-metabolic homeostasis due to its pivotal role in the orchestration of both the nutritional status and the activation of the obesity-induced inflammatory response [5,15]. Accordingly, we have analysed the modulatory effects of foods containing biologically active compounds on the

regulation of the obesity-induced inflammatory response. Thus, the supplementation of the cafeteria diet with GSPE, DHA-OR or the combination of these compounds in cafeteria diet-induced obese rats provoked a phenotypic switch in the immunological profile of mesenteric adipose tissue, promoting the resolution of the adipose tissue-triggered inflammation through the inhibition of pro-inflammatory biomarkers transcription, such as *IL-6*, *Tnf-α* and *iNOS,* and inducing the transcription of *IL-10,* an anti-inflammatory cytokine. Notably supplementing the CAF diet with any of the bioactive compounds, administered alone or together, restored the obesity-induced overexpression of circulating inflammatory biomarkers to concentrations similar to those determined for the STD diet-fed rats. Thus, the systemic anti-inflammatory effects of GSPE, DHA-OR and the combination of these compounds, includes the diminution in the secretion of the chemokine MCP-1, orosomucoid and C3, as markers of the acute-phase response.

Furthermore, the secretion of immunomodulating adipokines, including adiponectin and leptin, was slightly modulated. Thus, GSPE supplementation highlighted an increase in the concentration of adiponectin in plasma, which exerts multiple anti-inflammatory actions in multiple cells and tissues, including the promotion of macrophage polarisation towards an alternative anti-inflammatory phenotype [28]. However, supplementation with the combination of GSPE and DHA-OR promoted increased circulating leptin levels. Notably, the supplementation of CAF diet with the combination of GSPE and DHA-OR induced a significant increase in adiposity, resulting in increased circulating leptin levels. This increase in adiposity, however, was not associated with inflammation-triggered adipose tissue dysfunction. Indeed, the GSPE and DHA-OR combination improved some of the anti-inflammatory effects observed after supplementation with these bioactives individually, such as in the inhibition of *Tnf-α* and the induction of *IL-10* gene expression. Leptin regulation is of great of interest because in addition to its central role as an endocrine hormone for the regulation of energy metabolism, leptin also exerts pleiotropic actions on multiple organs [29]. Thus, we showed that the diet-induced increment of adiposity is reflected by a rise in circulating leptin levels, which is detected by the cognate leptin receptor located in the hypothalamus, mediating the central regulation of energy metabolism [30,31]. Nevertheless, in obesity-induced hyperleptinaemia, leptin signalling is attenuated [32], resulting in leptin resistance. However, leptin also plays a pivotal role in the regulation of both innate and adaptive immunity, affecting not only the cell-mediated immune response but also the functionality of lymphoid organs. In innate immunity, leptin promotes the phagocytic function of macrophages and increases the production of pro-inflammatory cytokines and reactive oxygen species (ROS). In addition, in adaptive immunity leptin associates nutritional status with T cell function [33,34]. Furthermore, leptin is essential for the lymphoid organ homeostasis [35]. Thus, considering the results observed for GSPE and DHA-OR diet supplementation, increased leptin levels might reflect an increase in the activation of the immune response; that is, in combination, GSPE and DHA-OR might act as stimulators of the immune response.

In addition, the immunomodulatory effects of GSPE and DHA supplementation are not limited to the modulation of the immune response at a molecular level, but these bioactive compounds also modulate cell-mediated immunity. Thus, the supplementation of the CAF diet with biologically active components induced a decrease in the number of macrophages recruited to adipose tissue resulting from diet-induced obesity. Notably, the combination between GSPE and DHA-OR also modulated the T cell lymphocyte profile in adipose tissue by increasing the recruitment of Treg cells. This small subset of $CD4^+$ lymphocytes plays an important role in the control of inappropriate immune responses through a potent immunosuppressive role in inflammatory disorders [36]. Indeed, the immunomodulatory effects of Treg cells are based on the production of the anti-inflammatory cytokine IL-10, the modulation of the effector T cell response and the promotion of the anti-inflammatory M2 macrophage phenotypic polarisation [37,38]. Thus, we can associate the significant increase in the Treg cell subset in the adipose tissues of CAF-fed rats, whose diets were supplemented with the combination of GSPE and DHA-OR, with the significant increase in IL-10 secretion and the anti-inflammatory phenotypic profile. Thus, the combination of these two biologically active compounds might improve anti-inflammatory effects through the promotion of Treg cell recruitment in adipose tissue [39].

Moreover, obesity has also been associated with the induction of immunological disturbances and an impairment of the immune system, both functionally and quantitatively. Thus, the impairment of immune function affects the responsiveness of primary and secondary lymphoid organs, including the thymus and spleen, to perturbations in homeostasis. Furthermore, obesity-induced quantitative impairment of immunity is evidenced as T lymphocytopenia, promoting a decrease in T lymphocytes, including both helper ($CD4^+$) and cytotoxic ($CD8^+$) T cells, in peripheral blood, spleen and thymus [40–42].

Thus, the thymus is a primary lymphoid organ, vital for the homeostatic maintenance of immunity, associated with a central role in thymocyte differentiation and maturation into functional T lymphocytes [43]. Moreover, obesity accelerates thymic aging, characterised by compromising the generation of naïve T cells and the replacement of lymphoidstromal thymic zones with adipose tissue. Furthermore, diet-induced obesity reduces T cell repertoire diversity leading to immunosenescence [44,45]. Thus, considering the obesity-induced impairment of thymus functionality, the results suggest that the CAF diet *per se* modulates thymus morphology, as reflected by the visual appearance of the thymus from CAF diet-fed rats, independently of supplementation with bioactive compounds, compared with STD diet-fed rats. This change in the morphology suggests that the CAF diet could promote thymus atrophy by increasing perithymic adipose tissue [44]. Furthermore, we assessed the immunomodulatory role of GSPE and DHA-OR, counteracting the obesity-induced modulatory effects in terms of the morphology and functionality of the thymus. Thus, the supplementation of the CAF diet with the combination of GSPE and DHA-OR not only significantly increased the weight of the thymus but also increased the thymocyte counts. These results suggest that supplementation with these bioactive compounds promoted weight increase not only as a consequence of a CAF diet-induced ectopic fat accumulation

on the thymus [15], but rather might reflect increased thymocyte counts. However, GSPE supplementation also improved T cell maturation through the significant modulation of the expression of *F4/80*, a selective marker of macrophages, and the cytokine *IL-10*. Thus, whereas thymic macrophages are crucial in T cell maturation through the expression of the major histocompatibility complex molecules (MHC), whose recognition is the basis of T lymphocyte selection and maturation [46,47], IL-10 regulates T cell maturation; indeed, a decrease *in IL-10* expression in the thymus is associated with T cell immunodeficiency [48]. Moreover, we characterised the immunostimulatory effects of GSPE, particularly in combination with DHA-OR, on improving thymus functionality through the induction of thymopoiesis as consequence of a greater pool of progenitor $CD4^+CD8^+$ T cells, counteracting the obesity-induced immunodeficiency state resulting from defects in T-cell generation in thymus [13,44].

The spleen is the largest secondary lymphoid organ involved in blood filtration, the regulation of the immune-metabolic-endocrine network, and the integration of innate and adaptive immune responses [49]. Thus, the proper functionality of the spleen is key for responses against homeostatic perturbations. Thus, bioactive food components, such as GSPE and/or DHA, significantly counteract the obesity-induced loss of splenic functionality. Notably, the GSPE and DHA-OR combination, increases spleen weight, but not splenocytes counts and improves the capacity of the splenocytes to appropriately respond to obesity-induced stimuli through the increased transcription of *IL-6* and *Tnf-α*, as biomarkers involved in the reactivity against the presence of obesity-related systemic biomarkers [50]. Moreover, supplementation with bioactive compounds increases the number of infiltrated macrophages, which plays a key role in host immunity, linking innate and adaptive immune systems based on the recognition of harmful stimuli through the expression of a large variety of pathogen receptor molecules [51]. Furthermore, supplementing the CAF diet with these bioactives also modulated T cell subsets in the spleen, particularly inducing a slight increase in the total numbers of $CD8^+$ T cell population and changing the relative proportion of helper and cytotoxic T lymphocytes.

In addition, T cell counts within the PBMCs were also modulated through supplementation with bioactive compounds. The bioactive supplementation of the CAF diet with GSPE, alone or in combination with DHA-OR, resulted in increased counts of circulating PBMCs and $CD4^+$ and $CD8^+$ T cells. Remarkably, the relative composition of the T cell subset in peripheral blood changed as a consequence of the increased frequency of $CD8^+$ T lymphocytes. Notably, the bioactive-mediated stimulation of new T cell generation, associated with higher counts of $CD4^+CD8^+$ T lymphocytes in thymus, was reflected as an increase in the circulating pools of both $CD4^+$ and $CD8^+$ T lymphocytes, counteracting obesity-induced lymphopenia.

Notably, the combination of GSPE and DHA-OR, considered as biologically active components, not only showed anti-inflammatory properties but also improved the immunostimulatory effects observed when these compounds were administered individually.

In addition, these results provide the first demonstration that the administration of both bioactive compounds potentiates the immunomodulatory effects of the individual administration of these compounds. Thus, the GSPE-DHA combination not only suppressed obesity-induced systemic inflammation but also inhibited obesity-triggered adipose tissue inflammation principally by promoting a switch in immune cells towards an anti-inflammatory phenotype, reflected as an increase *IL-10* expression and Treg cell subpopulation. Furthermore, the GSPE-DHA combination also improved the functionality of lymphoid organs, including the thymus and the spleen, through the generation of new T lymphocytes and by improving the responsiveness to homeostatic perturbations. These results suggest that the supplementation of diet with the combination of procyanidins and DHA induces a healthy state through the promotion of the resolution of adipose tissue-driven inflammation and improvements in the ability to boost the obesity-induced weakened immune response.

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REFERENCES

1 Miranda, P. J., DeFronzo, R. A., Califf, R. M. and Guyton, J. R. (2005) Metabolic syndrome: definition, pathophysiology, and mechanisms. Am. Heart J., St. Louis, CV Mosby Co. **149**, 33–45.

2 Speakman, J. R. (2004) Obesity: the integrated roles of environment and genetics. J. Nutr., Am Soc Nutrition **134**, 2090S–2105S.

3 De Heredia, F. P., Gómez-Martínez, S. and Marcos, A. (2012) Obesity, inflammation and the immune system. Proc. Nutr. Soc., Cambridge Univ Press **1**, 1–7.

4 Matarese, G., Moschos, S. and Mantzoros, C. S. (2005) Leptin in immunology. J. Immunol., American Association of Immunologists **174**, 3137–42.

5 Hotamisligil, G. S. (2006) Inflammation and metabolic disorders. Nature, Nature Publishing Group **444**, 860–867.

6 Iyer, A., Fairlie, D. P., Prins, J. B., Hammock, B. D. and Brown, L. (2010) Inflammatory lipid mediators in adipocyte function and obesity. Nat. Rev. Endocrinol., Nature Publishing Group **6**, 71–82.

7 Wellen, K. E. and Hotamisligil, G. S. (2003) Obesity-induced inflammatory changes in adipose tissue. J. Clin. Invest., Am Soc Clin Investig **112**, 1785–1787.

8 Chawla, A., Nguyen, K. D. and Goh, Y. P. S. (2011) Macrophage-mediated inflammation in metabolic disease. Nat. Rev. Immunol., Nature Publishing Group.

9 Kanda, H., Tateya, S., Tamori, Y., Kotani, K., Hiasa, K., Kitazawa, R., Kitazawa, S., Miyachi, H., Maeda, S. and Egashira, K. (2006) MCP-1 contributes to macrophage infiltration into

adipose tissue, insulin resistance, and hepatic steatosis in obesity. J. Clin. Invest., Am Soc Clin Investig **116**, 1494.

10 Baumann, H. and Gauldie, J. (1994) The acute phase response. Immunol. Today **15**, 74.

11 Epstein, F. H., Gabay, C. and Kushner, I. (1999) Acute-phase proteins and other systemic responses to inflammation. N. Engl. J. Med., Mass Medical Soc **340**, 448–454.

12 La Cava, A. and Matarese, G. (2004) The weight of leptin in immunity. Nat. Rev. Immunol., Nature Publishing Group **4**, 371–379.

13 Howard, J. K., Lord, G. M., Matarese, G., Vendetti, S., Ghatei, M. A., Ritter, M. A., Lechler, R. I. and Bloom, S. R. (1999) Leptin protects mice from starvation-induced lymphoid atrophy and increases thymic cellularity in ob/ob mice. J. Clin. Invest. **104**, 1051–9.

14 Dixit, V. D. (2008) Adipose-immune interactions during obesity and caloric restriction: reciprocal mechanisms regulating immunity and health span. J. Leukoc. Biol. **84**, 882–92.

15 Kanneganti, T.-D. and Dixit, V. D. (2012) Immunological complications of obesity. Nat. Immunol., Nature Publishing Group **13**, 707–712.

16 Weinreich, M. A. and Hogquist, K. A. (2008) Thymic emigration: when and how T cells leave home. J. Immunol. **181**, 2265–70.

17 Gotoh, K., Inoue, M., Masaki, T., Chiba, S., Shimasaki, T., Ando, H., Fujiwara, K., Katsuragi, I., Kakuma, T. and Seike, M. (2012) A Novel Anti-inflammatory Role for Spleen-Derived Interleukin-10 in Obesity-Induced Inflammation in White Adipose Tissue and Liver. Diabetes, Am Diabetes Assoc **61**, 1994–2003.

18 Procaccini, C., Galgani, M., De Rosa, V. and Matarese, G. (2011) Intracellular metabolic pathways control immune tolerance. Trends Immunol., Elsevier.

19 Lumeng, C. N. and Saltiel, A. R. (2011) Inflammatory links between obesity and metabolic disease. J. Clin. Invest., American Society for Clinical Investigation **121**, 2111.

20 Martinez-Micaelo, N., González-Abuín, N., Ardèvol, A., Pinent, M. and Blay, M. T. (2012) Procyanidins and inflammation: Molecular targets and health implications. BioFactors, Wiley Online Library **38**, 257–265.

21 Terra, X., Pallarés, V., Ardèvol, A., Bladé, C., Fernández-Larrea, J., Pujadas, G., Salvadó, J., Arola, L. and Blay, M. (2011) Modulatory effect of grape-seed procyanidins on local and systemic inflammation in diet-induced obesity rats. J. Nutr. Biochem., Elsevier **22**, 380–387.

22 Yates, C. M., Calder, P. C. and Ed Rainger, G. (2014) Pharmacology and therapeutics of omega-3 polyunsaturated fatty acids in chronic inflammatory disease. Pharmacol. Ther. **141**, 272–82.

23 Serra, A., Macià, A., Romero, M.-P., Valls, J., Bladé, C., Arola, L. and Motilva, M.-J. (2010) Bioavailability of procyanidin dimers and trimers and matrix food effects in in vitro and in vivo models. Br. J. Nutr., Cambridge Univ Press **103**, 944.

24 Sampey, B. P., Vanhoose, A. M., Winfield, H. M., Freemerman, A. J., Muehlbauer, M. J., Fueger, P. T., Newgard, C. B. and Makowski, L. (2011) Cafeteria Diet Is a Robust Model of Human Metabolic Syndrome With Liver and Adipose Inflammation: Comparison to High-Fat Diet. Obesity, Wiley Online Library **19**, 1109–1117.

25 Schäffler, A. and Schölmerich, J. (2010) Innate immunity and adipose tissue biology. Trends Immunol., Elsevier **31**, 228–235.

26 Weisberg, S. P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R. L. and Ferrante, A. W. (2003) Obesity is associated with macrophage accumulation in adipose tissue. J. Clin. Invest., Am Soc Clin Investig **112**, 1796–1808.

27 Marti, A., Marcos, A. and Martinez, J. A. (2001) Obesity and immune function relationships. Obes. Rev. **2**, 131–140.

28 Ohashi, K., Parker, J. L., Ouchi, N., Higuchi, A., Vita, J. A., Gokce, N., Pedersen, A. A., Kalthoff, C., Tullin, S., Sams, A., et al. (2010) Adiponectin promotes macrophage polarization toward an anti-inflammatory phenotype. J. Biol. Chem. **285**, 6153–60.

29 Coelho, M., Oliveira, T. and Fernandes, R. (2013) Biochemistry of adipose tissue: an endocrine organ. Arch. Med. Sci. **9**, 191–200.

30 Lam, Q. L. K. and Lu, L. (2007) Role of leptin in immunity. Cell. Mol. Immunol. **4**, 1–13.

31 Wauman, J. and Tavernier, J. (2011) Leptin receptor signaling: pathways to leptin resistance. Front. Biosci. (Landmark Ed. **16**, 2771–93.

32 Lin, S., Thomas, T. C., Storlien, L. H. and Huang, X. F. (2000) Development of high fat diet-induced obesity and leptin resistance in C57Bl/6J mice. Int. J. Obes., Nature Publishing Group **24**, 639–646.

33 Matarese, G., Procaccini, C., De Rosa, V., Horvath, T. L. and La Cava, A. (2010) Regulatory T cells in obesity: the leptin connection. Trends Mol. Med. **16**, 247–56.

34 Faggioni, R., Feingold, K. R. and Grunfeld, C. (2001) Leptin regulation of the immune response and the immunodeficiency of malnutrition. FASEB J. **15**, 2565–71.

35 Zhang, F., Chen, Y., Heiman, M. and Dimarchi, R. (2005) Leptin: structure, function and biology. Vitam. Horm. **71**, 345–72.

36 Eller, K., Kirsch, A., Wolf, A. M., Sopper, S., Tagwerker, A., Stanzl, U., Wolf, D., Patsch, W., Rosenkranz, A. R. and Eller, P. (2011) Potential role of regulatory T cells in reversing obesity-linked insulin resistance and diabetic nephropathy. Diabetes **60**, 2954–62.

37 Wing, K. and Sakaguchi, S. (2010) Regulatory T cells exert checks and balances on self tolerance and autoimmunity. Nat. Immunol., Nature Publishing Group **11**, 7–13.

38 Liu, G., Ma, H., Qiu, L., Li, L., Cao, Y., Ma, J. and Zhao, Y. (2011) Phenotypic and functional switch of macrophages induced by regulatory CD4+CD25+ T cells in mice. Immunol. Cell Biol. **89**, 130–42.

39 Pereira, S. S., Teixeira, L. G., Aguilar, E. C., Oliveira, M. C. de, Savassi-rocha, A. L., Pelaez, J. M. N., Capettini, L. S. A., Diniz, M. T. C., Ferreira, A. V. M. and Alvarez-Leite, J. I. (2013) Modulation of Adipose Tissue Inflammation by Foxp3+ Treg Cells, Il-10 and Tgfβ In Metabolically Healthy Class Iii Obese Individuals. Nutrition, Elsevier.

40 Sheridan, P. A., Paich, H. A., Handy, J., Karlsson, E. A., Hudgens, M. G., Sammon, A. B., Holland, L. A., Weir, S., Noah, T. L. and Beck, M. A. (2012) Obesity is associated with impaired immune response to influenza vaccination in humans. Int. J. Obes. (Lond)., Macmillan Publishers Limited **36**, 1072–7.

41 Tanaka, S., Inoue, S., Isoda, F., Waseda, M., Ishihara, M., Yamakawa, T., Sugiyama, A., Takamura, Y. and Okuda, K. (1993) Impaired immunity in obesity: suppressed but reversible lymphocyte responsiveness. Int. J. Obes. Relat. Metab. Disord. **17**, 631–6.

42 Tanaka, S. i, Isoda, F., Yamakawa, T., Ishihara, M. and Sekihara, H. (1998) T lymphopenia in genetically obese rats. Clin. Immunol. Immunopathol. **86**, 219–25.

43 Dooley, J. and Liston, A. (2012) Molecular control over thymic involution: From cytokines and microRNA to aging and adipose tissue. Eur. J. Immunol., Wiley Online Library **42**, 1073–1079.

44 Yang, H., Youm, Y.-H., Vandanmagsar, B., Rood, J., Kumar, K. G., Butler, A. A. and Dixit, V. D. (2009) Obesity accelerates thymic aging. Blood **114**, 3803–12.

45 Linton, P. J. and Dorshkind, K. (2004) Age-related changes in lymphocyte development and function. Nat. Immunol. **5**, 133–9.

46 Wood, G. W. (1985) Macrophages in the thymus. Surv. Immunol. Res. **4**, 179–91.

47 Martín-Fontecha, A., Schuurman, H. J. and Zapata, A. (1994) Role of thymic stromal cells in thymocyte education: a comparitive analysis of different models. Thymus **22**, 201–13.

48 Rouleau, M., Cottrez, F., Bigler, M., Antonenko, S., Carballido, J. M., Zlotnik, A., Roncarolo, M. G. and Groux, H. (1999) IL-10 transgenic mice present a defect in T cell development reminiscent of SCID patients. J. Immunol., American Association of Immunologists **163**, 1420–7.

49 Tarantino, G., Scalera, A. and Finelli, C. (2013) Liver-spleen axis: intersection between immunity, infections and metabolism. World J. Gastroenterol. **19**, 3534–42.

50 Lamas, O., Martínez, J. A. and Marti, A. (2004) Decreased splenic mRNA expression levels of TNF-α and IL-6 in diet-induced obese animals. J. Physiol. Biochem. **60**, 279–283.

51 Den Haan, J. M. M. and Kraal, G. (2012) Innate immune functions of macrophage subpopulations in the spleen. J. Innate Immun. **4**, 437–45.

CHAPTER 7

Leptin signal transduction underlies the differential response to diet induced-obesity in rats

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In preparation

ABSTRACT

In the present study, we investigate the differential phenotypic response between Lewis (LEW) and Wistar Kyoto (WKY) inbred rats after a metabolic challenge based on the dietary energy density.

Although the cafeteria (CAF) diet promoted higher weight gain, a consequence of the increase of energy intake, in both strains, we found that the metabolic management of this energy surplus was significantly affected by the genetic background. Detailed phenotypic profiling showed a strain-specific pattern of energy metabolism and modulation of the metabolites profiles in plasma, revealing that while LEW rats are a strain prone to diet-induced obesity, WKY rats are resistant.

Genome-wide gene expression analysis confirmed the different transcriptional response in the circulating monocytes of LEW and WKY rats. By comparing differentially expressed genes between CAF-fed LEW and WKY rats, we identified the *Acss2* gene as key hub-gene within a network of highly co-expressed genes that were functionally enriched as nutrient sensors, the expression modulation of which could be underlying the phenotype.

The phenotypic and transcriptional analyses also pointed to the leptin-signalling pathway as a divergent point between the strain-specific adaptations to diet. Our results revealed that LEW rats (genetically prone to diet-induced obesity) were resistant to the central effects of leptin receptor stimulation. On the other hand, in WKY rats (resistant to diet-induced obesity) the transduction of central and peripheral leptin signalling was not attenuated by the diet. These results were mirrored by the activation of STAT3 as a result of the down-regulation of hypothalamic *Socs3* gene expression or primary *Mir223* gene expression down-regulation in peripheral monocytes.

INTRODUCTION

Obesity can result from the imbalance between the energy intake and expenditure and is associated with the development of an array of metabolic disorders collectively known as metabolic syndrome [1,2].

A prolonged excess of nutrient consumption results in the continuous saturation of the white adipose tissue's ability to store energy as fat, triggering a cytokine-driven response characterised by the secretion of pro-inflammatory chemokines that underlie an obesity-induced inflammatory response. Monocyte chemotactic protein-1 (MCP-1) is one of the most important adipokines due to its ability to recruit macrophages to the adipose tissue [3,4]. In addition, the continuous activation of the local inflammatory response underlies the chronic low-grade systemic inflammation that results in the widespread activation of the immune system [5]. Thus, white adipose tissue plays a key role in the regulation of metabolism, not only as the major site for energy storage but also as an endocrine organ that regulates the production of hormones such as leptin, indispensable for the regulation of immune and inflammatory responses [6–8]. Although the obesity-induced inflammatory response is triggered and takes place primarily in white adipose tissue, other organs are involved in obesity-induced inflammation. C-reactive protein (CRP) and orosomucoid are acute-phase proteins, which are produced mainly in the liver, and high circulating levels of CRP or orosomucoid are considered biomarkers of systemic inflammation and metabolic disturbances [9–12].

Leptin, a 16 kDa adipocyte-derived circulating cytokine, is produced in proportion to fat stores in order to regulate energy metabolism via its highly expressed cognate receptor in the central nervous system (CNS) [13]. In addition to its key role for the maintenance of energy homeostasis, leptin modulates both humoral and cell-mediated immunity, highlighting its pivotal role in linking nutritional status with the neuroendocrine and immune systems [14]. Leptin signalling is dependent on the presence of the long isoform of the leptin receptor (LerRb), which bears homology to members of the class I cytokine receptor (gp130) superfamily. Leptin stimulation activates the Janus-activated kinase (JAK)/signal transducers and activators of transcription (STAT) pathway, leading to the formation of a LepRb/JAK2 complex that, after trans-phosphorylation, results in STAT3 activation and the induction of SOCS3 expression; SOCS3 in turn negatively regulates leptin signalling by phosphorylating JAK2 [15–17]. The diet-induced excessive intake of energy or fat deposition leads to the development of resistance to leptin through the attenuation of hypothalamic STAT3 transcription factor activation by different molecular mechanisms, including an impaired transport of leptin across the blood-brain barrier or the presence of several negative regulators of leptin signalling, such as the protein-tyrosine phosphatase 1B (PTP1B), the tyrosine phosphatases SH2-containing protein tyrosine phosphatase-2 (SHP-2) or an enhanced expression of SOCS3, the negative feedback regulator of intracellular leptin signalling [18–20].

The reciprocal interactions between the most important organs and tissues involved in metabolism and immune cells have been described as key events in the pathogenesis of obesity and its associated diseases [7]. Therefore, the threats to the dynamic crosstalk between metabolic regulation and the immune system that are promoted by obesity may endanger the maintenance of homeostasis [11]. Monocytes, the blood-circulating precursors of tissue-differentiated macrophages and dendritic cells, are powerful stress-sensing immune cells that play central roles in the regulation of the inflammatory and innate immune response through the release of inflammatory cytokines and in the activation of the adaptive immune response [21]. Monocytes are of particular interest because, as circulating immune cells, they are exposed to the systemic environment, including the metabolic factors and pro-inflammatory cytokines produced and secreted by organs and tissues; therefore, the activity and expression profiles (i.e. transcriptomes) of monocytes may reflect the physiological state of the whole organism.

The susceptibility to obesity has an established underlying genetic component, but environmental influences, such as dietary effects, may be required to induce its manifestation [22–27]. Furthermore, although the interactions between genes and the environment that are associated with the development of obesity are not fully understood, the fact that some inbreed strains are susceptible to diet-induced obesity, whereas other are resistant, suggests that the genetic background contribute to the variability in obesogenic phenotypes between individuals [28–31]. Although different obesity models have been used to understand the genetic factors that underlie the regulation of energy homeostasis, diet-induced obesity models, such as the cafeteria diet (CAF)-induced obesity, are considered to be a robust model of the human metabolic syndrome and its related pathologies [32]. In a CAF diet, obesity is induced as consequence of hyperphagia resulting from the voluntary intake of highly palatable and energy-dense cafeteria-style foods present in Western-type diets instead of the standard chow [32]. Taking into account the distinct genetic background of inbred rat strains (phenotypically different) such as Lewis (LEW) and Wistar Kyoto (WKY) rats, and using the CAF diet-induced obesity model in these rats, both genetic and environmental factors can be controlled, providing a powerful tool for understanding the interactions between the genotype and the environment that underlie the development of complex traits such as metabolic syndrome. These inbred strains have been widely studied for their differential susceptibility to experimental glomerulonephritis through macrophage activation [33,34] and present a markedly distinct macrophage transcriptome, which result in differential macrophage activation [35,36].

Here, we studied the phenotypic and transcriptional responses of LEW and WKY rats, in response to CAF diet in order to determine the “gene-diet” interactions, focusing specifically on whole genome expression profiles in the circulating monocytes of these rats. We found that although the CAF diet promoted higher energy intake and weight gain for both strains, the metabolic and transcriptional management of this excess of energy was significantly influenced by the genetic background.

MATERIALS AND METHODS

Animals and experimental design

Male LEW (LEW/Crl) and WKY (WKY/NCrl) (Charles River, Margate, UK) rats, weighing 190 g and 130 g, respectively, were housed individually in a 22 ºC temperature-controlled room with a 12-h light/dark cycle. After an adaptation period, rats from each strain were randomly distributed into two experimental groups (n=5) and fed with either a standard chow diet (STD) (Panlab, Barcelona, Spain) or a cafeteria diet (CAF) composed of bacon, biscuits, pate, muffins, carrots, and milk with sugar for seven weeks. The daily total energy and distribution of the diets are described in Table 1.

Table 1. Total energy and diet composition.

	Standard diet	Cafeteria diet
Energy Intake (Kcal/day)	2.9	7.5
Protein (% energy)	21.7	10.4
Carbohydrate (% energy)	66.2	38.6
Fat (% energy)	9.6	50.8

After seven weeks of the indicated diet, the rats were fasted for 9 h and euthanised by exsanguination under anaesthesia. Blood was collected from abdominal aorta, and circulating monocytes were immediately isolated. The liver, hypothalamus, thymus, spleen and white adipose tissue depots (mesenteric, epididymal and perirenal) were rapidly removed, weighed, frozen in liquid nitrogen and stored at -80 ºC until analysis. All the procedures were performed with the approval of the Animal Ethics Committee of the Universitat Rovira i Virgili (Tarragona, Spain).

Measurement of adiposity and body weight gain

The effects of diet on body weight gain were determined by weekly measuring the weight difference between the current week and those weights measured at the beginning of the experiment. In this way, the effect of the different morphology of LEW and WKY rats was avoided. The adiposity index was computed at the end point as the sum of the weight of mesenteric, epididymal and perirenal fat pads and expressed as a percentage of total body weight.

Food intake

The rats were housed individually in metabolic cages for 48 h, and their food intake was assessed in week 5 of the experiment based on a measurement of the feeding pattern for each animal. The total energy intake and the contribution of each macronutrient to the total intake were normalised to the metabolic mass.

Energy metabolism

The energy metabolism components were measured, in at least 3 rats per group, using an indirect open-circuit calorimeter (Panlab Harvard Apparatus, Barcelona, Spain). The oxygen consumption (VO_2) and carbon dioxide production (VCO_2) of the rats were monitored simultaneously every 10 min in two metabolic chambers during the first 200 min of the postprandial period. The respiratory quotient was calculated from gas exchange measurements ($RQ=VCO_2/VO_2$), and the substrate oxidation values were calculated as previously described [37,38].

Measurement of biochemical parameters in plasma

After sacrifice, blood was collected, and heparinised plasma was obtained by centrifugation and stored at -80 ºC until analysis. Triglycerides and glucose levels were measured by enzymatic colorimetric methods (QCA, Barcelona, Spain). The concentration of β-hydroxybutyrate was determined using an ELISA kit provided by BEN (Milano, Italy), the non-esterified free fatty acid ELISA kit was purchased from WAKO (Neuss, Germany), the commercial kit for the determination of leptin was provided by EMD Millipore (Darmstadt, Germany), and the ELISA kits used to measure the plasma concentrations of MCP-1 (Abcam, Cambridge, USA) and Orosomucoid were purchased from GenWay (San Diego, USA).

Quantitative RT-PCR

Total RNA from the liver, mesenteric fat depot and hypothalamus was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Barcelona, Spain) and purified with RNeasy Mini Kit spin columns (Qiagen). cDNA was generated by reverse-transcription from total RNA using the reverse transcription reagent kit (Applied Biosystems, Madrid, Spain) and was subjected to RT-PCR amplification using SsoFast EvaGreen SuperMix (Bio-Rad). Relative gene expressions were normalised according to the cyclophilin peptidylprolyl isomerase A (*Ppia*) mRNA levels. The forward and reverse primers used for the different genes are described in Supplementary Table S1. The relative gene expression was normalised to the *Ppia* mRNA levels using the $2^{-\Delta\Delta Ct}$ method.

Isolation of circulating monocytes, RNA extraction and microarray preparation

Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation using HISTOPAQUE-1083 solution (SIGMA, Madrid). Monocytes were purified based on their adherence to plastic in serum-free RPMI-supplemented medium. Thus, after 90 min, the non-adherent cells were removed by several washes with warm PBS, and the total RNA from the adherent monocytes was extracted using the TRIzol reagent according to the manufacturer's instructions (Invitrogen, Barcelona, Spain) and purified using RNeasy microkits (Qiagen). The quality of total RNA isolated was determined using the Agilent 2100 Bioanalyser. The RNA Integrity Number (RIN) of RNA ranged from 9.10-10. One hundred and fifty nanograms of total RNA was amplified, labelled and hybridised to Rat Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA, USA) using the Ambion WT Expression kit (Life

Technologies) according to the manufacturer's instructions. The Affymetrix Rat Gene 1.0 ST arrays contain a subset of microRNA specific probes that align to the stem loop sequences allowing the analysis of the precursor forms of microRNA.

Microarray analysis of differential gene expression

The data from microarrays were normalised using the robust multi-array average (RMA) method [39], implemented in the Affymetrix Bioconductor package, and the differentially expressed genes were determined using the linear model implemented in the limma Bioconductor package. The Benjamini and Hochberg method was used to adjust P-values for multiple testing and control of the false discovery rate. Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis was performed using the GOstats Bioconductor package [40].

Gene co-expression network

A gene co-expression network was constructed from the gene expression profiles of the transcripts whose expression was differentially modulated (absolute fold change >1.5) when comparing the expression profiles of circulating monocytes from CAF diet-fed WKY and LEW rats. The co-expression network was inferred using a graphical Gaussian model (GGM) implemented in the R package GeneNet [41]. Briefly, a partial-correlation matrix was estimated by computing the partial correlation between the expression profiles of each gene pair. Bayesian posterior edge probability >0.95 (corresponding to a local false discovery rate <5%) was used to determine the significance of the resulting pairwise partial correlations. In the resulting co-expression network the nodes represent the set of genes that were differentially expressed and co-expressed between WKY and LEW rats, and the edges link the pairs of genes whose expression is not conditionally independent, defined as the pairwise partial correlation once the common effects of the other genes in the subset are removed [42]. To identify sub-clusters (modules) within the network we used the MCODE algorithm implemented in the clusterMaker plugin [43] of Cytoscape software [44], and the modules were functionally annotated with GO terms and KEGG pathways using the GOstats Bioconductor package [40]. The selected clusters were analysed using the cyto-Hubba plugin [45] to determine the hubs and bottlenecks that represent the key regulatory genes within the network.

Statistical analysis

The phenotypic data are expressed as the mean ± SEM. Strain and diet specifics effects (represented by a hash tag or an asterisk, respectively) were assessed using ANOVA or Student's t-tests, as appropriate. Differences were considered to be significant when the *p*-values were < 0.05. These calculations were performed using SPSS 17.0 software.

RESULTS

The cafeteria diet promotes voluntary hyperphagia and weight gain but a differential profile of adiposity in LEW and WKY rats

To investigate the role of the genetic background on the phenotypic response to a diet-induced obesity, two inbred rat strains, LEW and WKY, were fed with a cafeteria diet (CAF) for 7 weeks. The rats were allowed free access to highly palatable and energy-dense foods in addition to standard chow; animals of both genotypes showed significantly increased body weight in response to the CAF diet (Figure 1A and 1B).

This weight gain was accompanied by a significant increase in the total energy intake resulting from voluntary hyperphagia (Figure 1C). Regarding the distribution of energy obtained from food intake macronutrient, the cafeteria diet-induced enhance of energy intake was mainly due to a substantial increase in fat intake (Figure 1D). In addition, although the data were normalised to the metabolic weight, no differences were observed between the caloric intake of LEW and WKY (Figure 1E and 1F). When comparing the effects of the cafeteria diet on the contribution of each macronutrient to the total energy intake, the percentage of calories from fat intake shifted from the least important to the most important energy source, independent of the genetic background (Figure 1G and 1H).

Although the cafeteria diet caused no significant differences in carbohydrate intake, we observed that WKY rats fed with either standard chow or the cafeteria diet ingested significantly fewer carbohydrates than LEW rats under the same metabolic conditions.

Furthermore, when comparing the effects of CAF on body composition with respect to the standard diet (STD)-fed rats (Table 2), no significant strain differences were found for liver weight, however an increase in kidney weight was observed for CAF-fed WKY rats in comparison to the STD-fed rats. The CAF diet promoted an increase in thymus weight for both strains but did not affect spleen weight. Moreover, CAF-fed LEW and WKY rats showed greater adipose accumulation than the STD-fed rats. Therefore, even though the weights of the most relevant fad pads were increased equally for LEW rats, the WKY rats showed a different profile of adiposity: the CAF diet induced significant increases in adiposity only in the epididymal and perirenal fat pads but not the mesenteric fat pads.

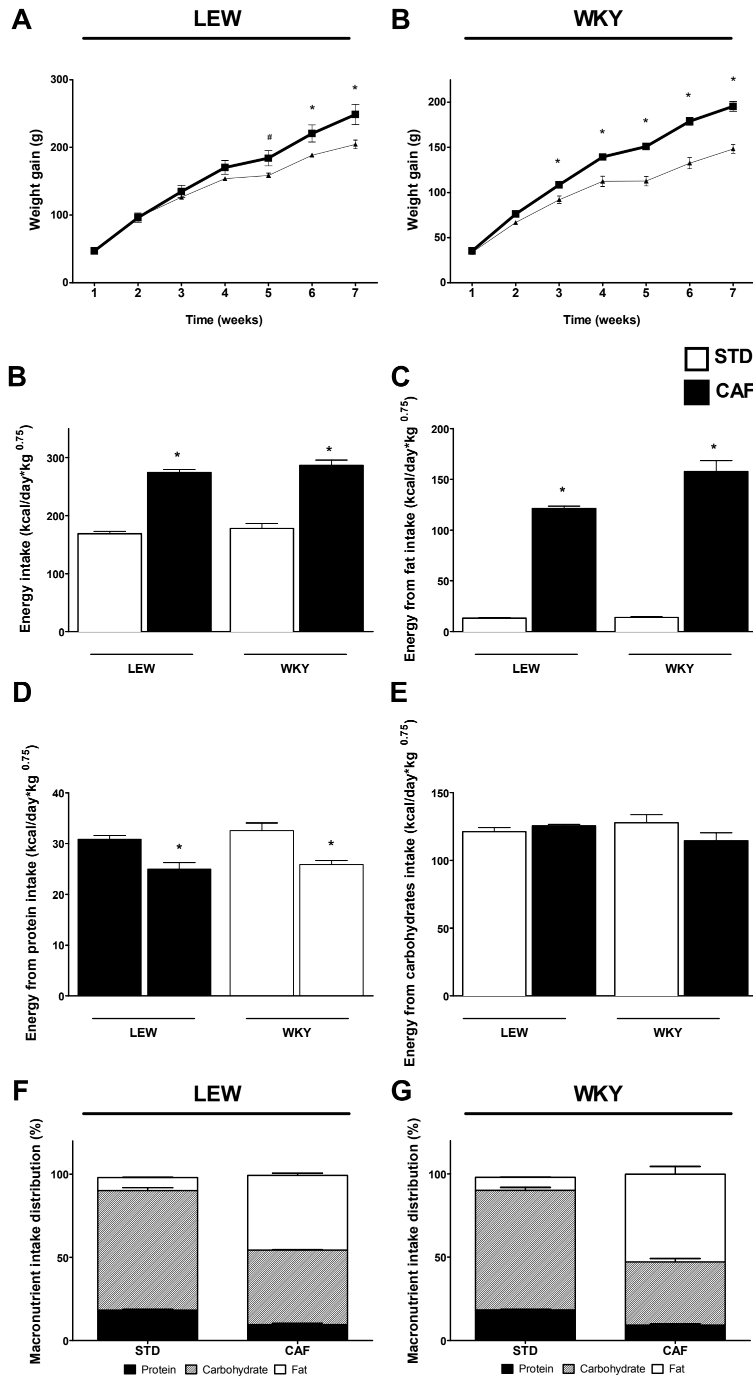


Figure 1. Body weight and food intake pattern in response to the STD and CAF diets. (A and B) Body weight gain changes in STD- and CAF-fed LEW and WKY rats, respectively. (C, D, G and H) The measured total energy intake and the energy intake from fat, protein and carbohydrates, respectively. (E and F) The distribution of the macronutrient intake based on the food consumption in STD- and CAF-fed LEW and WKY rats, respectively. *P<0.05 between the rats fed with the STD diet and the CAF diet within each strain, defined as the diet effect

Table 2. Cafeteria diet effect on adiposity and organs weight.

	LEW		WKY	
	Lean	**Cafeteria**	**Lean**	**Cafeteria**
Metabolic organs				
Liver weight (g)	11.09 ± 0.43	12.35 ± 0.32	7.16 ± 0.17	8.28 ± 1.55
Kidneys weight (g)	2.85 ± 0.05	2.97 ± 0.08	1.99 ± 0.07	2.47 ± 0.07
Immune-involved organs				
Spleen weight (g)	0.76 ± 0.06	0.63 ± 0.03	0.61 ± 0.02	0.59 ± 0.05
Thymus weight (g)	0.46 ± 0.02	0.58 ± 0.03	0.27 ± 0.01	0.44 ± 0.03
White adipose tissue depots				
Mesenteric fat (g)	3.93 ± 0.38	7.95 ± 0.47 *	2.27 ± 0.18	3.78 ± 0.49
Epididymal fat (g)	3.53 ± 0.16	11.32 ± 1.56 *	3.22 ± 0.07	6.98 ± 0.57 *
Perirenal fat (g)	4.98 ± 0.37	11.37 ± 1.01 *	4.29 ± 0.31	8.96 ± 0.41 *
Adiposity index (%)	3.05 ± 0.13	7.42 ± 0.64 *	3.40 ± 0.13	5.99 ± 0.25 *

Energy metabolism response to cafeteria diet is genotype dependent in the rat

To investigate the effects of cafeteria diet-induced overfeeding on the postprandial energy metabolism response, we determined the energy expenditure, metabolic rate and fuel oxidation in LEW and WKY rats.

An increase in energy expenditure was observed during the earliest times of postprandial phase independent of the genetic background and diet; this increase was greater and more sustained for LEW (STD and CAF) rats (Figure 2). Although the energy expenditure of CAF-fed LEW rats was higher than that of the corresponding STD-fed rats at some specific time points, no significant differences were observed for the postprandial energy expenditure, determined as the area under the curve (AUC) (Figure 2A). In contrast, for WKY rats, the CAF diet induced a shorter but greater increase in energy expenditure compared to the STD diet, and this expenditure remained high during most of the postprandial state. This resulted in a significant CAF-induced increase in the energy expenditure of WKY rats, measured as the AUC during the postprandial state (Figure 2B).

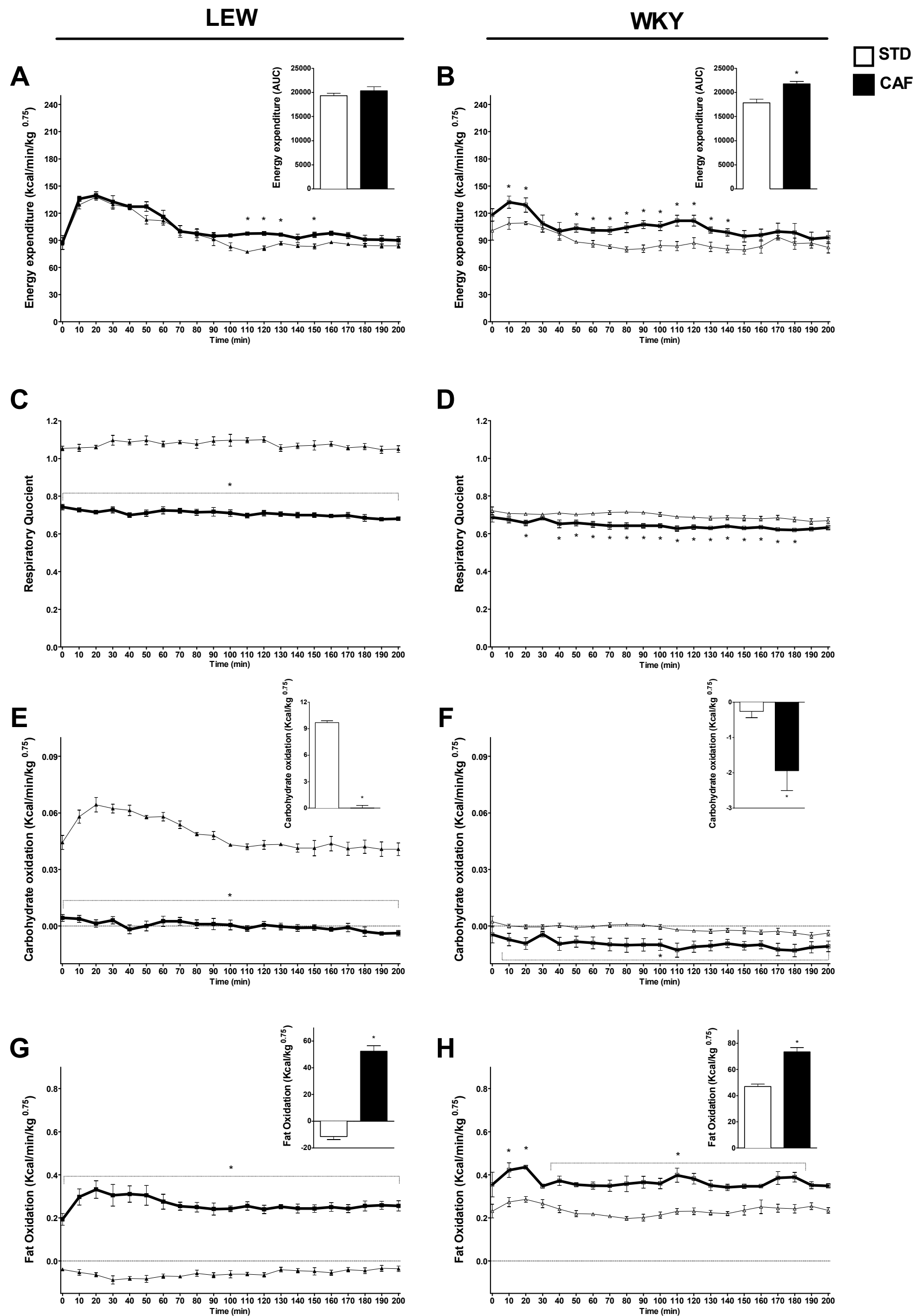


Figure 2. Postprandial metabolic responsiveness to diet. Energy expenditure, RQ and substrate oxidation were determined based on gaseous exchange measured by indirect calorimetry during the first 200 min of the postprandial period in STD- and CAF-fed LEW and WKY rats. (A and B) The energy expenditure for LEW and WKY rats; the inset graphs correspond to the area under the curve (AUC) measured during the 200 min recorded. (C and D) The respiratory quotients of LEW and WKY rats, respectively, calculated using the quotient VCO_2/VO_2. (E and F) Carbohydrate oxidation in response to the diet challenge (STD or CAF diets) for LEW and WKY rats. (G and H) Fat oxidation during the postprandial state after the consumption of the STD or CAF diet in LEW and WKY rats. *P<0.05 vs. STD diet-fed rats within each strain (diet effect).

The metabolic rate measured as the respiratory quotient (RQ) showed similarly a difference in the energy metabolism pattern between LEW and WKY rats. Although STD-fed LEW rats maintained a RQ around of 1.0-1.1, which can be associated primarily with carbohydrate oxidation and endogenous fatty acid synthesis (Figure 2C) (as expected from the predominance of carbohydrates as the main substrate source (Figure 2E) instead of the oxidation of fat (Figure 2G)). However the RQ determined in STD-fed WKY rats was lower when comparing with STD-fed LEW rats and significantly greater for most measured times than in CAF-fed WKY rats (Figure 2D). The RQ of STD-fed WKY rats suggested that fat is the principal fuel for energy production (Figure 2H) instead of carbohydrates (Figure 2F), indicating that the energy metabolism pattern of STD-fed WKY rats differed with respect to that observed in STD-fed LEW rats. The CAF-fed LEW and CAF-fed WKY rats showed a RQ of approximately 0.70 (Figure 2C and D), suggesting that CAF-diet promotes fat oxidation rather than carbohydrate oxidation (Figure 2E and F) as the most important fuel energetic source (Figure 2G and H).

The profile of diet-induced circulating metabolites is genotype-dependent in the rat

The phenotypic response to the cafeteria diet in LEW and WKY rats was assessed by determining the plasma levels of several metabolites previously described as biomarkers of metabolic homeostasis (see Methods).

Genotype-dependent differences were observed for the modulation of plasma levels of obesity-related biomarkers in the rat. Notably, although CAF induced a significant increase in the plasma levels of glucose, triglycerides, NEFAs, β-hydroxybutyrate and MCP-1 in LEW rats, no similarly strong induction was observed on the plasma levels of these biomarkers in WKY rats (Figure 3A-C and 3F). Furthermore, LEW rats showed higher circulating levels of NEFAs with both analysed diets, whereas the glucose and triglyceride levels were only significantly higher when comparing CAF-fed LEW and WKY rats. Moreover, the β-hydroxybutyrate and the orosomucoid plasma levels of STD-fed WKY rats were higher than those of CAF-fed WKY rats and were also significantly higher than those of STD-fed LEW rats.

The plasma leptin concentration was enhanced by the cafeteria diet, and although the diet-induced hyperleptinaemia was independent of the genetic background, the leptin concentrations were, for the different proposed diets, significantly higher for LEW rats than for WKY rats (Figure 3E).

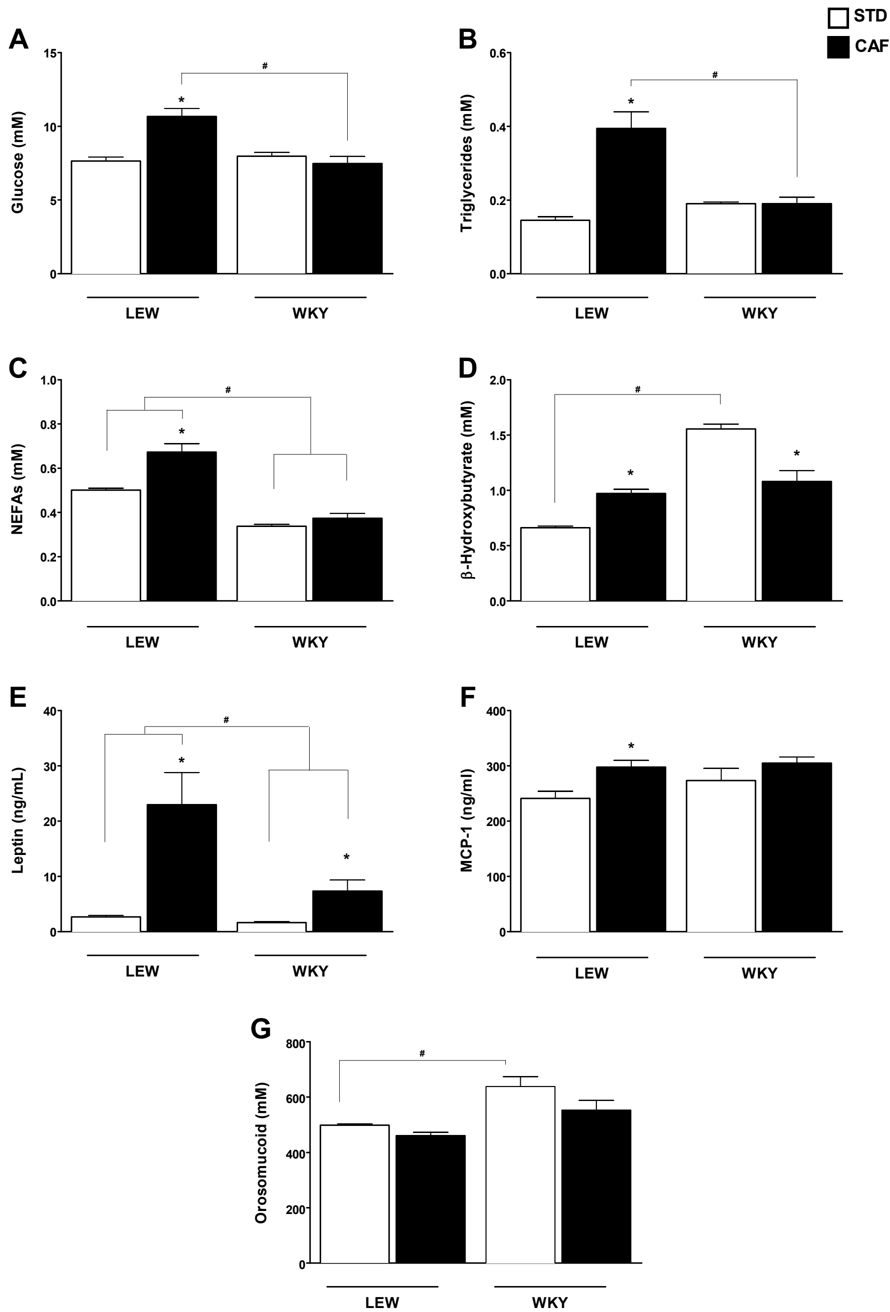


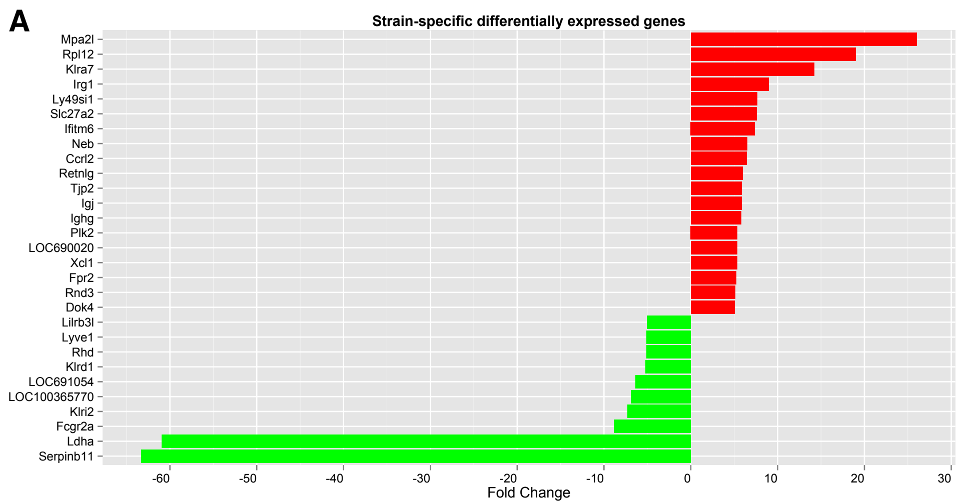
Figure 3. Circulating levels of the biomarkers involved in dietary responsiveness. The plasma concentration of glucose (A), triglycerides (B), NEFAs (C), β-Hydroxybutyrate (D), leptin (E), MCP-1 (F) and orosomucoid (G) were measured in STD- and CAF-fed LEW and WKY rats. The data represent the mean ± SEM. *P<0.05 between the rats fed with the STD diet and the CAF diet within each strain (the diet effect), #P<0.05 between the rats from the two strains fed with the same diet (STD or CAF; the strain effect).

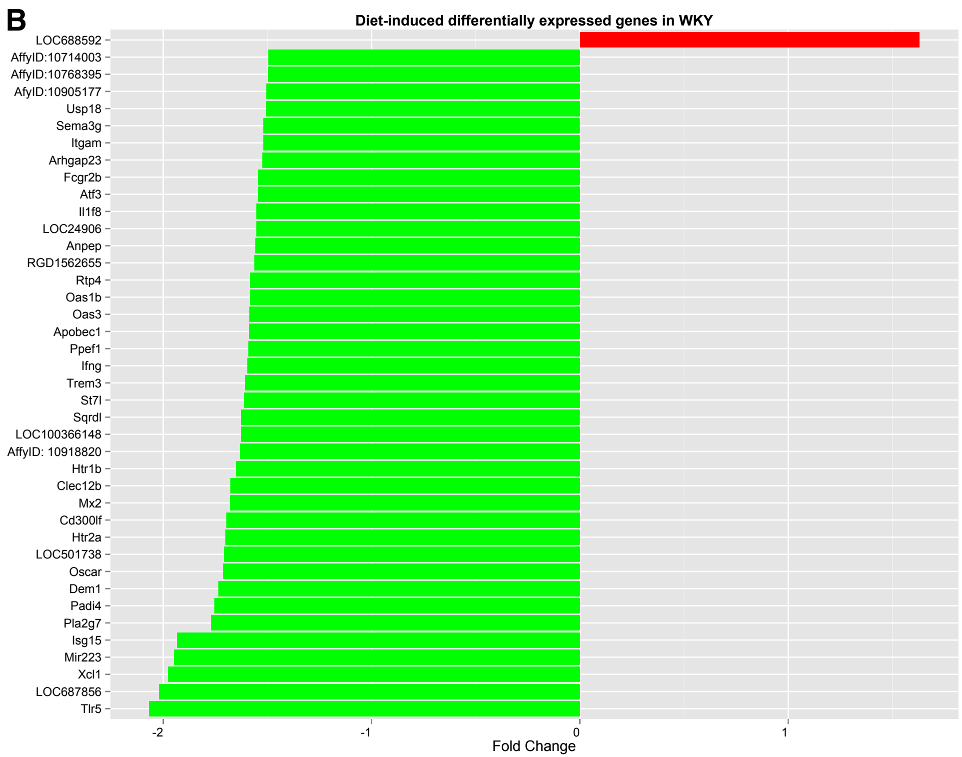
The effect of diet on monocytes gene expression is genotype dependent in the rat

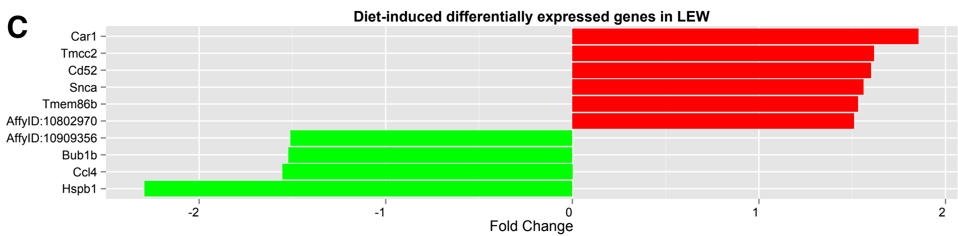
Once we determined that LEW and WKY responded phenotypically differently to diet-induced obesity challenges, we set out to investigate genome-wide the transcriptional response to diet in these strains. To explore the genes and pathways that underlie the metabolic-immunological crosstalk of the differential phenotypic response we carried out gene expression profiling of circulating monocytes from STD- and CAF-fed LEW and WKY rats. Microarray data were analysed in two ways. First, we compared the monocyte expression profiles of CAF-fed WKY and LEW rats in order to identify the genes that underlie the strain-dependent adaptation to diet-induced obesity. Second, we analysed the strain-specific modulation of the transcriptome of circulating monocytes by the CAF diet.

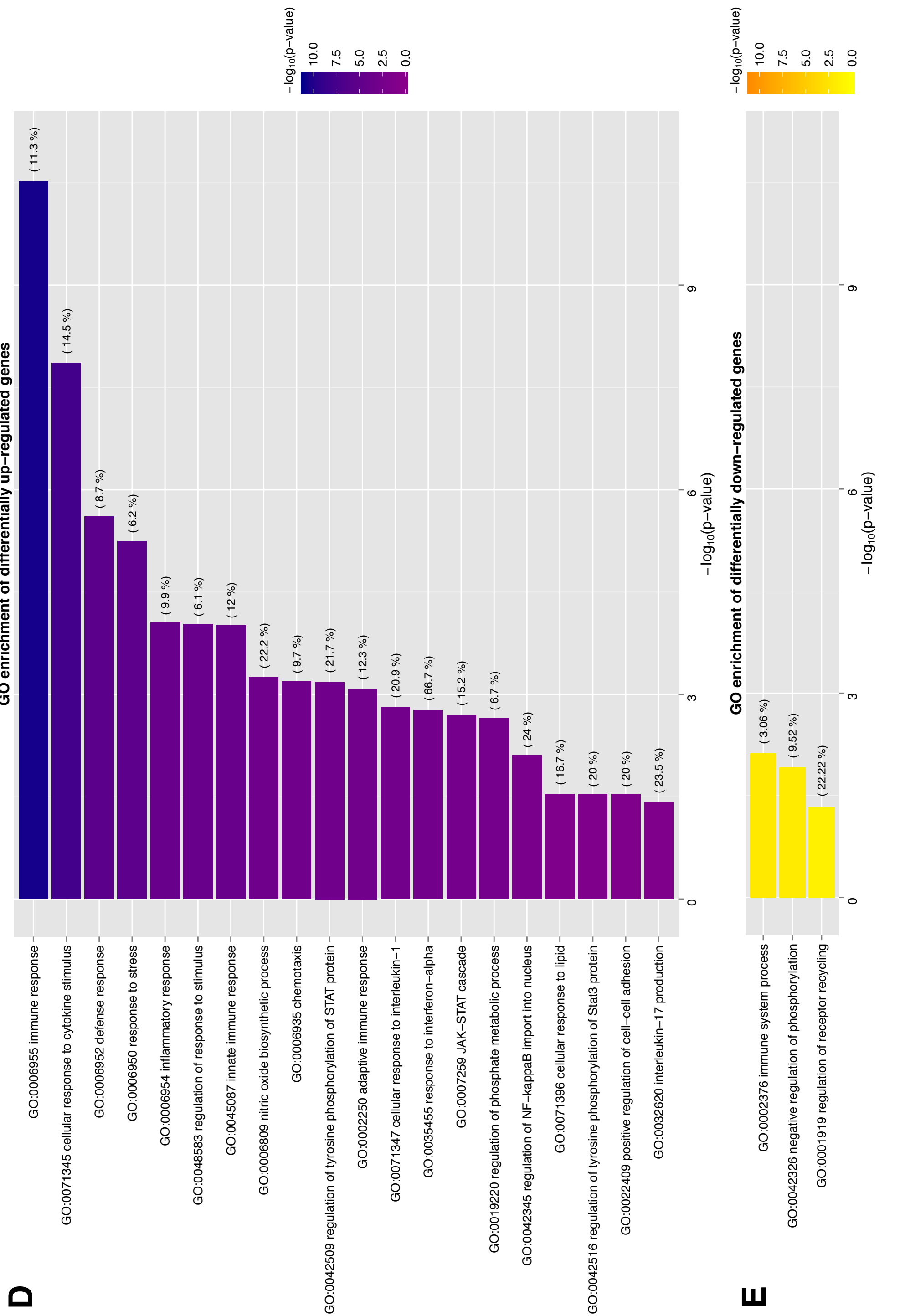
The expression profiles of circulating monocytes from WKY and LEW CAF-fed rats were first compared in order to determine the genetic basis of the strain-dependent response to obesity. This analysis identified 8,479 transcripts that were nominally differentially expressed ($p<0.01$) between the circulating monocytes from CAF-fed WKY and LEW rats; 75.5% of these transcripts were more highly expressed in WKY rats than in LEW rats (Supplementary Table S2). We prioritized a set of 29 transcripts that were significantly differentially expressed after correction for multiple testing (FDR <5%) and with fold change (FC) > 5 when comparing the expression profiles from WKY and LEW rats (Figure 4A). This subset of highly differentially expressed transcripts included macrophage activation 2 like (*Mpa2l*), a target gene of nuclear factor-κB (NF-κB) [46], that was 26-fold up-regulated in WKY and lactate dehydrogenase A (*Ldha)*, the gene encoding for the enzyme that catalyses the conversion of lactate to pyruvate during the glycolysis under anaerobic conditions in LEW rats (down-regulated in WKY with a fold change of -60.95). Furthermore, 6 of the 29 transcripts with high differential expression between WKY and LEW (*Klra7*, *Irg1*, *Ly49si1*, *Plk2*, *Rnd3*, *Lilrb3l*) were also identified previously as differentially expressed transcripts comparing the untreated bone marrow-derived macrophages of WKY and LEW rats [35].

Figure 4. Gene expression profile of circulating monocytes from STD- and CAF-fed LEW and WKY rats. (A) Strain-specific differentially expressed genes. Transcripts with more than 5-fold differences between CAF-fed WKY and LEW rats are shown; the red bars correspond to the transcripts that are overexpressed in WKY rats, and the green bars indicate the transcripts that are overexpressed in LEW rats. The full list of the differentially expressed genes is included in Supplementary Table S3. (B) Effect of the CAF diet on the gene expression profile of circulating monocytes from WKY rats. The full list of differentially expressed genes is given in Supplementary Table S8. (C) The effect of the CAF diet on the gene expression profile of circulating monocytes from LEW rats. The full list of differentially expressed genes is given in Supplementary Table S7. (D and E) A summary of the over-represented Gene Ontology Categories based on a Gene Set Enrichment analysis of the differentially expressed genes between CAF-fed WKY and LEW rats. The complete list of significantly over-represented terms is given in Supplementary Table S4.


A
Strain-specific differentially expressed genes
Mpa2l
Rpl12
Klra7
Irg1
Ly49si1
Slc27a2
Ifitm6
Neb
Ccrl2
Retnlg
Tjp2
Igj
Ighg
Plk2
LOC690020
Xcl1
Fpr2
Rnd3
Dok4
Lilrb3l
Lyve1
Rhd
Klrd1
LOC691054
LOC100365770
Klri2
Fcgr2a
Ldha
Serpinb11
-60
-50
-40
-30
-20
-10
0
10
20
30
Fold Change


B
Diet-induced differentially expressed genes in WKY
LOC688592
AffyID:10714003
AffyID:10768395
AfyID:10905177
Usp18
Sema3g
Itgam
Arhgap23
Fcgr2b
Atf3
Il1f8
LOC24906
Anpep
RGD1562655
Rtp4
Oas1b
Oas3
Apobec1
Ppef1
Ifng
Trem3
St7l
Sqrdl
LOC100366148
AffyID: 10918820
Htr1b
Clec12b
Mx2
Cd300lf
Htr2a
LOC501738
Oscar
Dem1
Padi4
Pla2g7
Isg15
Mir223
Xcl1
LOC687856
Tlr5
-2
-1
0
1
Fold Change

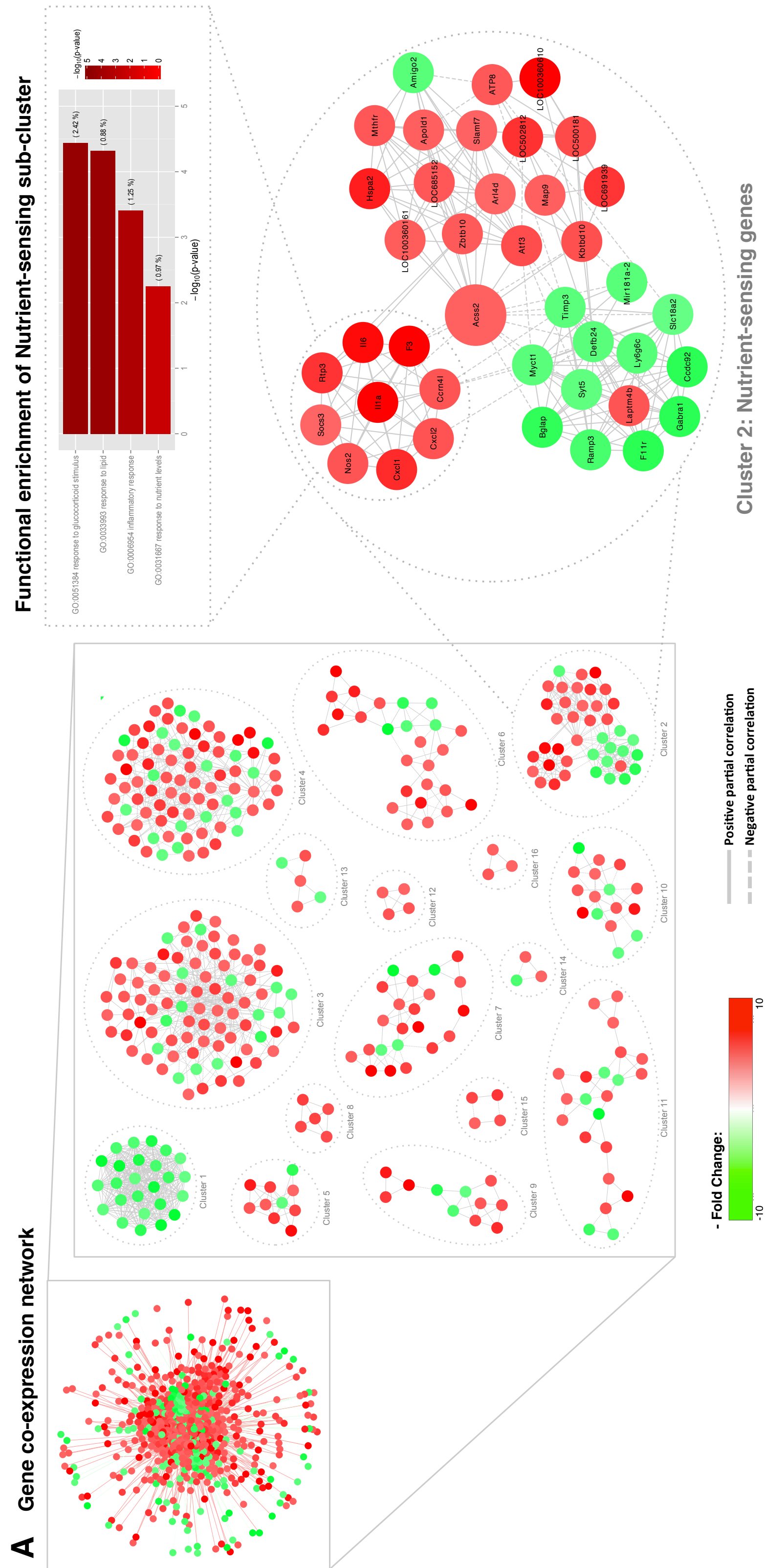

C
Diet-induced differentially expressed genes in LEW
Car1
Tmcc2
Cd52
Snca
Tmem86b
AffyID:10802970
AffyID:10909356
Bub1b
Ccl4
Hspb1
-2
-1
0
1
2
Fold Change

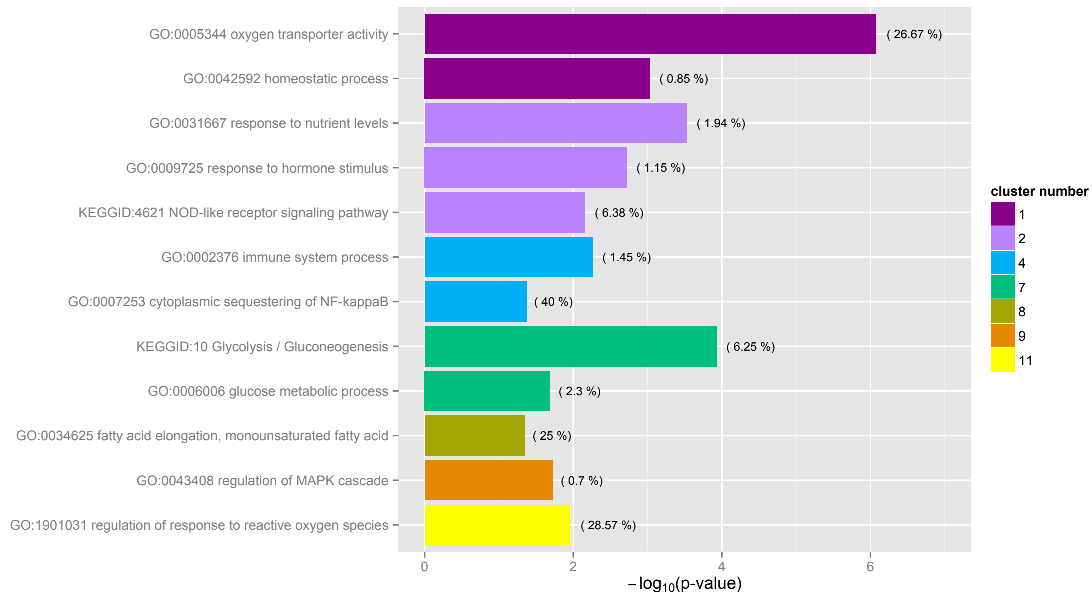

D
GO enrichment of differentially up−regulated genes
GO:0006955 immune response
GO:0071345 cellular response to cytokine stimulus
GO:0006952 defense response
GO:0006950 response to stress
GO:0006954 inflammatory response
GO:0048583 regulation of response to stimulus
GO:0045087 innate immune response
GO:0006809 nitric oxide biosynthetic process
GO:0006935 chemotaxis
GO:0042509 regulation of tyrosine phosphorylation of STAT protein
GO:0002250 adaptive immune response
GO:0071347 cellular response to interleukin−1
GO:0035455 response to interferon−alpha
GO:0007259 JAK−STAT cascade
GO:0019220 regulation of phosphate metabolic process
GO:0042345 regulation of NF−kappaB import into nucleus
GO:0071396 cellular response to lipid
GO:0042516 regulation of tyrosine phosphorylation of Stat3 protein
GO:0022409 positive regulation of cell−cell adhesion
GO:0032620 interleukin−17 production
(11.3 %)
(14.5 %)
(8.7 %)
(6.2 %)
(9.9 %)
(6.1 %)
(12 %)
(22.2 %)
(9.7 %)
(21.7 %)
(12.3 %)
(20.9 %)
(66.7 %)
(15.2 %)
(6.7 %)
(24 %)
(16.7 %)
(20 %)
(20 %)
(23.5 %)
0
3
6
9
−log10(p−value)
− log10(p−value)
10.0
7.5
5.0
2.5
0.0
E
GO enrichment of differentially down−regulated genes
GO:0002376 immune system process
GO:0042326 negative regulation of phosphorylation
GO:0001919 regulation of receptor recycling
(3.06 %)
(9.52 %)
(22.22 %)
0
3
6
9
−log10(p−value)
− log10(p−value)
10.0
7.5
5.0
2.5
0.0

To gain insights into the molecular processes and pathways underlying these transcriptional responses, we analysed a larger set of 954 transcripts that were more than 1.5 up- or down-regulated in CAF-fed WKY rats compared with CAF-fed LEW rats for enrichment of gene ontology (GO) terms. The GO terms were manually curated to remove redundant categories resulting from overlapping gene sets (Supplementary Table S3), and the most relevant terms that were significantly over-represented are shown in Figure 4D and E. The overexpressed genes in CAF-fed WKY rats were enriched for terms related to the activation of the inflammatory and immune responses resulting from the up-regulation of classical inflammatory mediators such as interferon gamma (*Ifng*, FC = 2.43), tumour necrosis factor (*Tnf*, FC = 2.11), nitric oxide synthase 2 (*Nos2*, FC = 1.68), interleukins (IL) including *IL1a* (FC = 2.66), *IL6* (FC = 2.41), *IL12a* (FC = 2.34), *IL18* (FC = 1.96) and *IL23a* (FC = 3.45) as well as genes involved in the regulation of eicosanoid production such as the gene encoding for cyclooxygenase 2 (prostaglandin-endoperoxide synthase 2 or *Ptgs2*, FC = 2.71) and the gene encoding the 5-lipoxygenase (arachidonate 5-lipoxygenase or *Alox*, FC = 2.70). Moreover, the overexpressed genes were also enriched for terms related to the positive regulation of the JAK-STAT cascade, specifically via the promotion of the tyrosine phosphorylation of STAT3. Nevertheless, the genes that were under-expressed in CAF-fed WKY rats compared to CAF-fed LEW rats were significantly enriched for very few gene ontologies, highlighting the functional gene enrichment in the immune system.

We then investigated gene regulatory networks using the gene expression profiles assessed in circulating monocytes (see Methods). In order to identify individual genes and the gene clusters whose co-regulation underlie the strain-specific responses to diet, gene co-expression networks were inferred based on the differentially expressed transcripts that showed a fold change greater than 1.5, independent of the direction of modulation between the circulating monocytes from CAF-fed WKY and LEW rats (Figure 5A).

Figure 5. The gene co-expression network of the genes that are differentially expressed in CAF-fed WKY and LEW rats. (A) A simplified representation of the network and the MCODE-based clusters; detailed information on the genes within each cluster is shown in Supplementary Figure S1. Cluster number 2, significantly enriched as nutrient sensor, is highlighted, and the significantly over-represented Gene Ontology categories from the selected sub-cluster are shown. The edge shape is related to the direction of the partial correlation; the continuous lines represent partial positive correlations, and dotted lines refer to partial negative correlation. The node colour represents the fold change between the expression of the corresponding transcript in CAF-fed WKY and LEW rats; red nodes correspond to genes that are overexpressed in WKY rats, and green nodes correspond to genes that are overexpressed in LEW rats. The highlighted node within cluster 2 represents a high-scoring differentially expressed gene. (B) Functional enrichment analysis of the MCODE-based co-expressed gene clusters. Significantly over-represented GO terms and KEEG pathways within each cluster are represented.


A
Gene co-expression network
Functional enrichment of Nutrient-sensing sub-cluster
GO:0051384 response to glucocorticoid stimulus
GO:0033993 response to lipid
GO:0006954 inflammatory response
GO:0031667 response to nutrient levels
(2.42 %)
(0.88 %)
(1.25 %)
(0.97 %)
−log10(p-value)
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6
Cluster 7
Cluster 8
Cluster 9
Cluster 10
Cluster 11
Cluster 12
Cluster 13
Cluster 14
Cluster 15
Cluster 16
Cluster 2: Nutrient-sensing genes
Amigo2
ATP8
LOC100360810
Mthfr
Apold1
Slamf7
LOC502812
LOC500181
Hspa2
LOC685152
Arl4d
Map9
LOC691930
LOC100360161
Zbtb10
Atf3
Kbtbd10
Mir181a-2
Acss2
Timp3
Slc18a2
Il6
F3
Rtp3
Il1a
Ccrn4l
Socs3
Cxcl2
Nos2
Cxcl1
Myct1
Defb24
Ly6g6c
Ccdc92
Syt5
Laptm4b
Gabra1
Bglap
Ramp3
F11r
- Fold Change:
-10
10
Positive partial correlation
Negative partial correlation



The resulting network is composed of a single connected component formed by 742 protein-coding genes linked by 8,215 edges. We then used the molecular complex detection (MCODE) cluster algorithm [47] to extract functional modules of densely interconnected genes. The biological relevance of the genes included in each of the 16 MCODE-derived clusters (the clustered genes are reported in Supplementary Figure S1) was tested with GO and the KEGG [48] analysis of the functional significance of biochemical pathways (Figure 5B). Although not all of the modules were enriched significantly for GO terms or KEGG pathways, the genes in cluster 2 (representing 40 annotated protein-coding genes), were functionally enriched for nutrient sensors (P-value = 2.94×10^{-4}), responders to hormone stimuli (P-value = 1.90×10^{-3}), and components of the NOD-like receptor signalling pathway (P-value = 6.84×10^{-3}). Topology analysis of cluster 2 identified the Acyl-CoA synthetase short-chain family member 2 (*Acss2*) as the most important *hub* within the cluster, that is the gene with the largest degree and the highest betweenness score within the cluster. The functional relevance of *Acss2* as regulator of nutrient sensing was initially investigated by analysing the overlap of Quantitative Trait Loci (QTLs) within the region encoding for the *Acss2* gene using previous QTL mapping results from the Rat Genome Database [49]. This analysis revealed that within the 29 overlapping QTLs, 8 are related to traits associated with the regulation of the metabolism, including a body weight QTL (Bw94), a glucose level QTL (Gluco39) and a serum leptin concentration QTL (Slep7) (Supplementary Table S4). Furthermore, we determined the association between the mRNA expression of *Acss2* in peripheral monocytes and metabolism-related traits (Supplementary Table S5), and found significant negative correlations between the expression of *Acss2* and the plasma levels of glucose (r = -0.71), triglycerides (r = -0.68), leptin (r = -0.61) and NEFAs (r = -0.61) and with the total fat (r = -0.59) and body weight (r = -0.59). Furthermore, a topological analysis inside

this nutrient sensing gene cluster revealed a functional sub-module consisting of 9 highly co-expressed genes; all of these nodes were overexpressed in CAF-fed WKY rats compared to the LEW-fed rats and significantly enriched for inflammatory response genes and genes that function in response to lipid and glucocorticoid stimuli.

We also determined the effects of diet on the monocyte gene expression profile from LEW and WKY rats (Figure 4C and 4B, respectively). We determined that the phenotypic response of LEW rats to the CAF diet resulted in moderate changes in monocyte gene expression, and although 228 transcripts were differentially up-regulated and 195 transcripts were significantly down-regulated (FDR<5%), the magnitude of gene expression modulation by the diet was moderate (Supplementary Table S6). Gene ontology and KEGG analysis of these differentially expressed transcripts did not show significant enrichment for obvious obesity-related pathways. In contrast, the modulation of the circulating monocyte expression profile by CAF diet in the WKY rats resulted in the diet-induced up-regulation of 483 transcripts and the down-regulation of 449 transcripts (Supplementary Table S7). Amongst the CAF diet-induced differentially expressed genes in WKY rats we identified the down-regulated *Mir223*. miR-223, the mature form of *Mir223,* directly targets STAT3 to regulate its activation [50]. Functional enrichment analyses showed that the up-regulated transcripts were significantly enriched for the Wnt signalling pathway among other non-obesity-related pathways, while the genes that were down-regulated by the CAF diet were functionally enriched for the peroxisome proliferator-activated receptor (PPAR) signalling pathway.

WKY, unlike LEW rats, are not susceptible to diet-induced leptin resistance

The results obtained regarding the phenotypic and transcriptional response to diet-induced obesity between LEW and WKY rats pointed to the diet-induced modulation of leptin and its signalling pathway as a possible divergent point between the two strains. To analyse the role of leptin in the differential phenotypic response to the cafeteria diet in LEW and WKY rats, the relationship between circulating leptin levels and metabolic-related traits was determined by means of correlation analysis (using non-parametric Spearman Rank Correlation coefficient).

In LEW rats, positive significant correlations were observed between plasma leptin levels and the main morphometric parameters related to the induction of leptin secretion, such as energy intake, body weight and the adiposity index. Furthermore, a strong correlation was observed between the plasma leptin levels and triglycerides (Supplementary Table S8).

Significant relationships between circulating leptin levels and the measured energy intake levels, body weight, adiposity and the expression of *Lep* in the mesenteric fat pad were also observed in WKY rats. However, non-significant correlations were observed between leptin levels and the plasma concentrations of triglycerides and glucose, respectively (Supplementary Table S8), suggesting strain-dependent relationships with secreted leptin in the rat.

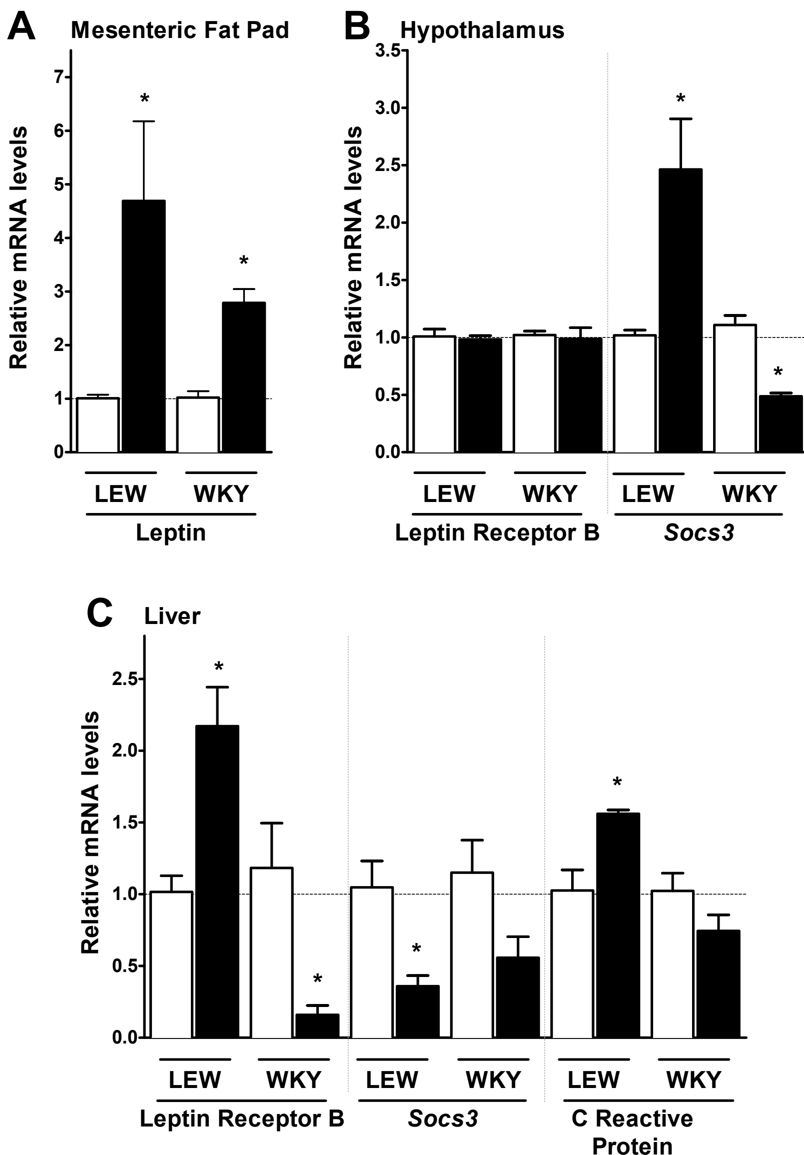
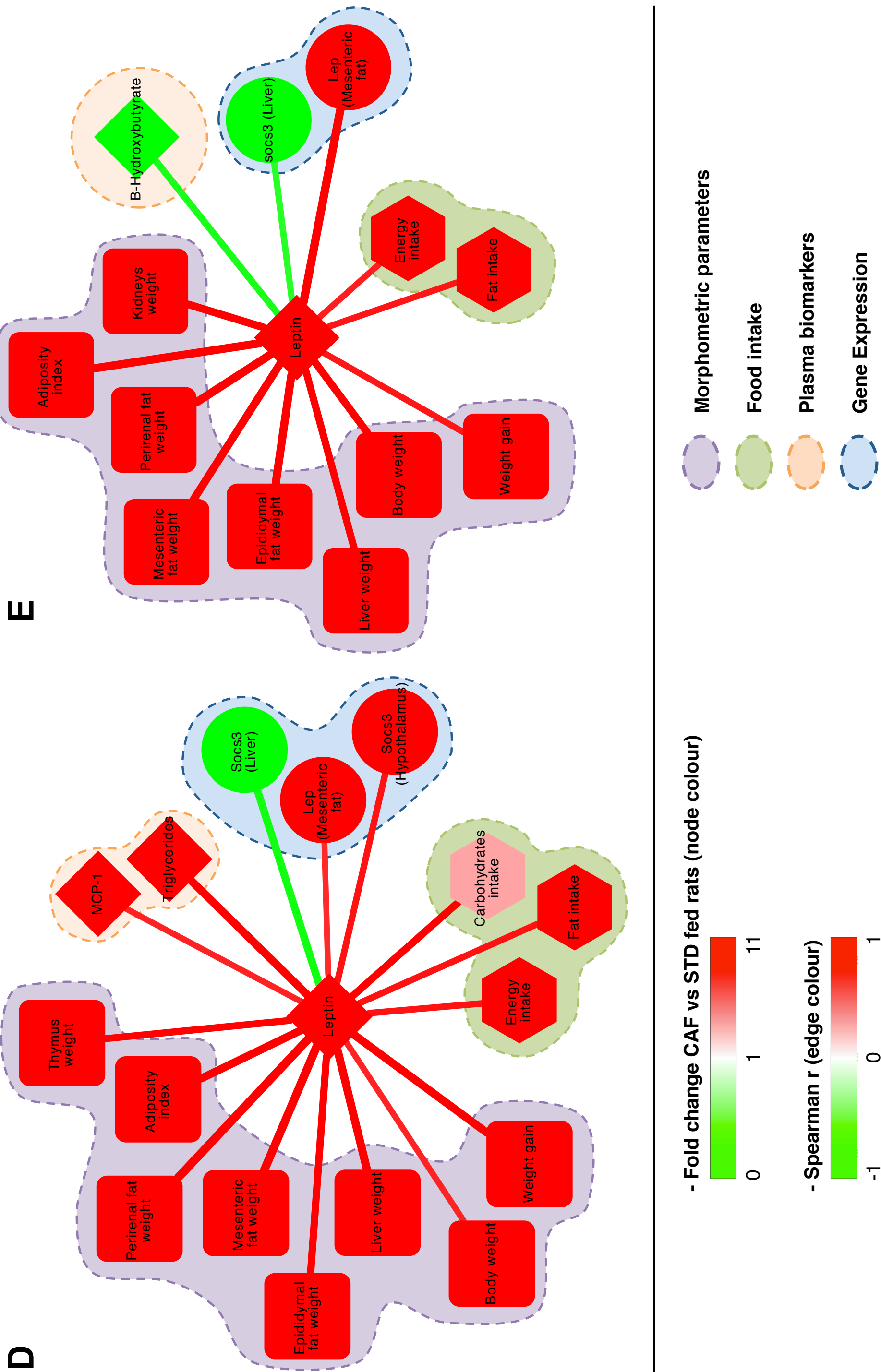


Figure 6. The expression of key genes involved in the regulation of leptin signalling. Gene expression was measured by RT-PCR. (A) The effect of the CAF diet on leptin gene expression in mesenteric fat pads from LEW and WKY rats. (B) The effect of diet on the expression of leptin receptor B, *Socs3* and C-reactive protein in the liver. (C) The effect of diet on the hypothalamic regulation of leptin receptor B and *Socs3* gene expression in LEW and WKY rats. *P<0.05 between the rats fed with the STD diet and the CAF diet within each strain (diet effect). (D and E) Association between leptin and biomarkers related with the development and progression of obesity. The network of significant non-parametric Spearman correlations (P<0.05) between the circulating leptin levels and morphometric parameters, food intake-related values, circulating metabolites and the expression of the genes involved in leptin signalling in LEW and WKY rats, respectively. The node colour represents the fold change between the levels of the corresponding parameter in CAF and STD-fed LEW and WKY rats; red nodes correspond to parameters that are up-regulated by CAF diet. The edge colour represents the significant Spearman correlation relationship.


D
Leptin
Thymus weight
Adiposity index
Perirenal fat weight
Mesenteric fat weight
Epididymal fat weight
Liver weight
Body weight
Weight gain
MCP-1
Triglycerides
Socs3 (Liver)
Lep (Mesenteric fat)
Socs3 (Hypothalamus)
Carbohydrates intake
Fat intake
Energy intake
E
Leptin
Adiposity index
Kidneys weight
Perirenal fat weight
Mesenteric fat weight
Epididymal fat weight
Liver weight
Body weight
Weight gain
B-Hydroxybutyrate
socs3 (Liver)
Lep (Mesenteric fat)
Energy intake
Fat intake
- Fold change CAF vs STD fed rats (node colour)
0
1
11
- Spearman r (edge colour)
-1
0
1
Morphometric parameters
Food intake
Plasma biomarkers
Gene Expression

The effects of genotype on the central and peripheral regulation of diet-induced leptin signalling

To assess whether the differential phenotypic response to diet-induced obesity in LEW and WKY rats was a consequence of a differential regulation of the leptin signalling pathway genes, the expression of key genes involved in the regulation of leptin signal transduction in metabolically active organs, including the mesenteric fat pad, the hypothalamus and the liver were measured.

While rats of both genotypes react to cafeteria diet by inducing the expression of leptin (*Lep*) in the mesenteric fat pad (Figure 6A), we found different expression patterns of the genes involved in the transduction of leptin signalling in the hypothalamus (Figure 6B) and liver (Figure 6C). While no diet-induced effects were observed for the gene expression of leptin receptor B (*LepRb*), the modulation of *Socs3* gene expression was inversely modulated by the cafeteria diet in LEW and WKY rats (Figure 6B). Therefore, the CAF diet promoted an increase in *Socs3* gene expression in LEW rats and a strong inhibition of *Socs3* gene expression in WKY rats.

Furthermore, the diet-induced modulation of leptin receptor B gene expression in the liver was also opposite for LEW and WKY rats; in LEW rats, the gene expression of *LepRb* was up-regulated, and in WKY rats, it was not (Figure 6C). Nonetheless, although *Socs3* gene expression in the liver was down-regulated by the CAF diet in both strains, this inhibition was only significant in LEW rats. The CAF-mediated modulation of *Crp* gene expression in the liver was also determined as a biomarker of diet-induced inflammation. The gene expression of *Crp* was only significantly up regulated by the CAF diet in LEW rats.

Finally, when comparing the significant relationships between plasma leptin levels and the measured metabolic traits, different diet-specific and genotype-specific effects were observed (Figure 6D and 6E). Strong relationships were observed between circulating leptin levels and the measured levels of energy and fat intake, final body weight, weight gain, the expression of *Lep* in mesenteric fat and the expression of *Socs3* in liver for both genetic backgrounds, suggesting that diet is the main modulator of these effects. However, the relatively high correlations observed between plasma leptin levels and thymus weight, carbohydrate intake, the expression of *Socs3* in the hypothalamus and the circulating levels of triglycerides and MCP-1 in LEW rats and the correlation between plasma leptin and β-hydroxybutyrate and kidney weight in WKY rats suggest strain-specific effects.

DISCUSSION

In the present study, we showed the differential genotypic and phenotypic response of two homogeneous genetic backgrounds, LEW and WKY rats, subjected to a metabolic challenge based on dietary energy density. We show that the CAF diet resulted in an increase in weight gain due to diet-induced hyperphagia and to the hedonic preference for fat intake, reflected primarily in the increased adiposity, independent of the strain.

Despite the fact that both genetic backgrounds showed hyperphagia and changes in body composition in response to the CAF diet challenge, the postprandial energy metabolism pattern and the peripheral substrate utilisation were different for both strains. The CAF diet-induced body weight increase of LEW rats was supported not only by the increase in the energy intake but also by the maintenance of the energy expenditure, as well as by the switch to fat instead of carbohydrates as the primarily oxidation source to obtain energy. This pattern of energy metabolism is consistent with the typical energetic response, which was previously observed in outbred rats in response to a CAF diet [51,52]. On the other hand, the lower metabolic rate observed in STD-fed WKY rats may be attributed to an ineffective use of carbohydrates as the major energy substrate, resulting instead in the β-oxidation of fatty acids as the principal fuel source. In fact, some degree of carbohydrate intolerance had been reported previously for lean WKY rats, although in that occasion rats were still not fully inbred [53,54]. Furthermore, the very high levels of circulating β-hydroxybutyrate, one of the main ketone bodies produced by the liver, determined in the plasma of STD-fed WKY rats supported this non-predominant oxidative glucose utilisation [55,56]. Moreover, the disparity observed between the circulating levels of ketone bodies in STD- and CAF-fed WKY rats may be an indication that the source of the dietary carbohydrates plays a key role in carbohydrate metabolism, as has been previously described for other rat strains [57]. Therefore, although no effect of diet on the amount of carbohydrate intake was observed, the complexity of the major carbohydrate form found in each type of diet was different. Thus, while the main carbohydrate found in the STD diet was starch, a polysaccharide, the most abundant carbohydrate found in the CAF diet was the sucrose, a disaccharide.

Plasma metabolic profiling also corroborated the disparity in the phenotypic response to CAF diet of both genetic backgrounds. Thus, while CAF diet challenge induced hyperglycaemia and hypertriglyceridaemia in LEW rats, no effect of diet was observed in WKY rats. Furthermore, although the expansion of the adipose tissue was observed as an increase in leptin circulating levels independent of the genetic background, the downstream response to this diet-induced hyperleptinaemia was strain-specific. Therefore, in LEW rats, the strong relationship between plasma leptin levels and the nutritional status-associated traits, including the food intake, adiposity and plasma triglycerides, may be associated with the metabolic phenotype resulting from central leptin resistance, a pivotal event underlying the development of obesity and its associated dysfunctions [58]. Nevertheless, although the secreted leptin levels showed close relationships with diet-induced traits involved in the upstream regulation of leptin signalling such as food intake and adiposity in WKY rats and the same pattern was observed in LEW rats, the metabolic traits that were influenced by the regulation of leptin signalling, such as triglycerides plasma levels, did not respond to a diet-induced increase in adiposity in the same way as leptin; in other words, in WKY rats, the diet-induced hyperleptinaemia was not reflected in an impairment of lipid metabolism. This finding can have important implications because of the role of leptin as an integral endocrine regulator of energy metabolism. Therefore, our data suggest continuous functional interactions between the leptin axis and other metabolic pathways, including the lipid

metabolism pathway. Leptin is involved, among other functions, in fat storage regulation and in the modulation of the glucose metabolism independent of its effects on energy balance, mediated by its underlying mechanisms as the regulator of fatty acid β-oxidation [59,60] or controller of peripheral tissue insulin sensitivity [61].

The diet-induced modulation of the inflammatory response was also strain-specific. Whereas the overfeeding-induced obesity promoted inflammation in LEW rats, as reflected by the increase in fat-derived MCP-1 circulating levels and the overexpression of *Crp* in liver (both believed to be robust biomarkers of diet-induced inflammation), the inflammatory response was not induced by diet in WKY rats. A physical interaction between CRP and leptin in plasma has been hypothesised; this interaction has been proposed to spatially impair the transmission of leptin across the blood-brain-barrier and to impede the activation of central leptin signalling [62,63].

The evidence about the influence of genetic components as determinants of the phenotypic response to diet [25,26] points to genetic components as the factors underlying the differential responsiveness to the dietary energy density between the LEW and WKY rats. The role of peripheral monocytes as professional stress-sensing cells that circulate throughout the body and the pivotal role of leptin in the regulation of energy metabolism and as an immunoregulator linking nutritional status to the immune response, suggest that monocytes and the regulation of their gene expression profiles are highly valuable for the determination of the host genetic factors influencing the plasticity of the response to diet-induced obesity. In this study, the genes and pathways that underlie the strain-specific responsiveness to the dietary energy density were determined by comparing the gene expression profiles of circulating monocytes from STD diet- and CAF diet-fed LEW and WKY rats using genome-wide microarrays. The differentially expressed genes underlying the strain-dependent response to the CAF diet were determined by comparing the expression profiles of monocytes from CAF-fed WKY and LEW rats. Notably, the genes that were significantly overexpressed in WKY rats were over-enriched for the activation of inflammatory and immune response GO categories. Indeed, genes encoding classical inflammatory mediators, including *Ifng*, *Tnf*, *Nos2*, several interleukins and regulators of eicosanoid production were significantly overexpressed in CAF-fed WKY rats. When the overexpressed genes in CAF-fed LEW rats were compared with those in WKY rats, the strong overexpression of *Ldha* stands out; *Ldha* may be involved in the differential energy metabolism patterns of both strains.

Moreover, a gene co-expression network analysis of the differentially expressed genes in monocytes identified a module of highly interconnected genes enriched not only for nutrient sensing genes but also for response to hormone stimuli and the NOD-like receptor-signalling pathway. *Acss2* was identified as the most highly connected hub gene and given its degree of interconnectivity modification of the expression of *Acss2* might have a large impact on the biological functions of the cluster [64]. *Acss2* encodes a protein member of the Acyl-CoA synthetase short-chain family, an enzyme that catalyses the ligation of the acetate derived from the β-oxidation of fatty acids to CoA to produce acetyl-CoA for oxidation through the

citric acid cycle (TCA); in this way, *Acss2* is an essential enzyme for energy expenditure under ketogenic conditions [65]. QTLs related to complex obesity-related traits such as body weight and glucose and leptin levels were found to overlap within *Acss2*. *Acss2* gene expression has been proposed [66] as an accurate diagnostic biomarker of glucose intolerance, being negatively correlated with glucose plasma levels. Additionally, *Acss2* gene expression in WBC has been linked with hepatic concentrations of TCA-involved metabolites, including citrate, malate, succinate and fumarate, whose production depends directly on the ACSS2 protein activity. Consistent with these results, here we found that, in addition to the overexpression of *Acss2* in CAF-fed WKY rats, which showed significantly lower glucose levels that CAF-fed LEW rats, there is a significant and strong correlation between *Acss2* gene expression in circulating monocytes and the corresponding glucose plasma levels. *Acss2* levels also significantly correlate with plasma leptin levels and the body weight, traits related to the overlapping QTLs, and with the plasma levels of triglycerides and NEFAs. Therefore, our results confirm that a low expression of *Acss2* in circulating monocytes is an indicator of impaired glucose metabolism. Furthermore, *Acss2* is involved in *de novo* lipogenesis, and in the regulation of the triglyceride storage capacity of adipose tissue and also plays a key role as a nutrient sensor. *Acss2* expression is regulated by sterol regulatory element-binding proteins (SREBPs), transcription factors, which are believed to be key regulators of nutritional homeostasis [67]. SREBPs, especially SREBP-1, which is abundantly expressed in monocytes, have also been described as a link between the lipid metabolism and the innate immune response through its direct regulation of core genes involved in the inflammasome activation [68], supporting the significant over-representation of the NOD-like receptor signalling pathway in the co-expressed genes within the *Acss2*-regulated nutrient sensing cluster. Thus, our gene expression and regulatory network results suggest that *Acss2*, a nutrient sensing protein and a diagnostic biomarker of glucose resistance, is a key regulator gene for the differential responsiveness to dietary energy density and a pivotal genetic link for the integration of metabolic and immune homeostasis.

We found that the phenotypic response of LEW rats to the CAF diet was translated to a moderate modification of the gene expression profile of the circulating monocytes from LEW rats, and any obvious obesity-related ontology or pathway was enriched. Otherwise, although the phenotypic response to the CAF diet in WKY rats was apparently non-existent, a substantial set of genes was differentially modulated by the diet. Between the CAF diet-induced differentially expressed genes highlights the down-regulation of *Mir223* expression. miR-223, the mature form of *Mir223,* is a microRNA that is highly expressed in monocytes and directly targets STAT3 to regulate its activation; the down-regulation of miR-223 expression, such as was induced by the CAF diet in monocytes from WKY rats, is associated with the activation of STAT3 [50]. This regulatory function of miR-223 may be an underlying mechanism and corroborates our results regarding the non-attenuation of JAK/STAT signalling pathway induced by high leptin levels observed in WKY rats.

Taken together, all the phenotypic and transcriptomics results with respect to the differential response of LEW and WKY rats to a diet-induced energy surplus indicate that the regulation of the leptin signalling axis might underlie the strain-specific response.

Our data confirm, in a genotype-independent manner, what has been previously hypothesized: an overfeeding-induced increase in adiposity promotes the adipocyte-mediated up-regulation of leptin transcription, increasing the circulating leptin levels [17]. Once it is secreted, plasma leptin can be sensed by the central nervous system, and more specifically by the hypothalamus, where leptin recognition by its receptor (LepRb) elicits the JAK2-mediated activation of STAT3, a transcription factor that is essential for leptin functionality. Remarkably, our analysis of leptin receptor signal transduction in the hypothalamus revealed important differences in the regulation of downstream leptin signalling between LEW and WKY rats. Although no diet-specific effects were observed regarding the amount of *LepRb* expressed in the hypothalamus, the modulation of hypothalamic *Socs3* transcription was dependent not only on the diet but also on the genetic background. This strain-specific regulation of *Socs3* expression by the CAF diet is highly relevant for the functionality of leptin signalling because *Socs3* acts as a negative-feedback regulator of leptin signal transduction by inhibiting JAK tyrosine kinase activity via its binding to the Src homology-2 (SH2) domain of the phosphorylated leptin receptor [19]. Thus, while the enhanced expression of *Socs3* in the hypothalamus of CAF-fed LEW rats confirms the attenuation of central leptin signalling, a hallmark feature of leptin resistance [18,19,69,70], the down-regulation of *Socs3* expression observed in CAF-fed WKY rats is associated with an increase in central leptin sensitivity. Therefore the hyperleptinaemia derived from the overfeeding-induced adipose tissue expansion in WKY rats was not translated to an attenuation of the leptin signalling, but the opposite. This finding can have potential health implications because the suppression of hypothalamic *Socs3* expression has been proposed as a potential therapy to confer resistance to diet-induced obesity [19] and may partially explain the non-responsiveness to the dietary energy density observed in CAF fed WKY rats.

Furthermore, the effect of diet on the peripheral regulation of leptin signalling was also strain-specific, and the impairment of central leptin signalling in CAF-fed LEW rats was also observed in the liver by the significant up-regulation of *LepRb* transcription and the down-regulation of *Socs3,* a phenomenon that is associated with an increase in liver insulin sensitivity and with the promotion of the lipogenesis-induced inflammatory response and the obesity [71]. Nonetheless, in CAF-fed WKY rats, *LepRb* gene expression was down-regulated by the diet, whereas *Socs3* expression was slightly but not significantly decreased; this peripheral inactivation of *LepRb* in CAF-fed animals compared with the STD-fed ones is consistent with the over-repression of SOCS3 in hypothalamus and is supported by previous results [70].

Our data proposed SOCS3 and its differential regulation as an important underlying event in the genotype-dependent responsiveness to the dietary energy density observed between LEW and WKY rats. In fact, the molecular mechanisms that underlie the SOCS3-mediated

attenuation of leptin intracellular signalling are based on the binding of SOCS3 to phosphorylated Tyr_{985} in the leptin receptor and the direct binding of SOCS3 to JAK2, blocking its docking domain and preventing its phosphorylation as well as the phosphorylation and then the activation of STAT3 [15,19,20]. We can therefore hypothesize that the maintenance of STAT3 activation through its phosphorylation consequence of SOCS3 or miR-223 down-regulation is an essential step for the phenotypic response to diet. Moreover, *LepRb* and its intracellular tyrosine residues may also play a pivotal role in the regulation of STAT3 activation under chronic leptin stimulation [69]. Indeed, single-gene mutations in the leptin receptor, which are also known as the fa-gene, have been reported to be involved in the pathophysiology of obesity. The obese phenotype expressed by the Zucker Fatty rats (fa/fa) results from a missense mutation in the extracellular domain of the rat leptin receptor diminishing therefore the number of leptin receptors [72,73]. Interestingly, another rat model of obesity, the Wistar Kyoto fatty (WKY/NDrt-fa), is a congenic strain of WKY rats that resulted from backcrossing the fa-gene from the Zucker rats into the WKY/N background [74]. In fact, the selection of WKY rats as the recipient strain of the Zucker fa-gene was justified due to the glucose metabolism observed in lean WKY rats characterized by glucose intolerance accompanied by exaggerated insulin secretion [53,54].

Although no strain-dependent differences were observed when comparing the biometric and energy intake response to the CAF diet in LEW and WKY rats, the metabolic management of the excess of energy were different in the two strains. Our results confirm that the phenotypic and genetic responses associated with obesity are more complex than a simple balance between energy intake and expenditure and suggest that the genetic background plays a prominent role.

In summary our integrated genetic, phenotypic and monocyte gene expression analyses in LEW and WKY rats subjected to metabolic challenge, show that the leptin axis, and specifically the LepRb-mediated regulation of STAT3 activation under conditions of chronic ligand stimulation, plays a pivotal role in the genotype-dependent adaptation to diet-induced obesity.

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The authors declare no competing financial interest.

REFERENCES

1 Eckel, R. H., Grundy, S. M. and Zimmet, P. Z. (2005) The metabolic syndrome. Lancet, Elsevier **365**, 1415–1428.

2 Miranda, P. J., DeFronzo, R. A., Califf, R. M. and Guyton, J. R. (2005) Metabolic syndrome: definition, pathophysiology, and mechanisms. Am. Heart J., St. Louis, CV Mosby Co. **149**, 33–45.

3 Iyer, A., Fairlie, D. P., Prins, J. B., Hammock, B. D. and Brown, L. (2010) Inflammatory lipid mediators in adipocyte function and obesity. Nat. Rev. Endocrinol., Nature Publishing Group **6**, 71–82.

4 Osborn, O. and Olefsky, J. M. (2012) The cellular and signaling networks linking the immune system and metabolism in disease. Nat. Med., Nature Publishing Group **18**, 363–374.

5 Lumeng, C. N. and Saltiel, A. R. (2011) Inflammatory links between obesity and metabolic disease. J. Clin. Invest., American Society for Clinical Investigation **121**, 2111.

6 Galic, S., Oakhill, J. S. and Steinberg, G. R. (2010) Adipose tissue as an endocrine organ. Mol. Cell. Endocrinol., Elsevier **316**, 129–139.

7 Kanneganti, T.-D. and Dixit, V. D. (2012) Immunological complications of obesity. Nat. Immunol., Nature Publishing Group **13**, 707–712.

8 Sun, K., Kusminski, C. M. and Scherer, P. E. (2011) Adipose tissue remodeling and obesity. J. Clin. Invest., American Society for Clinical Investigation **121**, 2094.

9 Fournier, T., Medjoubi-N, N. and Porquet, D. (2000) Alpha-1-acid glycoprotein. Biochim. Biophys. Acta **1482**, 157–71.

10 Alfadda, A. A., Fatma, S., Chishti, M. A., Al-Naami, M. Y., Elawad, R., Mendoza, C. D. O., Jo, H. and Lee, Y. S. (2012) Orosomucoid serum concentrations and fat depot-specific mRNA and protein expression in humans. Mol. Cells **33**, 35–41.

11 Hotamisligil, G. S. (2006) Inflammation and metabolic disorders. Nature, Nature Publishing Group **444**, 860–867.

12 Fröhlich, M., Imhof, A., Berg, G., Hutchinson, W. L., Pepys, M. B., Boeing, H., Muche, R., Brenner, H. and Koenig, W. (2000) Association between C-reactive protein and features of the metabolic syndrome: a population-based study. Diabetes Care, American Diabetes Association **23**, 1835–1839.

13 Myers, M. G., Cowley, M. A. and Münzberg, H. (2008) Mechanisms of leptin action and leptin resistance. Annu.Rev.Physiol., Annual Reviews **70**, 537–556.

14 La Cava, A. and Matarese, G. (2004) The weight of leptin in immunity. Nat. Rev. Immunol., Nature Publishing Group **4**, 371–379.

15 Bjørbæk, C., El-Haschimi, K., Frantz, J. D. and Flier, J. S. (1999) The role of SOCS-3 in leptin signaling and leptin resistance. J. Biol. Chem., ASBMB **274**, 30059–30065.

16 Frederich, R. C., Löllmann, B., Hamann, A., Napolitano-Rosen, A., Kahn, B. B., Lowell, B. B. and Flier, J. S. (1995) Expression of ob mRNA and its encoded protein in rodents. Impact of nutrition and obesity. J. Clin. Invest., American Society for Clinical Investigation **96**, 1658.

17 Frederich, R. C., Hamann, A., Anderson, S., Löllmann, B., Lowell, B. B. and Flier, J. S. (1995) Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. Nat. Med., Nature Publishing Group **1**, 1311–1314.

18 Howard, J. K., Cave, B. J., Oksanen, L. J., Tzameli, I., Bjørbæk, C. and Flier, J. S. (2004) Enhanced leptin sensitivity and attenuation of diet-induced obesity in mice with haploinsufficiency of Socs3. Nat. Med., Nature Publishing Group **10**, 734–738.

19 Mori, H., Hanada, R., Hanada, T., Aki, D., Mashima, R., Nishinakamura, H., Torisu, T., Chien, K. R., Yasukawa, H. and Yoshimura, A. (2004) Socs3 deficiency in the brain elevates leptin

sensitivity and confers resistance to diet-induced obesity. Nat. Med., Nature Publishing Group **10**, 739–743.

20 El-Haschimi, K., Pierroz, D. D., Hileman, S. M., Bjørbaek, C. and Flier, J. S. (2000) Two defects contribute to hypothalamic leptin resistance in mice with diet-induced obesity. J. Clin. Invest. **105**, 1827–32.

21 Parihar, A., Eubank, T. D. and Doseff, A. I. (2010) Monocytes and macrophages regulate immunity through dynamic networks of survival and cell death. J. Innate Immun., Karger Publishers **2**, 204–215.

22 Barsh, G. S., Farooqi, I. S. and O'Rahilly, S. (2000) Genetics of body-weight regulation. Nature, Nature Publishing Group **404**, 644–651.

23 Bouchard, C. (2008) Gene–environment interactions in the etiology of obesity: defining the fundamentals. Obesity, Wiley Online Library **16**, S5–S10.

24 Ordovas, J. M. (2008) Genotype–phenotype associations: modulation by diet and obesity. Obesity, Wiley Online Library **16**, S40–S46.

25 Pérusse, L. and Bouchard, C. (2000) Gene-diet interactions in obesity. Am. J. Clin. Nutr., American Society for Nutrition **72**, 1285s–1290s.

26 Speakman, J. R. (2004) Obesity: the integrated roles of environment and genetics. J. Nutr., Am Soc Nutrition **134**, 2090S–2105S.

27 Hetherington, M. M. and Cecil, J. E. (2009) Gene-environment interactions in obesity, Karger Publishers.

28 Koza, R. A., Nikonova, L., Hogan, J., Rim, J.-S., Mendoza, T., Faulk, C., Skaf, J. and Kozak, L. P. (2006) Changes in gene expression foreshadow diet-induced obesity in genetically identical mice. PLoS Genet., Public Library of Science **2**, e81.

29 Surwit, R. S., Feinglos, M. N., Rodin, J., Sutherland, A., Petro, A. E., Opara, E. C., Kuhn, C. M. and Rebuffe-Scrive, M. (1995) Differential effects of fat and sucrose on the development of obesity and diabetes in C57BL/6J and AJ mice. Metabolism, Elsevier **44**, 645–651.

30 Hu, C. C., Qing, K. and Chen, Y. (2004) Diet-Induced Changes in Stearoyl-CoA Desaturase 1 Expression in Obesity-Prone and-Resistant Mice. Obes. Res., Wiley Online Library **12**, 1264–1270.

31 Parks, B. W., Nam, E., Org, E., Kostem, E., Norheim, F., Hui, S. T., Pan, C., Civelek, M., Rau, C. D., Bennett, B. J., et al. (2013) Genetic control of obesity and gut microbiota composition in response to high-fat, high-sucrose diet in mice. Cell Metab. **17**, 141–52.

32 Sampey, B. P., Vanhoose, A. M., Winfield, H. M., Freemerman, A. J., Muehlbauer, M. J., Fueger, P. T., Newgard, C. B. and Makowski, L. (2011) Cafeteria Diet Is a Robust Model of Human Metabolic Syndrome With Liver and Adipose Inflammation: Comparison to High-Fat Diet. Obesity, Wiley Online Library **19**, 1109–1117.

33 Behmoaras, J., Bhangal, G., Smith, J., McDonald, K., Mutch, B., Lai, P. C., Domin, J., Game, L., Salama, A., Foxwell, B. M., et al. (2008) Jund is a determinant of macrophage activation and is associated with glomerulonephritis susceptibility. Nat. Genet. **40**, 553–9.

34 Behmoaras, J., Smith, J., D'Souza, Z., Bhangal, G., Chawanasuntoropoj, R., Tam, F. W. K., Pusey, C. D., Aitman, T. J. and Cook, H. T. (2010) Genetic loci modulate macrophage activity and glomerular damage in experimental glomerulonephritis. J. Am. Soc. Nephrol. **21**, 1136–44.

35 Maratou, K., Behmoaras, J., Fewings, C., Srivastava, P., D'Souza, Z., Smith, J., Game, L., Cook, T. and Aitman, T. (2011) Characterization of the macrophage transcriptome in glomerulonephritis-susceptible and -resistant rat strains. Genes Immun. **12**, 78–89.

36 D'Souza, Z., McAdoo, S. P., Smith, J., Pusey, C. D., Cook, H. T., Behmoaras, J. and Aitman, T. J. (2013) Experimental crescentic glomerulonephritis: a new bicongenic rat model. Dis. Model. Mech. **6**, 1477–86.

37 Ferrannini, E. (1988) The theoretical bases of indirect calorimetry: a review. Metabolism. **37**, 287–301.

38 Arch, J. R. S., Hislop, D., Wang, S. J. Y. and Speakman, J. R. (2006) Some mathematical and technical issues in the measurement and interpretation of open-circuit indirect calorimetry in small animals. Int. J. Obes. (Lond). **30**, 1322–31.

39 Irizarry, R. A., Hobbs, B., Collin, F., Beazer-Barclay, Y. D., Antonellis, K. J., Scherf, U. and Speed, T. P. (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics **4**, 249–64.

40 Falcon, S. and Gentleman, R. (2007) Using GOstats to test gene lists for GO term association. Bioinformatics **23**, 257–8.

41 Schäfer, J. and Strimmer, K. (2005) An empirical Bayes approach to inferring large-scale gene association networks. Bioinformatics **21**, 754–64.

42 Opgen-Rhein, R. and Strimmer, K. (2007) From correlation to causation networks: a simple approximate learning algorithm and its application to high-dimensional plant gene expression data. BMC Syst. Biol. **1**, 37.

43 Morris, J. H., Apeltsin, L., Newman, A. M., Baumbach, J., Wittkop, T., Su, G., Bader, G. D. and Ferrin, T. E. (2011) clusterMaker: a multi-algorithm clustering plugin for Cytoscape. BMC Bioinformatics **12**, 436.

44 Cline, M. S., Smoot, M., Cerami, E., Kuchinsky, A., Landys, N., Workman, C., Christmas, R., Avila-Campilo, I., Creech, M., Gross, B., et al. (2007) Integration of biological networks and gene expression data using Cytoscape. Nat. Protoc., Nature Publishing Group **2**, 2366–82.

45 Lin, C.-Y., Chin, C.-H., Wu, H.-H., Chen, S.-H., Ho, C.-W. and Ko, M.-T. (2008) Hubba: hub objects analyzer--a framework of interactome hubs identification for network biology. Nucleic Acids Res. **36**, W438–43.

46 Rothgiesser, K. M., Erener, S., Waibel, S., Lüscher, B. and Hottiger, M. O. (2010) SIRT2 regulates NF-κB dependent gene expression through deacetylation of p65 Lys310. J. Cell Sci. **123**, 4251–8.

47 Bader, G. D. and Hogue, C. W. V. (2003) An automated method for finding molecular complexes in large protein interaction networks. BMC Bioinformatics **4**, 2.

48 Kanehisa, M. and Goto, S. (2000) KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res. **28**, 27–30.

49 De la Cruz, N., Bromberg, S., Pasko, D., Shimoyama, M., Twigger, S., Chen, J., Chen, C.-F., Fan, C., Foote, C., Gopinath, G. R., et al. (2005) The Rat Genome Database (RGD): developments towards a phenome database. Nucleic Acids Res. **33**, D485–91.

50 Chen, Q., Wang, H., Liu, Y., Song, Y., Lai, L., Han, Q., Cao, X. and Wang, Q. (2012) Inducible microRNA-223 down-regulation promotes TLR-triggered IL-6 and IL-1β production in macrophages by targeting STAT3. PLoS One **7**, e42971.

51 Rothwell, N. J. and Stock, M. J. (1982) Energy expenditure of'cafeteria'-fed rats determined from measurements of energy balance and indirect calorimetry. J. Physiol., Physiological Soc **328**, 371–377.

52 Spiegelman, B. M. and Flier, J. S. (2001) Obesity and the regulation of energy balance. Cell, Cell Press **104**, 531–543.

53 Ikeda, H., Shino, A., Matsuo, T., Iwatsuka, H. and Suzuoki, Z. (1981) A new genetically obese-hyperglycemic rat (Wistar fatty). Diabetes **30**, 1045–50.

54 Kava, R., Peterson, R. G., West, D. B. and Greenwood, M. R. C. (1990) Wistar Diabetic Fatty Rat. ILAR J. **32**, 9–13.

55 Laffel, L. (1999) Ketone bodies: a review of physiology, pathophysiology and application of monitoring to diabetes. Diabetes. Metab. Res. Rev., Wiley Online Library **15**, 412–426.

56 McGarry, J. D. and Foster, D. W. (1980) Regulation of hepatic fatty acid oxidation and ketone body production. Annu. Rev. Biochem., Annual Reviews 4139 El Camino Way, PO Box 10139, Palo Alto, CA 94303-0139, USA **49**, 395–420.

57 Michaelis, O. E., Ellwood, K. C., Tulp, O. L. and Greenwood, M. R. C. (1986) Effect of feeding sucrose or starch diets on parameters of glucose tolerance in the LA/N-corpulent rat. Nutr. Res., pp 95–99.

58 Zhang, Y. and Scarpace, P. J. (2006) The role of leptin in leptin resistance and obesity. Physiol. Behav. **88**, 249–56.

59 Minokoshi, Y., Kim, Y.-B., Peroni, O. D., Fryer, L. G. D., Müller, C., Carling, D. and Kahn, B. B. (2002) Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. Nature, Nature Publishing Group **415**, 339–43.

60 Unger, R. H. (2003) The physiology of cellular liporegulation. Annu. Rev. Physiol. **65**, 333–47.

61 Morton, G. J. and Schwartz, M. W. (2011) Leptin and the central nervous system control of glucose metabolism. Physiol. Rev. **91**, 389–411.

62 Chen, K., Li, F., Li, J., Cai, H., Strom, S., Bisello, A., Kelley, D. E., Friedman-Einat, M., Skibinski, G. A., McCrory, M. A., et al. (2006) Induction of leptin resistance through direct interaction of C-reactive protein with leptin. Nat. Med. **12**, 425–32.

63 Rutter, M. K., Sattar, N., Tajar, A., O'Neill, T. W., Lee, D. M., Bartfai, G., Boonen, S., Casanueva, F. F., Finn, J. D., Forti, G., et al. (2013) Epidemiological evidence against a role for C-reactive protein causing leptin resistance. Eur. J. Endocrinol. **168**, 101–6.

64 Carter, S. L., Brechbühler, C. M., Griffin, M. and Bond, A. T. (2004) Gene co-expression network topology provides a framework for molecular characterization of cellular state. Bioinformatics **20**, 2242–50.

65 Luong, A., Hannah, V. C., Brown, M. S. and Goldstein, J. L. (2000) Molecular characterization of human acetyl-CoA synthetase, an enzyme regulated by sterol regulatory element-binding proteins. J. Biol. Chem. **275**, 26458–66.

66 Wopereis, S., Radonjic, M., Rubingh, C., Erk, M. van, Smilde, A., Duyvenvoorde, W. van, Cnubben, N., Kooistra, T., Ommen, B. van and Kleemann, R. (2012) Identification of prognostic and diagnostic biomarkers of glucose intolerance in ApoE3Leiden mice. Physiol. Genomics **44**, 293–304.

67 Osborne, T. F. (2000) Sterol regulatory element-binding proteins (SREBPs): key regulators of nutritional homeostasis and insulin action. J. Biol. Chem. **275**, 32379–82.

68 Im, S.-S., Yousef, L., Blaschitz, C., Liu, J. Z., Edwards, R. A., Young, S. G., Raffatellu, M. and Osborne, T. F. (2011) Linking lipid metabolism to the innate immune response in macrophages through sterol regulatory element binding protein-1a. Cell Metab. **13**, 540–9.

69 Knobelspies, H., Zeidler, J., Hekerman, P., Bamberg-Lemper, S. and Becker, W. (2010) Mechanism of attenuation of leptin signaling under chronic ligand stimulation. BMC Biochem. **11**, 2.

70 Olofsson, L. E., Unger, E. K., Cheung, C. C. and Xu, A. W. (2013) Modulation of AgRP-neuronal function by SOCS3 as an initiating event in diet-induced hypothalamic leptin resistance. Proc. Natl. Acad. Sci., National Acad Sciences **110**, E697–E706.

71 Sachithanandan, N., Fam, B. C., Fynch, S., Dzamko, N., Watt, M. J., Wormald, S., Honeyman, J., Galic, S., Proietto, J., Andrikopoulos, S., et al. (2010) Liver-specific suppressor of cytokine signaling-3 deletion in mice enhances hepatic insulin sensitivity and lipogenesis resulting in fatty liver and obesity. Hepatology **52**, 1632–42.

72 Yamashita, T., Murakami, T., Iida, M., Kuwajima, M. and Shima, K. (1997) Leptin receptor of Zucker fatty rat performs reduced signal transduction. Diabetes **46**, 1077–80.

73 Phillips, M. S., Liu, Q., Hammond, H. A., Dugan, V., Hey, P. J., Caskey, C. J. and Hess, J. F. (1996) Leptin receptor missense mutation in the fatty Zucker rat. Nat. Genet. **13**, 18–9.

74 Peterson, R. G., Little, L. A. and Neel, M.-A. (1990) WKY Fatty Rat as a Model of Obesity and Non-insulin-dependent Diabetes Mellitus. ILAR J. **32**, 13–15.

4. General discussion

The body's homeostasis is constantly threatened with various types of pathogenic and host-derived stimuli. A wide-ranging immune response is responsible for preserving the body against these homeostatic disturbances. The immune response and metabolism are interdependent systems, resulting in an interface that is known as immunometabolism. The optimal function and regulation of immunometabolism is crucial for human health.

Through nutrition the organism is continuously challenged with foreign components and other immunologically relevant molecules. These nutrients and food compounds have biological and immunological importance in all stages of life. They are essential for the survival of the organism, influencing the body's physiology from absorption through metabolism until excretion [110]. Therefore, our hypothesis postulated that molecular signals contained in food could modulate the function of immunometabolism through regulation of the immune system. In this sense, the nutritional immunotherapy concept refers to the promotion of homeostatic balance through natural food-based compounds, optimising the body's own immune system to induce, enhance or suppress the immune response and restore the system back to homeostasis.

To achieve this purpose, it is first necessary to have greater insight about the intricacies of the immune response and how it could be modulated by the nutritional profile. Taking this into account, the **first part** of this thesis is focused on the analysis of whether the immune system, and specifically its effector cells, the macrophages, is able to sense different dietary patterns through a challenge with different bioactive food compounds.

Consequently, to understand the magnitude of immune regulation by nutrition, it is important to characterise the macrophages and their role as innate immune effector cells. Macrophages are found in all tissues throughout the body, and the primary function of macrophages and their precursors, blood monocytes, is to detect harmful stimuli and orchestrate an adequate activation and resolution of the inflammatory response to protect the host. This situates macrophages as vital cells for the proper functioning of the immune system [111]. Furthermore, macrophages are not only pivotal cells for the immune system but also have central functions for the regulation of homeostasis. Macrophage plasticity is very great and the microenvironment plays a key role in the phenotypic switch, so once stimuli are sensed by the macrophage, the molecular response differs greatly depending on the precise nature of the challenge.

For that reason, studying the influence of the nutritional profile on macrophage activity can be considered a powerful *in vitro* model to elucidate the potential role of dietary patterns in the preservation of immuno-homeostasis. Therefore, the research included in this part aimed to identify whether macrophages could be targeted by the diet. We analysed the two most important signalling pathways involved in sensing immunological disturbances and activating the inflammatory response, the NF-κB and the NLRP3 inflammasome pathways. We further sought to determine the molecular mechanisms that underlie the nutritional modulation of macrophage activity.

In this way, according to the **first objective** proposed, human macrophages were challenged with bioactive food compounds, including the pure forms of dimeric and trimetric procyanidins (B_1, B_2 and C_1), an n-3 PUFA (the DHA) or the combination of each individual procyanidin with DHA, to determine the ability of the tested compounds to modulate the activation of the inflammatory response. Procyanidins and DHA have been previously described as important immuno-active food compounds [112–115]. Afterwards, macrophages were stimulated with bacterial LPS, mimicking innate immune response activation. The capacity of the food bioactive compounds to interfere with the activation of macrophages was assessed through the modulation of cyclooxygenase-dependent eicosanoid production as well as through measurement of TLR4 signal transduction, NF-κB transcription factor activation and transcriptional regulation.

In this sense, we first evaluated whether procyanidins, in pure dimeric and trimeric forms, and DHA could specifically modulate the synthesis of PGE_2, one the most important pleiotropic mediators of inflammation. COX1 and COX2 are the enzymes responsible for catalysing the first committed steps in the production of prostaglandins from arachidonic acid. Using a cell-free assay, we demonstrated the different abilities of the tested procyanidins and DHA to interact with both COX isoforms and alter the affinity of the substrate, arachidonic acid. DHA is a potent and selective inhibitor of the COX1 isoform with an IC_{50} of 13.5 μM, being able to modulate the affinity between AA and COX1 by binding at a different site than AA, resulting in its mixed inhibitory behaviour. The B_1 procyanidin is also a specific inhibitor of COX1 activity, with an IC_{50} of 8.0 μM, although in this case, B_1 competes directly with AA to bind in the active site of the enzyme. Moreover, B_2 and C_1 procyanidins are strong and competitive selective inhibitors of the COX2 isoform, with an IC_{50} of 9.7 μM and 3.3 μM, respectively. In addition, this specific behaviour of the compounds to decrease the catalytic efficiency of prostaglandin synthesis was also corroborated by measuring the secreted levels of PGE_2 in human primary macrophages. Both COX isoforms have been extensively studied because they are considered pivotal for the widespread actions of the inflammatory response. Therefore, COX1 and COX2 are the pharmacological targets of the non-steroidal anti-inflammatory drugs (NSAIDs).

On the other hand, the cell membrane pattern recognition receptor TLR4 is fundamental for recognition of harmful stimuli and activation of innate immunity. TLR4, besides being considered the host sensor of LPS, is also considered a pivotal sensor for DAMPs recognition [10,116]. Therefore, we analysed the capacity of bioactive food compounds to

modulate one of the most fundamental pathways of innate immunity, the TLR4 signal transduction pathway that results in the activation of the transcription factor NF-κB. NF-κB has been shown to tightly regulate gene networks involved in the differentiation, proliferation and activation of macrophage effector pathways. Therefore, modulation of the activation of NF-κB involves the simultaneous regulation of the transcription of its target genes and their downstream pathways.

We demonstrated that B_1, B_2 and C_1 procyanidins, and DHA alone or in combination with procyanidins, prevented activation of the transcription factor NF-κB at both early and late stages within the NF-κB signalling pathway. The bioactive food compounds interfere with activation of the NF-κB transcription factor by several molecular mechanisms. On the one hand, bioactive food compounds can promote the cytoplasmic retention of the NF-κB classical heterodimer (p65:p50) and therefore inhibit its nuclear translocation by two mechanisms. First, they can mediate the down-regulation of IKKβ activity, which was determined by the inhibition of pIκBα phosphorylation, and second, they can promote the up-regulation of p105. p105 has dual functions within the NF-κB pathway, as it is both the precursor of the p50 subunit and is capable of retaining in the cytoplasm all the different NF-κB subunits. Therefore, the overexpression of p105 could be associated not only with a decreased proteolytic formation of p50 but also with an increased cytoplasmic retention of NF-κB. On the other hand, the bioactive food compounds, and more specifically DHA, stimulate the nuclear translocation of the NF-κB p50:p50 homodimer instead of the p65:p50 heterodimer. The subunits that compose the NF-κB transcription factor are fundamental because while the p65 subunit possesses a transactivation domain, the p50 subunit does not [117]; therefore, the presence of the p50:p50 NF-κB homodimer could decrease the transcriptional activity of the NF-κB heterodimer. The repression of NF-κB nuclear translocation and transcription of its target pro-inflammatory genes was also supported by the bioactive compound-induced down-regulation of the binding activity of the p65 subunit to the corresponding κB consensus sequence found in the promoter and enhancer regions of several pro-inflammatory genes, as well as by the inhibition of secreted levels of the IL-6 pro-inflammatory cytokine, one of the target genes for the NF-κB transcription factor.

From the results derived from the first objective, we concluded that macrophages, the effector cells of the innate immune system, sense the presence of bioactive food compounds in the intercellular microenvironment. All the tested bioactive compounds, pure procyanidins, DHA and DHA-procyanidins combinations, have anti-inflammatory properties. They exert these properties by inhibiting the production of prostaglandins through interfering directly and specifically with cyclooxygenases, the rate-limiting enzymes, as well as by the suppression of the activation and nuclear translocation of NF-κB in LPS-activated macrophages. Furthermore, the molecular mechanisms underlying the immunosuppressive functions of the bioactive compounds were not the same, which make us consider that the intracellular modulation of the inflammatory response is compound-dependent and that intrinsic

factors, including chemical properties or the nutritional profile, could determine the role of the bioactive compounds as functional modulators of the immune response.

Once we determined that bioactive food compounds modulate the activation of the inflammatory response, we wanted to determine, through the **second objective**, the specificity of macrophages to sense different nutritional profiles. For this purpose we evaluated the activation of the NLRP3 inflammasome pathway under different conditions. In the first condition, we evaluated the ability of the procyanidin B_2 to modulate inflammasome activation, and in the second condition we challenged macrophages with four different fatty acids to analyse the capacity of the inflammasome to sense different dietary compositions.

The determination of how the innate immune system senses noxious stimuli is a central question in immunology. Whereas TLR4, a Toll-like receptor, detects the presence of noxious molecular patterns extracellularly, NLRP3, a NOD-like receptor, recognises intracellular stimuli.

The inflammasome is the putative metabolic sensor that links the inflammatory response with metabolism [118]. Inflammasome activation is the result of a two-step process. In the first step, a priming signal induces the NF-κB-mediated transcription of the limiting inflammasome components, NLRP3 and the immature form of IL-1β, pro-IL-1β. During the second step, an activation signal promotes conformational changes that induce NLRP3 inflammasome assembly and drives the activation of caspase-1, which mediates the proteolytic maturation and secretion of IL-1β, which is considered a potent pro-inflammatory cytokine that initiates inflammation and metabolic dysfunction.

Given the potential shown by the procyanidin B_2 as an inhibitor of NF-κB activation, along with the fact that NF-κB activation is the first step required for inflammasome activation, added to the role of TLR4 and NLRP3 as the most important sensors for the innate immune system to detect and activate the inflammatory response, we hypothesised that procyanidin B_2 could also inhibit the activation of the inflammasome and thus the secretion of IL-1β. Accordingly, macrophages were primed with LPS with or without the presence of B_2, and subsequently the inflammasome was specifically activated using ATP. The procyanidin B_2 was chosen because, according to our previous results, procyanidin B_2 and procyanidin C_1 were the most immunologically active forms within the pure forms of procyanidins tested. However, the bioavailability of procyanidins is largely influenced by their degree of polymerisation, and due to the dimeric structure of B_2 versus the trimeric of C_1, procyanidin B_2 is favoured for absorption and thus its plasma concentration is higher [119,120]. Whereas dimeric procyanidins can be directly absorbed in the small intestine, the absorption of the trimeric form is controversial and, unlike B_2, there is not a clear consensus regarding its unmodified presence in plasma [121].

Procyanidin B_2 inhibited the activation of the inflammasome at both early and late stages, which included the repression of NF-κB-mediated transcription of NLRP3 and pro-IL-1β, two limiting components for inflammasome assembly, as well as the activation of caspase-1 and

the subsequent release of the cytokine IL-1β. Therefore, we concluded that procyanidin B_2 inhibits the activation of the inflammatory response by blocking the activation of NF-κB and the NLRP3 inflammasome, both of which play central roles in sensing noxious stimuli and orchestrating inflammatory responses by rapidly inducing a variety of pro-inflammatory mediators.

Within the context of the **second** proposed **objective**, we evaluated whether the inflammasome could differentially sense the presence of dietary fatty acids. Elevated concentrations of free fatty acids are one of the hallmarks of diet-induced obesity. This continuous lipid overload promotes a fatty acid spillover in metabolic and non-metabolic tissues, which is associated with the recruitment and activation of macrophages [122]. Thus, recruited macrophages are exposed to intercellular environments with high concentrations of free fatty acids [123]. In addition, the fatty acids contained in the blood and in tissues are mainly derived from the diet [124]. Dietary lipids or free fatty acids are more than just nutrients for energetic purposes; they are also signalling molecules. It is noteworthy that free fatty acids can affect the immune system directly through gene modulation or interference in the production of other inflammatory mediators such as the eicosanoids [110]. Dietary fatty acids can also be incorporated into cell membranes, and depending on the dietary lipid composition, the cellular membrane structure will be affected in terms of its fluidity, lipid raft formation, cell signalling and antigen presentation. The ability to be incorporated into the cell membrane is dependent upon each fatty acid, which in turn will determine the bioactivity of the fatty acid [110].

Therefore, taking these considerations into account, we determined the effects of four dietary fatty acids, two saturated fatty acids (palmitate and stearic acid), one n-3 fatty acid (DHA) and one n-6 (arachidonic acid), on the modulation of inflammasome activation in macrophages. The sensing capacity of the inflammasome was evaluated, using the dietary fatty acids as a priming signal in ATP-activated macrophages, by measuring the capacity of fatty acids to interfere with the LPS-induced inflammasome activation, and by measuring the ability of the dietary fatty acids to modulate the activation of the inflammasome.

From our results we determined that the chemical properties of dietary fatty acids, in terms of their degree of saturation, are fundamental for their immunological activity. In this way, the stimulation of macrophages with both saturated fatty acids tested (palmitate and stearic acid) or arachidonic acid was enough to prime the macrophages and stimulate, together with ATP as an additional activation signal, the activation of the inflammasome and the subsequent secretion of IL-1β. The capacity of the dietary fatty acids to interfere with LPS-induced priming and activation of inflammation highlights the role of stearic acid because it potentiates the pro-inflammatory effects of LPS.

Surprisingly, the role of arachidonic acid in the modulation of inflammasome priming and activation was dependent on the intracellular environment, that is, using arachidonic acid as a priming signal and ATP as an activating signal, the inflammasome became primed and activated and the macrophages secreted increased levels of IL-1β. However, with LPS in the

cellular milieu, the immunomodulatory effects of arachidonic acid were the reverse, decreasing the activation of the NF-κB-mediated priming step and also decreasing the secretion of IL-1β. Therefore, the immunomodulatory action of arachidonic acid could be mediated by its incorporation into cell membranes, interfering in membrane receptor function and in LPS-TLR4 signal transduction [125].

DHA was not able to induce activation of the inflammasome, and when macrophages were stimulated with LPS, DHA inhibited the activation of the inflammasome mainly through the inhibition of NF-κB activation rather than directly affecting the second step of inflammasome activation.

We also showed that palmitate was the only tested dietary fatty acid with a direct engagement of immune sensors, being able to enhance the secretion of IL-1β. However, although there is evidence indicating that palmitate is a TLR4 ligand able to activate NF-κB, it is not clear if palmitate is also able to bind to the NLRP3 inflammasome and directly activate the caspase-1 mediated maturation of IL-1β. Furthermore, the interaction between macrophages and palmitate is associated with the production of reactive oxygen species (ROS), one of the described DAMPs for inflammasome activation [126].

Taking together all the results included within the second objective, we concluded that bioactive food compounds promote functional changes in innate effector immune cells, as well as that bioactive food compounds can modulate the activation of inflammation and the innate immune system. In addition, our results demonstrated that the immunological activity of food compounds is bioactive-dependent and therefore, macrophages and their receptors, including TLR4 and NLRP3, may recognise specific bioactive food motifs.

Once we determined the pivotal role of the nutritional profile in the modulation of innate immunity, through the modulation of the activation of the inflammatory response in human macrophages, we set out to extrapolate our objectives to an *in vivo* model to incorporate the complex and interdependent functional connections between the immune system and metabolism. In obesity, the immune response, nutrition and metabolism are closely interconnected. Accordingly, the **second part** of this thesis is focused on understanding the role of the nutritional profile in the immunometabolic balance and in the maintenance of homeostasis. To achieve this objective, Wistar rats were immunometabolically challenged using highly palatable and energy dense foods, also known as the cafeteria (CAF) diet.

By feeding the CAF diet, which is considered a robust model of human metabolic syndrome [99], the body weight and the adiposity of the rats increased. Our results confirmed that in a diet-induced obesity context, the adipose tissue is a powerful immune organ, and the functions of adipocytes exceed the mere storage of surplus energy. It has been described [122] that when adipocytes store too much lipid, they begin to expand and rapidly proliferate, causing a lack of oxygen that leads to hypoxic conditions. As a consequence, fat cells die and release their lipid cargo. This process features a shift in the immunological profile of

adipose tissue, turning it from anti-inflammatory to proinflammatory, which is manifested by the up-regulation of *IL-6* and *Tnf-α* pro-inflammatory gene expression and the down-regulation of *IL-10*, an anti-inflammatory cytokine. As the fat cells die, macrophages travel to the over-expanded fat depots to remove the lipid droplets. Increased circulating levels of the chemotactic protein MCP-1 and expression of the F4/80 gene, a selective macrophage marker in adipose tissue, support this adipose-mediated recruitment of macrophages. However, as macrophages are not as efficient as adipocytes at storing lipids, they begin to spill over into other tissues, such as the liver or the pancreas [122]. The inappropriate accumulation of lipids amplifies the local low-grade inflammation into a systemic immunometabolic imbalance through the modulation of the circulating pattern of immunoregulatory factors. This imbalance results in increased circulating levels of acute phase proteins, such as complement factor C3 and orosomucoid, as well as changes in adipokines, such as elevated leptin and lowered adiponectin levels.

It has been widely described that diet-induced obesity is accompanied by a low-grade inflammatory response in metabolic tissues, a fact considered a pivotal feature of chronic metabolic disorders [123,127,128]. The role of the thymus and spleen, a primary and secondary lymphoid organ, respectively, in the regulation of this metaflammation is unknown. For this reason, in our **third objective** we evaluated the effect of diet-induced obesity in the thymus and the spleen.

The thymus is a vital organ for maintenance of the homeostasis of the peripheral immune system, as it is responsible for the maturation and output of *de novo* T cells. In the thymus, no differences were found in terms of weight, although the thymic appearance was profoundly influenced by the CAF diet, indicating that the obesity-induced lipid spillover can affect not only metabolic tissues but also the thymus. Moreover, the existence of immunometabolic interactions in the thymus has been postulated, leading to massive architectural changes over time that result from the replacement of thymocytes by adipocytes and leading to a thymic atrophy-induced immunodeprivation [129]. Diet-induced obesity also affected the functionality of the thymus by the alteration of the expression pattern of genes involved in the proliferation and *de novo* generation of T cells, including decreased *IL-6* and *IL-10* cytokine gene expression and the expression of the macrophage marker *F4/80*, which performs crucial functions for T cell maturation [130]. The obesity-induced decreased generation of *de novo* T cells, also known as lymphopenia, has deleterious consequences for the host because it results in a T cell pool containing dominant memory T cell clones. This limits the diversity and the repertoire of T cells, which impairs the establishment of a functional and effective immune system [131].

In addition, the spleen is the largest secondary lymphoid organ in the body and plays an important role not only for blood filtration but also for the immune-metabolic-endocrine network. The spleen is where the interaction between bloodstream metabolites and immune cells occurs [50]. Furthermore, an interplay also exists between the spleen and the liver for the regulation of immunometabolism [132]. We showed that diet-induced obesity resulted in a decrease in splenic weight, leading to a reduction in macrophages counts, as evidenced

by a decrease in *F4/80* gene expression. We also showed that obesity impairs the immune response by decreasing oxidative burst activity and the activation of macrophages by reducing the production of reactive oxygen species (ROS), events associated with increased expression levels of the *Ucp2* gene and decreased *IL-6* and *Crp* gene expression, both inflammatory biomarkers. Furthermore, diet-induced obesity supresses the splenic expression of *IL-10*, a marker of B cells. The down-regulation of *IL-10* gene expression in the spleen has been related to the promotion of chronic inflammation in adipose tissue, the liver and the hypothalamus [133].

From the results elucidated by this third objective, we concluded that diet-induced obesity disturbs immunometabolic homeostasis, affecting the immune function and gene expression of adipose tissue and also of lymphoid organs. In this way, whereas obesity induced an inflammatory response in adipose tissue, the functionality of lymphoid organs, in terms of their capacity to sense homeostatic disturbances and trigger responses to restore homeostasis, is weakened.

In accordance with our results originating from the last objective, we hypothesised that nutrition can target the immunometabolic axis, impairing its functionality; nevertheless, the main objective of our research was not only to know the impact of an unhealthy diet on immune homeostasis but also to determine the potential of foods and bioactive compounds to be used as tools to promote the proper function of the immune system and therefore to improve health through nutrition. Accordingly, with our **fourth objective** we proposed to counteract the immunometabolic impairment derived from an obesity-induced homeostasis imbalance through the dietary intake of food extracts rich in bioactive food compounds with known immunomodulatory properties.

For this purpose, using the previously described *in vivo* model of unbalanced immunometabolic homeostasis resulting from intake of the CAF diet, we assessed the potential of nutritional immunotherapy by supplementing the diet of unhealthy obese rats with the extracts of foods containing immunologically active compounds, including the grape seed procyanidin extract (GSPE), an oil rich in DHA (DHA-OR) and the combination of both. As we determined previously, diet-induced obesity triggered a local inflammatory response in the adipose tissue. Therefore, we first analysed the effects of the bioactive supplements on the immune profile of adipose tissue, to corroborate the anti-inflammatory properties observed *in vitro* for both the pure forms of procyanidins and DHA. Thus, we showed that supplementation of the cafeteria diet with GSPE, DHA-OR or the combination of both bioactive compounds supressed the obesity-triggered inflammatory response. Supplementation induced a phenotypic shift of the pro-inflammatory immunological profile of the adipose tissue, promoted the resolution of inflammation through the down-regulation of gene expression of inflammatory mediators such as *IL-6*, *Tnf-α*, and *iNOS*, and induced transcription of the *IL-10* gene.

Additionally, it is noteworthy that the immunomodulatory effects of the bioactive compounds are not limited to the modulation of immunity at a molecular level, as cell-mediated immunity was also influenced. Therefore, the presence and recruitment of macrophages to the adipose tissue of the bioactive-supplemented rats was decreased, as is reflected by the down-regulation of *F4/80* gene expression and also by the decrease in circulating levels of the chemotactic factor MCP-1. In agreement with our results, the profile of T cells within adipose tissue was also influenced by supplement intake, promoting the recruitment of Tregs, the immunosuppressive $CD4^+$ lymphocytes, characterised by the production of IL-10 and the promotion of the anti-inflammatory M2 macrophage phenotype [134,135].

Furthermore, the local anti-inflammatory properties of the bioactive compounds were also observed at the systemic level by the down-regulation of the circulating levels of acute inflammation biomarkers, such as complement C3 and orosomucoid, to levels similar to those determined for rats fed a standard diet.

Moreover, we determined the potential of bioactive compound-based nutritional immunotherapy to counteract the obesity-induced weakened immune system by analysing the immunomodulatory effects of the bioactive compounds on thymus and spleen functionality. We also analysed their regulatory effects on T cell populations in the thymus, the spleen and as part of the circulating PBMCs population. Notably, the bioactive compounds, and more strongly the combination of GSPE and DHA, induced the generation of naïve T cells, determined by increased counts of both thymocytes and also $CD4^+CD8^+$ T cells in the thymus. Furthermore, this striking immunostimulatory effect in the thymus was also reflected in the quantification of increased numbers of both $CD4^+$ and $CD8^+$ T cells within the population of circulating PBMCs. The bioactive compounds, particularly GSPE, also neutralised obesity-induced thymic atrophy.

The cafeteria diet supplemented with GSPE and DHA, and particularly the GSPE-DHA combination, increased the size of splenocytes and also the capacity of the spleen to counteract obesity-induced immunometabolic dysfunction, reflected by increased transcription of *IL-6* and *Tnf-α*, both immune response boosters, as well as increased numbers of immune cells, including macrophages and $CD8^+$ T cells, which play a key role in linking the innate and adaptive immune responses.

The immunostimulatory properties of the bioactive compounds are based on their capacity to promote the generation of *de novo* T cells, as well as their role in boosting splenic function to counteract obesity-induced immunotolerance. They furthermore improve the capacity of the immune system to sense noxious stimuli and enhance the responsiveness of the immune system against obesity-triggered homeostatic disturbances.

Additionally, the intake of GSPE and DHA in combination improved the immunomodulatory effects observed when supplementing the bioactive compounds separately. Indeed, although adiposity and circulating leptin levels increased, the local and systemic inflammatory responses were supressed and the functionality of the thymus and spleen was enhanced. In

this way, leptin could be playing a key role in the stimulation of the immune system. According to our results, the rats that were supplemented with the GSPE-DHA combination had higher circulating leptin levels as well as a major infiltration of Treg cells and IL-10 secretion in the adipose tissue. In addition, this group of rats also showed significantly higher counts of thymocytes and naïve T cell generation in circulation.

Targeting immunometabolism through the intake of food extracts rich in procyanidins and DHA, both immunologically bioactive compounds, improves health by targeting the immunometabolic axis and counteracting the obesity-induced homeostatic imbalance. We can conclude that in the context of obesity-induced unhealthy rats, the combination of both tested bioactive compounds, GSPE and DHA, provides added value to the anti-inflammatory effects observed for each bioactive compound individually. So, whereas DHA is mainly characterised by its anti-inflammatory properties and GSPE by its capacity to boost the immune response, in combination they improve both properties, increasing both anti-inflammatory and immunostimulatory effectiveness.

Obesity is not a simple consequence of the positive energy imbalance resulting from the difference between energy intake and expenditure. There is strong evidence that obesity is a complex phenotype resulting from the interaction of both genetic and environmental factors [107,109,136–138]. Metabolic challenge in combination with nutrigenomics approaches, such as the transcriptomics, is a powerful tool to understand the phenotypic response and gene-diet interactions. In addition, much greater complexity is revealed when the phenotypic effect is compared between the different genetic backgrounds of inbred strains.

Therefore, in our **fifth objective**, two rat strains were simultaneously exposed to a defined environmental (dietary) challenge to determine the molecular and genetic factors involved in the phenotypic response to obesity. For this reason, we subjected the LEW and WKY rats, two phenotypically different inbred rat strains, to a diet challenge based on dietary energy content. After 7 weeks of challenge, either with the standard (STD) or CAF diet, the physiological response at the organism level, energetic metabolism, modulation of the plasma metabolites and the dietary influence on the transcriptional profile of circulating monocytes were carefully analysed.

As expected, offering the CAF diet to rats resulted, independently of genetic background, in a voluntary hyperphagia that led to a high increase in energy intake resulting mainly from the preference for fat intake. CAF diet-induced body weight gain was accompanied by greater increases of whole body fat mass. However, the distribution of visceral fat mass was different between both strains. While LEW rats exhibited a significant increase in the weight of mesenteric, epididymal and perirenal fat pads, WKY only had increased weight of the epididymal and perirenal fat pads, with varying weights of the mesenteric fat pad. According to previous studies, the inappropriate localisation of accumulated fat can cause health problems [122].

The postprandial energy metabolism pattern and peripheral substrate utilisation in response to the increased energy intake were also strain-specific. In LEW rats, the diet-induced weight gain of the CAF group was supported by increased energy intake and the maintenance of the expended energy, as well as through the carbohydrate-to-fat switch as the primary oxidation fuel source, showing a pattern of energy metabolism in accordance with those observed previously for diet-induced obesity in rats [139,140]. However, despite the fact that diet-induced weight gain in WKY rats was supported by an increase of energy intake, energy expenditure was also higher for the rats that were fed the CAF diet.

The influence of genetic background in the obesity phenotype was also supported through the determination of a strain-specific differential profile of plasma metabolites between CAF fed LEW and WKY rats. While the CAF diet challenge induced hyperglycaemia, hypertriglyceridemia and high levels of circulating free fatty acids in LEW rats, the concentrations of the corresponding metabolites were unaltered by the diet in WKY rats. Regarding the diet-induced modulation of immunometabolic mediators, the CAF diet promoted higher plasma leptin levels in a genotype-independent manner. However, the circulating levels of MCP-1, a chemoattractant that reflects the activation of the local inflammatory response and the obesity-induced recruitment of monocytes to adipose tissue [141], were only augmented in LEW rats fed the CAF diet.

To gain insight into the complex interactions between genetic and dietary factors in obesity, we focused on the genotype-dependent modulation of the whole genome expression profiles of the circulating monocytes in LEW and WKY rats. The usefulness of PBMCs for nutrition and obesity studies has been previously highlighted, suggesting that these circulating cells are a potential source of homeostatic imbalance markers associated with obesity, as well as suggesting that the modulation of the gene expression of PBMCs reflects the transcriptional changes in many solid tissues, including liver, adipose tissue and muscle [31,32]. However, in these studies the PBMCs were considered a black box and the variability of the cellular pool was not taken into account, which, as we have demonstrated in our fourth objective, could be significantly affected by the nutritional profile. For this reason, we isolated only the monocyte fraction from the pool of PBMCs to increase robustness and eliminate the variability derived from the great phenotypic complexity of the immune cells.

Circulating monocytes are considered the peripheral sensors of immunometabolic homeostasis, and we used different approaches to identify the genetic basis for the differential phenotypic response to diet-induced obesity. First, through the comparison of the transcriptome of monocytes between the CAF diet fed LEW and WKY rats, we determined that the modulation of the gene expression profile was also genotype-dependent. Notably, the genes overexpressed in WKY monocytes were functionally enriched into inflammatory and immune responses, as well as into the positive regulation of the JAK-STAT cascade through the promotion of the tyrosine phosphorylation of STAT3. However, the genes over-expressed in CAF-diet-fed LEW rats were not as numerous as in WKY rats, highlighting the functional gene enrichment of the immune system.

Second, mediating the gene co-expression network based on the genes whose expressions were modulated genotype-specifically by the CAF diet, the power to detect gene-gene interactions genome-wide was enhanced. The clustering of the co-expressed genes revealed a sub-network of genes functionally enriched as nutrient sensors, responders to hormonal stimuli and components of the NOD-like receptor-signalling pathway. Remarkably, the Acyl-CoA synthetase short-chain family member 2 (*Acss2*) was identified as the hub within the gene sub-network. Additionally, *Acss2* is considered a nutrient-sensor gene, and besides overlapping with the metabolism-related quantitative trait locus (QTLs), we corroborated that *Acss2* gene expression in peripheral monocytes is inversely associated with the plasma levels of glucose, triglycerides, leptin and NEFAs as well as with body and adiposity weights, being considered as a biomarker of glucose intolerance [142]. The expression of *Acss2* is regulated by the sterol regulatory element-binding proteins (SREBPs) transcription factors, which are key regulators of energy homeostasis and links between lipid metabolism and the innate immune response, directly mediating the regulation of the genes involved in inflammasome activation [143].

Third, by the comparison of the gene expression profiles between the monocytes of CAF diet fed rats of each strain with the respective genotype-STD diet control groups, we found a moderate diet-induced modulation of the monocyte transcriptome in LEW rats. Surprisingly, although the phenotypic response of WKY rats to the CAF diet challenge was apparently non-existent, a substantial set of genes was modulated differentially by the diet. Among all the differentially expressed genes due to the CAF diet, a highlight was the down-regulation of *Mir223* gene expression, the immature form of miR-223. A decrease in miR-223 expression resulted in the activation of STAT3 in macrophages, which is directly targeted by miR-223, and in JAK2-STAT3 signal transduction [144].

Taking into account the strain-specific phenotypic response, as well as the differential modulation of gene expression profiles by diet induced-obesity, the evidence suggested that the leptin signalling pathway might be underlying these differences. Despite the fact that the expression of the leptin gene in adipose tissue and the plasma leptin levels were genotype-independently increased by the CAF diet, the subsequent metabolic events underlying the complexity of the obesity phenotype were strain-specific. It is noteworthy that, in accordance with the early events associated with obesity-induced immunometabolic homeostatic imbalance, chronic overfeeding results in overexpansion of adipose tissue and hyperleptinemia. This chronic stimulation of central leptin signalling leads to the attenuation of signal transduction and resistance to the immunomodulatory effects of leptin. To better understand the role of leptin signalling in the strain-specific phenotypic and genotypic response to diet-induced obesity, the expression of the pivotal genes involved in the central and peripheral regulation of the leptin signal transduction pathway were analysed. We found that, while no diet-related effects were observed regarding the modulation of the leptin receptor (*LepRb*) in the hypothalamus, the regulation of *Socs3* gene expression, the negative feedback regulator of leptin signal transduction, was dependent not only on the genetic background but also on the diet. While the diet-induced up-regulation of *Socs3* gene

expression in the hypothalamus of the LEW rats supported their central leptin resistance, the *Socs3* down-regulation in the hypothalamus of the CAF diet-induced obese WKY rats confirms the non-attenuation of central leptin signalling. The suppression of hypothalamic *Socs3* gene expression has been postulated as a potential mechanism underlying the resistance to diet-induced obesity [145].

In conclusion, the phenotypic and transcriptome characterisation of the response of LEW and WKY rats to a hyper-caloric diet challenge outlined the pivotal role of the genetic background within the complex interaction between the genetic and the dietary factors. Our analyses revealed that whereas LEW rats were prone to diet-induced obesity, the WKY rats were resistant and that this disparity is closely related to a differential regulation of central leptin signal transduction.

In summary, we conclude from our studies that the nutritional profile is a powerful tool to target the immunometabolic axis and that bioactive food compounds, such as the procyanidins and docosahexaenoic n-3 fatty acid, improve immune system efficiency, thus promoting homeostasis and a healthy state.

The main conclusions obtained from this thesis are:

1. NF-κB and NLRP3 inflammasome signalling pathways enable macrophages to sense their environment and shape immune responses.

2. LPS-induced macrophage activation can be supressed by bioactive food compounds.

- B_1, B_2 and C_1 procyanidins and DHA inhibit the activation and nuclear translocation of the transcription factor NF-κB.
- Bioactives compounds interfere with the production of prostaglandins by direct and specific modulation of COX activity. While B_1 procyanidin and DHA are strong and selective inhibitors of COX1, the B_2 and C_1 procyanidins are selective suppressors of COX2 activity.

3. NLRP3 inflammasome activation depends on dietary composition.

4. The immunological activity of food compounds is dependent on bioactive compounds.

- The pure procyanidins tested (B_1, B_2 and C_1) and the PUFAs (DHA and arachidonic acid) are anti-inflammatory compounds. However, SFAs (palmitate and stearic acid) are pro-inflammatory dietary compounds.

5. Diet induced obesity significantly affects immunometabolic homeostasis.

- Triggering adipose tissue-mediated inflammation.
- Impairing the immune function of the thymus and spleen.

6. Nutritional immunotherapy allows improved health through targeting immunometabolic function with bioactive food compounds.

- GSPE and DHA counteract obesity-induced inflammation in adipose tissue as well as weakened immunity.
- The administration of GSPE and DHA together provides added value to the immunomodulatory effects observed for each bioactive compound individually.
    - Supressing adipose tissue-triggered inflammatory response.
    - Boosting the immune system to resolve the obesity-induced homeostatic disturbance.

7. The genetic background plays a pivotal role in the complex interaction between the genetic and dietary factors that result in the phenotypic expression of obesity-associated traits.

- While LEW rats are prone to diet-induced obesity, WKY rats are resistant.

5. References

1 Mathis, D. and Shoelson, S. E. (2011) Immunometabolism: an emerging frontier. Nat. Rev. Immunol. **11**, 81.

2 Ferrante, A. W. (2013) Macrophages, fat, and the emergence of immunometabolism. J. Clin. Invest. **123**, 4992–3.

3 Wolowczuk, I., Verwaerde, C., Viltart, O., Delanoye, A., Delacre, M., Pot, B. and Grangette, C. (2008) Feeding our immune system: impact on metabolism. Clin. Dev. Immunol. **2008**, 639803.

4 Schäffler, A. and Schölmerich, J. (2010) Innate immunity and adipose tissue biology. Trends Immunol. **31**, 228–35.

5 Parkin, J. and Cohen, B. (2001) An overview of the immune system. Lancet **357**, 1777–89.

6 Chaplin, D. D. (2010) Overview of the immune response. J. Allergy Clin. Immunol. **125**, S3–23.

7 Dranoff, G. (2004) Cytokines in cancer pathogenesis and cancer therapy. Nat. Rev. Cancer, Nature Publishing Group **4**, 11–22.

8 Newton, K. and Dixit, V. M. (2012) Signaling in innate immunity and inflammation. Cold Spring Harb. Perspect. Biol., Cold Spring Harbor Lab **4**.

9 Medzhitov, R. (2008) Origin and physiological roles of inflammation. Nature, Nature Publishing Group **454**, 428–435.

10 Akira, S., Uematsu, S. and Takeuchi, O. (2006) Pathogen recognition and innate immunity. Cell **124**, 783–801.

11 Mills, K. H. G. (2011) TLR-dependent T cell activation in autoimmunity. Nat. Rev. Immunol., Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved. **11**, 807–22.

12 Calay, E. S. and Hotamisligil, G. S. (2013) Turning off the inflammatory, but not the metabolic, flames. Nat. Med., Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved. **19**, 265–7.

13 Kumar, H., Kawai, T. and Akira, S. (2011) Pathogen recognition by the innate immune system. Int. Rev. Immunol. **30**, 16–34.

14 Netea, M. G., van Deuren, M., Kullberg, B. J., Cavaillon, J.-M. and Van der Meer, J. W. M. (2002) Does the shape of lipid A determine the interaction of LPS with Toll-like receptors? Trends Immunol. **23**, 135–9.

15 Hayden, M. S., West, A. P. and Ghosh, S. (2006) NF-kappaB and the immune response. Oncogene **25**, 6758–80.

16 Li, Q. and Verma, I. M. (2002) NF-kappaB regulation in the immune system. Nat. Rev. Immunol. **2**, 725–34.

17 Oeckinghaus, A. and Ghosh, S. (2009) The NF-kappaB family of transcription factors and its regulation. Cold Spring Harb. Perspect. Biol. **1**, a000034.

18 Gerondakis, S., Fulford, T. S., Messina, N. L. and Grumont, R. J. (2014) NF-κB control of T cell development. Nat. Immunol., Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved. **15**, 15–25.

19 Strowig, T., Henao-Mejia, J., Elinav, E. and Flavell, R. (2012) Inflammasomes in health and disease. Nature, Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved. **481**, 278–86.

20 Schroder, K. and Tschopp, J. (2010) The inflammasomes. Cell, Elsevier **140**, 821–832.

21 Guarda, G. and So, A. (2010) Regulation of inflammasome activity. Immunology **130**, 329–36.

22 Kersse, K., Bertrand, M. J. M., Lamkanfi, M. and Vandenabeele, P. (2011) NOD-like receptors and the innate immune system: coping with danger, damage and death. Cytokine Growth Factor Rev. **22**, 257–76.

23 Henao-Mejia, J., Elinav, E., Strowig, T. and Flavell, R. A. (2012) Inflammasomes: far beyond inflammation. Nat. Immunol., Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved. **13**, 321–4.

24 Marrack, P., McKee, A. S. and Munks, M. W. (2009) Towards an understanding of the adjuvant action of aluminium. Nat. Rev. Immunol., Nature Publishing Group **9**, 287–93.

25 Funk, C. D. (2001) Prostaglandins and leukotrienes: advances in eicosanoid biology. Science **294**, 1871–5.

26 Chandrasekharan, N. V, Dai, H., Roos, K. L. T., Evanson, N. K., Tomsik, J., Elton, T. S. and Simmons, D. L. (2002) COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression. Proc. Natl. Acad. Sci. U. S. A. **99**, 13926–31.

27 Colville-Nash, P. R. and Gilroy, D. W. (2000) Cyclooxygenase enzymes as targets for therapeutic intervention in inflammation. Drug News Perspect. **13**, 587–97.

28 Gupta, R. A. and Dubois, R. N. (2001) Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. Nat. Rev. Cancer, Nature Publishing Group **1**, 11–21.

29 Smith, W. L. (2008) Nutritionally essential fatty acids and biologically indispensable cyclooxygenases. Trends Biochem. Sci. **33**, 27–37.

30 Geissmann, F., Manz, M. G., Jung, S., Sieweke, M. H., Merad, M. and Ley, K. (2010) Development of monocytes, macrophages, and dendritic cells. Science **327**, 656–61.

31 O'Grada, C. M., Morine, M. J., Morris, C., Ryan, M., Dillon, E. T., Walsh, M., Gibney, E. R., Brennan, L., Gibney, M. J. and Roche, H. M. (2014) PBMCs reflect the immune component of the WAT transcriptome-Implications as biomarkers of metabolic health in the postprandial state. Mol. Nutr. Food Res. **58**, 808–20.

32 Kohane, I. S. and Valtchinov, V. I. (2012) Quantifying the white blood cell transcriptome as an accessible window to the multiorgan transcriptome. Bioinformatics **28**, 538–45.

33 Geissmann, F., Jung, S. and Littman, D. R. (2003) Blood monocytes consist of two principal subsets with distinct migratory properties. Immunity **19**, 71–82.

34 Parihar, A., Eubank, T. D. and Doseff, A. I. (2010) Monocytes and macrophages regulate immunity through dynamic networks of survival and cell death. J. Innate Immun., Karger Publishers **2**, 204–215.

35 Murray, P. J. and Wynn, T. A. (2011) Protective and pathogenic functions of macrophage subsets. Nat. Rev. Immunol. **11**, 723–37.

36 Austyn, J. M. and Gordon, S. (1981) F4/80, a monoclonal antibody directed specifically against the mouse macrophage. Eur. J. Immunol. **11**, 805–15.

37 Odegaard, J. I. and Chawla, A. (2011) Alternative macrophage activation and metabolism. Annu. Rev. Pathol. **6**, 275–97.

38 Mosser, D. M. and Edwards, J. P. (2008) Exploring the full spectrum of macrophage activation. Nat. Rev. Immunol., Nature Publishing Group **8**, 958–969.

39 Gordon, S. and Taylor, P. R. (2005) Monocyte and macrophage heterogeneity. Nat. Rev. Immunol. **5**, 953–64.

40 McGaha, T. L., Chen, Y., Ravishankar, B., van Rooijen, N. and Karlsson, M. C. I. (2011) Marginal zone macrophages suppress innate and adaptive immunity to apoptotic cells in the spleen. Blood **117**, 5403–12.

41 Stout, R. D., Jiang, C., Matta, B., Tietzel, I., Watkins, S. K. and Suttles, J. (2005) Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. J. Immunol. **175**, 342–9.

42 Stout, R. D. and Suttles, J. (2004) Functional plasticity of macrophages: reversible adaptation to changing microenvironments. J. Leukoc. Biol. **76**, 509–13.

43 Stone, J. D., Chervin, A. S. and Kranz, D. M. (2009) T-cell receptor binding affinities and kinetics: impact on T-cell activity and specificity. Immunology **126**, 165–76.

44 Miller, J. F. A. P. (2011) The golden anniversary of the thymus. Nat. Rev. Immunol., Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved. **11**, 489–95.

45 Zhu, J. and Paul, W. E. (2008) CD4 T cells: fates, functions, and faults. Blood **112**, 1557–69.

46 Zhu, J., Yamane, H. and Paul, W. E. (2010) Differentiation of effector CD4 T cell populations (*). Annu. Rev. Immunol. **28**, 445–89.

47 Harty, J. T., Tvinnereim, A. R. and White, D. W. (2000) CD8+ T cell effector mechanisms in resistance to infection. Annu. Rev. Immunol. **18**, 275–308.

48 LeBien, T. W. and Tedder, T. F. (2008) B lymphocytes: how they develop and function. Blood, American Society of Hematology **112**, 1570–80.

49 Blackburn, C. C. and Manley, N. R. (2004) Developing a new paradigm for thymus organogenesis. Nat. Rev. Immunol., Nature Publishing Group **4**, 278–89.

50 Tarantino, G., Savastano, S., Capone, D. and Colao, A. (2011) Spleen: A new role for an old player? World J. Gastroenterol. **17**, 3776–84.

51 Alemany, M. (2013) Adjustment to dietary energy availability: from starvation to overnutrition. RSC Adv., The Royal Society of Chemistry **3**, 1636.

52 Galic, S., Oakhill, J. S. and Steinberg, G. R. (2010) Adipose tissue as an endocrine organ. Mol. Cell. Endocrinol., Elsevier **316**, 129–139.

53 Coelho, M., Oliveira, T. and Fernandes, R. (2013) Biochemistry of adipose tissue: an endocrine organ. Arch. Med. Sci. **9**, 191–200.

54 Schäffler, A., Schölmerich, J. and Salzberger, B. (2007) Adipose tissue as an immunological organ: Toll-like receptors, C1q/TNFs and CTRPs. Trends Immunol. **28**, 393–9.

55 Hruskova, Z. and Biswas, S. K. (2013) A new "immunological" role for adipocytes in obesity. Cell Metab., Elsevier **17**, 315–7.

56 Vandanmagsar, B., Youm, Y.-H., Ravussin, A., Galgani, J. E., Stadler, K., Mynatt, R. L., Ravussin, E., Stephens, J. M. and Dixit, V. D. (2011) The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance. Nat. Med., Nature Publishing Group **17**, 179–188.

57 Ouchi, N., Parker, J. L., Lugus, J. J. and Walsh, K. (2011) Adipokines in inflammation and metabolic disease. Nat. Rev. Immunol. **11**, 85–97.

58 Chawla, A., Nguyen, K. D. and Goh, Y. P. S. (2011) Macrophage-mediated inflammation in metabolic disease. Nat. Rev. Immunol., Nature Publishing Group.

59 Hotamisligil, G. S. (2006) Inflammation and metabolic disorders. Nature, Nature Publishing Group **444**, 860–867.

60 Gregor, M. F. and Hotamisligil, G. S. (2011) Inflammatory mechanisms in obesity. Annu. Rev. Immunol., Annual Reviews **29**, 415–45.

61 Hotamisligil, G. S., Shargill, N. S. and Spiegelman, B. M. (1993) Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science **259**, 87–91.

62 Greenberg, A. S. and Obin, M. S. (2006) Obesity and the role of adipose tissue in inflammation and metabolism. Am. J. Clin. Nutr., Am Soc Nutrition **83**, 461S–465S.

63 Cai, D., Yuan, M., Frantz, D. F., Melendez, P. A., Hansen, L., Lee, J. and Shoelson, S. E. (2005) Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. Nat. Med. **11**, 183–90.

64 Saghizadeh, M., Ong, J. M., Garvey, W. T., Henry, R. R. and Kern, P. A. (1996) The expression of TNF alpha by human muscle. Relationship to insulin resistance. J. Clin. Invest. **97**, 1111–6.

65 Ehses, J. A., Perren, A., Eppler, E., Ribaux, P., Pospisilik, J. A., Maor-Cahn, R., Gueripel, X., Ellingsgaard, H., Schneider, M. K. J., Biollaz, G., et al. (2007) Increased number of islet-associated macrophages in type 2 diabetes. Diabetes **56**, 2356–70.

66 De Souza, C. T., Araujo, E. P., Bordin, S., Ashimine, R., Zollner, R. L., Boschero, A. C., Saad, M. J. A. and Velloso, L. A. (2005) Consumption of a fat-rich diet activates a proinflammatory response and induces insulin resistance in the hypothalamus. Endocrinology **146**, 4192–9.

67 Rodríguez-Hernández, H., Simental-Mendía, L. E., Rodríguez-Ramírez, G. and Reyes-Romero, M. A. (2013) Obesity and inflammation: epidemiology, risk factors, and markers of inflammation. Int. J. Endocrinol. **2013**, 678159.

68 La Cava, A. and Matarese, G. (2004) The weight of leptin in immunity. Nat. Rev. Immunol., Nature Publishing Group **4**, 371–379.

69 Friedman, J. M. and Halaas, J. L. (1998) Leptin and the regulation of body weight in mammals. Nature **395**, 763–70.

70 Gainsford, T., Willson, T. A., Metcalf, D., Handman, E., McFarlane, C., Ng, A., Nicola, N. A., Alexander, W. S. and Hilton, D. J. (1996) Leptin can induce proliferation, differentiation, and functional activation of hemopoietic cells. Proc. Natl. Acad. Sci. U. S. A. **93**, 14564–8.

71 Sanchez-Margalet, V. and Martin-Romero, C. (2001) Human leptin signaling in human peripheral blood mononuclear cells: activation of the JAK-STAT pathway. Cell. Immunol. **211**, 30–6.

72 Dixit, V. D., Mielenz, M., Taub, D. D. and Parvizi, N. (2003) Leptin induces growth hormone secretion from peripheral blood mononuclear cells via a protein kinase C- and nitric oxide-dependent mechanism. Endocrinology **144**, 5595–603.

73 Friedman, J. M. (2002) The function of leptin in nutrition, weight, and physiology. Nutr. Rev., Wiley Online Library **60**, S1–S14.

74 Frederich, R. C., Löllmann, B., Hamann, A., Napolitano-Rosen, A., Kahn, B. B., Lowell, B. B. and Flier, J. S. (1995) Expression of ob mRNA and its encoded protein in rodents. Impact of nutrition and obesity. J. Clin. Invest., American Society for Clinical Investigation **96**, 1658.

75 Myers Jr, M. G., Leibel, R. L., Seeley, R. J. and Schwartz, M. W. (2010) Obesity and leptin resistance: distinguishing cause from effect. Trends Endocrinol. Metab., Elsevier **21**, 643–651.

76 Münzberg, H. and Myers, M. G. (2005) Molecular and anatomical determinants of central leptin resistance. Nat. Neurosci., Nature Publishing Group **8**, 566–70.

77 Gong, Y., Ishida-Takahashi, R., Villanueva, E. C., Fingar, D. C., Münzberg, H. and Myers, M. G. (2007) The long form of the leptin receptor regulates STAT5 and ribosomal protein S6 via alternate mechanisms. J. Biol. Chem. **282**, 31019–27.

78 Tartaglia, L. A. (1997) The Leptin Receptor. J. Biol. Chem. **272**, 6093–6096.

79 Banks, A. S., Davis, S. M., Bates, S. H. and Myers, M. G. (2000) Activation of downstream signals by the long form of the leptin receptor. J. Biol. Chem. **275**, 14563–72.

80 El-Haschimi, K., Pierroz, D. D., Hileman, S. M., Bjørbaek, C. and Flier, J. S. (2000) Two defects contribute to hypothalamic leptin resistance in mice with diet-induced obesity. J. Clin. Invest. **105**, 1827–32.

81 Caro, J. F., Kolaczynski, J. W., Nyce, M. R., Ohannesian, J. P., Opentanova, I., Goldman, W. H., Lynn, R. B., Zhang, P. L., Sinha, M. K. and Considine, R. V. (1996) Decreased cerebrospinal-fluid/serum leptin ratio in obesity: a possible mechanism for leptin resistance. Lancet **348**, 159–61.

82 Vaisse, C., Halaas, J. L., Horvath, C. M., Darnell, J. E., Stoffel, M. and Friedman, J. M. (1996) Leptin activation of Stat3 in the hypothalamus of wild-type and ob/ob mice but not db/db mice. Nat. Genet. **14**, 95–7.

83 Howard, J. K., Cave, B. J., Oksanen, L. J., Tzameli, I., Bjørbæk, C. and Flier, J. S. (2004) Enhanced leptin sensitivity and attenuation of diet-induced obesity in mice with haploinsufficiency of Socs3. Nat. Med., Nature Publishing Group **10**, 734–738.

84 Kaszubska, W., Falls, H. D., Schaefer, V. G., Haasch, D., Frost, L., Hessler, P., Kroeger, P. E., White, D. W., Jirousek, M. R. and Trevillyan, J. M. (2002) Protein tyrosine phosphatase 1B negatively regulates leptin signaling in a hypothalamic cell line. Mol. Cell. Endocrinol. **195**, 109–18.

85 Banks, W. A. (2004) The many lives of leptin. Peptides **25**, 331–8.

86 Chen, K., Li, F., Li, J., Cai, H., Strom, S., Bisello, A., Kelley, D. E., Friedman-Einat, M., Skibinski, G. A., McCrory, M. A., et al. (2006) Induction of leptin resistance through direct interaction of C-reactive protein with leptin. Nat. Med. **12**, 425–32.

87 Rutter, M. K., Sattar, N., Tajar, A., O'Neill, T. W., Lee, D. M., Bartfai, G., Boonen, S., Casanueva, F. F., Finn, J. D., Forti, G., et al. (2013) Epidemiological evidence against a role for C-reactive protein causing leptin resistance. Eur. J. Endocrinol. **168**, 101–6.

88 Mori, H., Hanada, R., Hanada, T., Aki, D., Mashima, R., Nishinakamura, H., Torisu, T., Chien, K. R., Yasukawa, H. and Yoshimura, A. (2004) Socs3 deficiency in the brain elevates leptin sensitivity and confers resistance to diet-induced obesity. Nat. Med., Nature Publishing Group **10**, 739–743.

89 Müller, M. and Kersten, S. (2003) Nutrigenomics: goals and strategies. Nat. Rev. Genet. **4**, 315–22.

90 Kau, A. L., Ahern, P. P., Griffin, N. W., Goodman, A. L. and Gordon, J. I. (2011) Human nutrition, the gut microbiome and the immune system. Nature, Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved. **474**, 327–36.

91 Hotamisligil, G. S. and Erbay, E. (2008) Nutrient sensing and inflammation in metabolic diseases. Nat. Rev. Immunol. **8**, 923–34.

92 Kersten, S. (2010) Regulation of nutrient metabolism and inflammation. Results Probl. Cell Differ. **52**, 13–25.

93 Fielding, B. A., Callow, J., Owen, R. M., Samra, J. S., Matthews, D. R. and Frayn, K. N. (1996) Postprandial lipemia: the origin of an early peak studied by specific dietary fatty acid intake during sequential meals. Am. J. Clin. Nutr., Am Soc Nutrition **63**, 36–41.

94 Glass, C. K. and Olefsky, J. M. (2012) Inflammation and lipid signaling in the etiology of insulin resistance. Cell Metab. **15**, 635–45.

95 Watt, M. J., Hevener, A., Lancaster, G. I. and Febbraio, M. A. (2006) Ciliary neurotrophic factor prevents acute lipid-induced insulin resistance by attenuating ceramide accumulation and phosphorylation of c-Jun N-terminal kinase in peripheral tissues. Endocrinology, Endocrine Society **147**, 2077–85.

96 Aljada, A., Mohanty, P., Ghanim, H., Abdo, T., Tripathy, D., Chaudhuri, A. and Dandona, P. (2004) Increase in intranuclear nuclear factor kappaB and decrease in inhibitor kappaB in mononuclear cells after a mixed meal: evidence for a proinflammatory effect. Am. J. Clin. Nutr. **79**, 682–90.

97 Nguyen, M. T. A., Favelyukis, S., Nguyen, A.-K., Reichart, D., Scott, P. A., Jenn, A., Liu-Bryan, R., Glass, C. K., Neels, J. G. and Olefsky, J. M. (2007) A subpopulation of macrophages infiltrates hypertrophic adipose tissue and is activated by free fatty acids via Toll-like receptors 2 and 4 and JNK-dependent pathways. J. Biol. Chem. **282**, 35279–92.

98 Mariathasan, S., Weiss, D. S., Newton, K., McBride, J., O'Rourke, K., Roose-Girma, M., Lee, W. P., Weinrauch, Y., Monack, D. M. and Dixit, V. M. (2006) Cryopyrin activates the inflammasome in response to toxins and ATP. Nature **440**, 228–32.

99 Sampey, B. P., Vanhoose, A. M., Winfield, H. M., Freemerman, A. J., Muehlbauer, M. J., Fueger, P. T., Newgard, C. B. and Makowski, L. (2011) Cafeteria Diet Is a Robust Model of Human Metabolic Syndrome With Liver and Adipose Inflammation: Comparison to High-Fat Diet. Obesity, Wiley Online Library **19**, 1109–1117.

100 Larsen, L. F., Bladbjerg, E. M., Jespersen, J. and Marckmann, P. (1997) Effects of dietary fat quality and quantity on postprandial activation of blood coagulation factor VII. Arterioscler. Thromb. Vasc. Biol. **17**, 2904–9.

101 Cordain, L., Eaton, S. B., Sebastian, A., Mann, N., Lindeberg, S., Watkins, B. A., O'Keefe, J. H. and Brand-Miller, J. (2005) Origins and evolution of the Western diet: health implications for the 21st century. Am J Clin Nutr **81**, 341–354.

102 Abbott, S. K., Else, P. L., Atkins, T. A. and Hulbert, A. J. (2012) Fatty acid composition of membrane bilayers: importance of diet polyunsaturated fat balance. Biochim. Biophys. Acta **1818**, 1309–17.

103 Ratnayake, W. M. N. and Galli, C. (2009) Fat and Fatty Acid Terminology, Methods of Analysis and Fat Digestion and Metabolism: A Background Review Paper. Ann. Nutr. Metab., Karger Publishers **55**, 8–43.

104 Stevenson, D. E. and Hurst, R. D. (2007) Polyphenolic phytochemicals--just antioxidants or much more? Cell. Mol. Life Sci. **64**, 2900–16.

105 Howitz, K. T. and Sinclair, D. A. (2008) Xenohormesis: sensing the chemical cues of other species. Cell **133**, 387–91.

106 Perusse, L. and Bouchard, C. (1999) Genotype-Environment Interaction in Human Obesity. Nutr. Rev., Wiley Online Library **57**, 31–38.

107 Pérusse, L. and Bouchard, C. (2000) Gene-diet interactions in obesity. Am. J. Clin. Nutr., American Society for Nutrition **72**, 1285s–1290s.

108 Bouchard, C., Tremblay, A., Després, J.-P., Nadeau, A., Lupien, P. J., Thériault, G., Dussault, J., Moorjani, S., Pinault, S. and Fournier, G. (1990) The response to long-term overfeeding in identical twins. N. Engl. J. Med., Mass Medical Soc **322**, 1477–1482.

109 Bouchard, C. (2008) Gene–environment interactions in the etiology of obesity: defining the fundamentals. Obesity, Wiley Online Library **16**, S5–S10.

110 Faria, A. M. C., Gomes-Santos, A. C., Gonçalves, J. L., Moreira, T. G., Medeiros, S. R., Dourado, L. P. A. and Cara, D. C. (2013) Food components and the immune system: from tonic agents to allergens. Front. Immunol. **4**, 102.

111 Hunter, M., Wang, Y., Eubank, T., Baran, C., Nana-Sinkam, P. and Marsh, C. (2009) Survival of monocytes and macrophages and their role in health and disease. Front. Biosci. (Landmark Ed. **14**, 4079–102.

112 Terra, X., Montagut, G., Bustos, M., Llopiz, N., Ardèvol, A., Bladé, C., Fernández-Larrea, J., Pujadas, G., Salvadó, J. and Arola, L. (2009) Grape-seed procyanidins prevent low-grade

inflammation by modulating cytokine expression in rats fed a high-fat diet. J. Nutr. Biochem., Elsevier **20**, 210–218.

113 Terra, X., Valls, J., Vitrac, X., Mérrillon, J.-M., Arola, L., Ardèvol, A., Bladé, C., Fernández-Larrea, J., Pujadas, G. and Salvadó, J. (2007) Grape-seed procyanidins act as antiinflammatory agents in endotoxin-stimulated RAW 264.7 macrophages by inhibiting NFkB signaling pathway. J. Agric. Food Chem., ACS Publications **55**, 4357–4365.

114 Terra, X., Pallarés, V., Ardèvol, A., Bladé, C., Fernández-Larrea, J., Pujadas, G., Salvadó, J., Arola, L. and Blay, M. (2011) Modulatory effect of grape-seed procyanidins on local and systemic inflammation in diet-induced obesity rats. J. Nutr. Biochem., Elsevier **22**, 380–387.

115 Pallarès, V., Calay, D., Cedó, L., Castell-Auví, A., Raes, M., Pinent, M., Ardévol, A., Arola, L. and Blay, M. (2012) Additive, antagonistic, and synergistic effects of procyanidins and polyunsaturated fatty acids over inflammation in RAW 264.7 macrophages activated by lipopolysaccharide. Nutrition, Elsevier **28**, 447–457.

116 Shi, H., Kokoeva, M. V, Inouye, K., Tzameli, I., Yin, H. and Flier, J. S. (2006) TLR4 links innate immunity and fatty acid–induced insulin resistance. J. Clin. Invest., Am Soc Clin Investig **116**, 3015–3025.

117 Saccani, S., Pantano, S. and Natoli, G. (2003) Modulation of NF-kappaB activity by exchange of dimers. Mol. Cell **11**, 1563–74.

118 Schertzer, J. D. and Steinberg, G. R. (2014) Immunometabolism: the interface of immune and metabolic responses in disease. Immunol. Cell Biol.

119 Appeldoorn, M. M., Vincken, J.-P., Gruppen, H. and Hollman, P. C. H. (2009) Procyanidin dimers A1, A2, and B2 are absorbed without conjugation or methylation from the small intestine of rats. J. Nutr. **139**, 1469–73.

120 Serra, A., Macià, A., Romero, M.-P., Valls, J., Bladé, C., Arola, L. and Motilva, M.-J. (2010) Bioavailability of procyanidin dimers and trimers and matrix food effects in in vitro and in vivo models. Br. J. Nutr., Cambridge Univ Press **103**, 944.

121 Monagas, M., Urpi-Sarda, M., Sánchez-Patán, F., Llorach, R., Garrido, I., Gómez-Cordovés, C., Andres-Lacueva, C. and Bartolomé, B. (2010) Insights into the metabolism and microbial biotransformation of dietary flavan-3-ols and the bioactivity of their metabolites. Food Funct., The Royal Society of Chemistry **1**, 233–53.

122 Owens, B. (2014) Cell physiology: The changing colour of fat. Nature, Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved. **508**, S52–S53.

123 Iyer, A., Fairlie, D. P., Prins, J. B., Hammock, B. D. and Brown, L. (2010) Inflammatory lipid mediators in adipocyte function and obesity. Nat. Rev. Endocrinol., Nature Publishing Group **6**, 71–82.

124 Hodson, L., Skeaff, C. M. and Fielding, B. A. (2008) Fatty acid composition of adipose tissue and blood in humans and its use as a biomarker of dietary intake. Prog. Lipid Res., Elsevier **47**, 348–380.

125 Calder, P. C., Bond, J. A., Harvey, D. J., Gordon, S. and Newsholme, E. A. (1990) Uptake and incorporation of saturated and unsaturated fatty acids into macrophage lipids and their effect upon macrophage adhesion and phagocytosis. Biochem.J **269**, 807–814.

126 Shrestha, C., Ito, T., Kawahara, K., Shrestha, B., Yamakuchi, M., Hashiguchi, T. and Maruyama, I. (2013) Saturated fatty acid palmitate induces extracellular release of histone H3: A possible mechanistic basis for high-fat diet-induced inflammation and thrombosis. Biochem. Biophys. Res. Commun., Elsevier.

127 Fantuzzi, G. (2005) Adipose tissue, adipokines, and inflammation. J. Allergy Clin. Immunol. **115**, 911.

128 Wellen, K. E. and Hotamisligil, G. S. (2003) Obesity-induced inflammatory changes in adipose tissue. J. Clin. Invest., Am Soc Clin Investig **112**, 1785–1787.

129 Dixit, V. D. (2010) Thymic fatness and approaches to enhance thymopoietic fitness in aging. Curr. Opin. Immunol. **22**, 521–8.

130 Wood, G. W. (1985) Macrophages in the thymus. Surv. Immunol. Res. **4**, 179–91.

131 Lynch, H. E., Goldberg, G. L., Chidgey, A., Van den Brink, M. R. M., Boyd, R. and Sempowski, G. D. (2009) Thymic involution and immune reconstitution. Trends Immunol. **30**, 366–73.

132 Tarantino, G., Scalera, A. and Finelli, C. (2013) Liver-spleen axis: intersection between immunity, infections and metabolism. World J. Gastroenterol. **19**, 3534–42.

133 Gotoh, K., Inoue, M., Masaki, T., Chiba, S., Shimasaki, T., Ando, H., Fujiwara, K., Katsuragi, I., Kakuma, T., Seike, M., et al. (2012) A novel anti-inflammatory role for spleen-derived interleukin-10 in obesity-induced hypothalamic inflammation. J. Neurochem. **120**, 752–64.

134 Pereira, S. S., Teixeira, L. G., Aguilar, E. C., Oliveira, M. C. de, Savassi-rocha, A. L., Pelaez, J. M. N., Capettini, L. S. A., Diniz, M. T. C., Ferreira, A. V. M. and Alvarez-Leite, J. I. (2013) Modulation of Adipose Tissue Inflammation by Foxp3+ Treg Cells, Il-10 and Tgfβ In Metabolically Healthy Class Iii Obese Individuals. Nutrition, Elsevier.

135 Matarese, G., Procaccini, C., De Rosa, V., Horvath, T. L. and La Cava, A. (2010) Regulatory T cells in obesity: the leptin connection. Trends Mol. Med. **16**, 247–56.

136 Barsh, G. S., Farooqi, I. S. and O'Rahilly, S. (2000) Genetics of body-weight regulation. Nature, Nature Publishing Group **404**, 644–651.

137 Ordovas, J. M. (2008) Genotype–phenotype associations: modulation by diet and obesity. Obesity, Wiley Online Library **16**, S40–S46.

138 Speakman, J. R. (2004) Obesity: the integrated roles of environment and genetics. J. Nutr., Am Soc Nutrition **134**, 2090S–2105S.

139 Rothwell, N. J. and Stock, M. J. (1982) Energy expenditure of'cafeteria'-fed rats determined from measurements of energy balance and indirect calorimetry. J. Physiol., Physiological Soc **328**, 371–377.

140 Spiegelman, B. M. and Flier, J. S. (2001) Obesity and the regulation of energy balance. Cell, Cell Press **104**, 531–543.

141 Kanda, H., Tateya, S., Tamori, Y., Kotani, K., Hiasa, K., Kitazawa, R., Kitazawa, S., Miyachi, H., Maeda, S. and Egashira, K. (2006) MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. J. Clin. Invest., Am Soc Clin Investig **116**, 1494.

142 Wopereis, S., Radonjic, M., Rubingh, C., Erk, M. van, Smilde, A., Duyvenvoorde, W. van, Cnubben, N., Kooistra, T., Ommen, B. van and Kleemann, R. (2012) Identification of prognostic and diagnostic biomarkers of glucose intolerance in ApoE3Leiden mice. Physiol. Genomics **44**, 293–304.

143 Osborne, T. F. (2000) Sterol regulatory element-binding proteins (SREBPs): key regulators of nutritional homeostasis and insulin action. J. Biol. Chem. **275**, 32379–82.

144 Chen, Q., Wang, H., Liu, Y., Song, Y., Lai, L., Han, Q., Cao, X. and Wang, Q. (2012) Inducible microRNA-223 down-regulation promotes TLR-triggered IL-6 and IL-1β production in macrophages by targeting STAT3. PLoS One **7**, e42971.

145 Bian, J., Zhang, li, Bai, xuemei, Zhao, Y. and Liu, Z. (2013) Down-regulation of SOCS3 gene in hypothalamus attenuates diet-induced obesity in young rats. Int. J. Pediatr. Endocrinol., BioMed Central Ltd **2013**, O46.

6. Annex

Abbreviations

APC Antigen-presenting cell

Asc Apoptosis associated speck-like protein

CAF Cafeteria

COX Cyclooxygenase

DAMP Damage-associated molecular pattern

DC Dendritic cell

DHA Docosahexaenoic acid

ERK Extracellular signal-regulated protein kinase

FFA Free fatty acid

Foxp3 Forkhead-winged-helix transcription factor 3

GSPE Grape seed procyanidin extract

IFN-γ Interferon γ

Ig Immunoglobulin

IKK IκB kinase

IL Interleukin

iNOS Inducible nitric oxide synthase

JAK Janus kinase

JNK c-Jun N-terminal kinase

LPS Lipopolysaccharide

LRR Leucine-rich repeat

MAPK Mitogen-activated protein kinase

MCP-1 Macrphage chemotactic protein-1

miR MicroRNA

NEFA Non-esterified fatty acid

NF-κB Nuclear factor κB

NLR Nod-like receptor

NLRP3 Nod-like receptor protein 3

NO Nitric oxide

NSAID Non-steroidal anti-inflammatory drug

PAMP Pathogen-associated molecular pattern

PBMC Peripheral blood mononuclear cell

PGE_2 Prostaglandin E_2

PLA_2 Phospholipase A2

PPAR-γ Peroxisome-proliferator-receptor-activated γ

PRR Pattern recognition receptor

PUFA Polyunsaturated fatty acid

RHD Rel homology domain

ROS Reactive oxygen species

SFA Saturated fatty acid

STAT Signal transducer and activator of transcription

TIR Toll/IL-1 receptor

TLR Toll-like receptor

TNF-α Tumour necrosis factor α

Resum

La immunoteràpia nutricional es basa en fomentar la salut mitjançant el consum de compostos bioactius presents de manera natural en els aliments, com les procianidines, un tipus de flavonoid, i/o l'àcid docosahexaenoic (DHA), un àcid gras omega-3, optimitzant la funcionalitat del propi sistema immune i millorant així el seu paper com a responsable de la preservació de l'organisme enfront desestabilitzadors de la homeòstasis. La recerca que aquesta tesi abasta es centra en el paper del perfil nutricional en la regulació de la interacció immunitat-metabolisme (immunometabolisme).

Amb aquesta finalitat, en la primera part del treball presentat en aquesta tesi, es va determinar si els macròfags, les cèl·lules efectores de la immunitat innata, poden percebre de manera diferencial la composició de la dieta. Hem demostrat que els compostos bioactius dels aliments modulen, a nivell molecular, l'activació dels macròfags. A més, aquest efecte immunomodulador és dependent del compost, on factors intrínsecs com les propietats químiques o nutritives són determinants per la seva bioactivitat.

La segona part va tenir com a objectiu determinar el paper primordial del patró nutricional en la regulació de la interacció entre el sistema immune i el metabolisme. Utilitzant un model d'obesitat induïda per la dieta es va inferir que, mentre una dieta hipercalòrica provoca un deteriorament de l'immunometabolisme, el consum de bioactius com les procianidines i/o el DHA, pot enfortir-ne la relació.

A la tercera part, es va analitzar el paper de les interaccions gen-dieta en l'expressió fenotípica dels trets associats amb la obesitat. Per aquest motiu, dos races de rates congènites, fenotípicament diferents, van ser sotmeses a un desequilibri immunometabòlic com a conseqüència de la ingesta d'una dieta hipercalòrica. Es va deduir que les interaccions entre els factors genètics i nutricionals són fonamentals per a la susceptibilitat d'un genotip a l'obesitat induïda per la dieta.

Hem establert que el perfil nutricional és una eina poderosa per orientar la funcionalitat de l'eix immunometabòlic. A més, arribem a la conclusió que compostos bioactius presents en els aliments poden millorar-ne l'eficiència d'aquest eix, promovent així un estat saludable.

Agraïments

Primer que tot agrair a la Dra. Mayte Blay per haver acceptat la direcció d'aquesta Tesi, pel seu temps i suport durant aquesta etapa. També donar les gràcies a tots els membres del grup de recerca en Nutrigenòmica de la Universitat Rovira i Virgili, i en especial a tots els companys de viatge, perquè la dèria per la ciència compartida passa de ser una bogeria a ser una festa del coneixement.

Dr. Enrico Petretto and Dr. Jacques Behmoaras, I am very grateful for the opportunity to work in your group, for your involvement in this project and for the best tiramisu in London. Also thank you to the members of your team for their help during my stay in London and for your advice.

Als de casa, perquè tant el component genètic com l'ambient juguen un paper fonamental en l'expressió del fenotip. Així que moltes gràcies pel material genètic i per l'ambient que m'heu proporcionat, espero que el fenotip pugui arribar a estar alguna vegada a l'alçada.

Agrair a tothom que d'una manera o altre ha contribuït en aquesta Tesi. I recordeu, tal com deia l'escriptor britànic Miles Kington, "*el coneixement és saber que el tomàquet és una fruita, la sabiesa és no afegir-lo a una macedònia*"…

Curriculum Vitae

Neus B. Martínez Micaelo was born on August 7th, 1983, in Reus, Catalonia. After completing her pre-university education, she obtained a BEng. in Agronomic Engineering at Universitat Rovira i Virgili (Tarragona), a MEng in Food Science Engineering at Univesitat de Lleida (Lleida) and a BSc in Biotechnology at Universitat Rovira i Virgili. During her MEng training, she was awarded a personal research grant to study the role of corn and soy genetic backgrounds in the production of bioethanol at Iowa State University (Iowa, USA), and she wrote her dissertation on the electrochemical identification of the zinc concentration in wines under the supervision of Dr. Josep Galceran and Dr. Encarna Companys in the Chemistry Department at Universitat de Lleida. She obtained her MSc degree in Nutrition and Metabolism at Universitat Rovira i Virgili. She was appointed as a PhD student at the same university in the Nutrigenomics research group of the Department of Biochemistry and Biotechnology under the supervision of Dr. Mayte Blay. During her PhD, she completed a European phase in the Integrative Genomics and Medicine Group of the MRC Institute of Clinical Sciences at Imperial College London, supervised by Dr. Enrico Petretto and Dr. Jacques Behmoaras. The results of this research are presented in this thesis.

List of Publications

Full Papers

Martinez-Micaelo, N., Gonzalez-Abuin, N., Pinent, M., Ardevol, A., Blay, M. (2014) Procyanidin B_2 inhibits inflammasome-mediated IL-1β production in lipopolysaccharide-stimulated human macrophages. (Submitted)

Martinez-Micaelo, N., Gonzalez-Abuin, N., Pinent, M., Ardevol, A., Blay, M. (2014) Dietary fatty acid composition is sensed by the NLRP3 inflammasome in human macrophages. (Submitted)

Martinez-Micaelo, N., Gonzalez-Abuin, N., Pinent, M., Ardevol, A., Blay, M. (2014) The role of the peripheral lymphoid organs, thymus and spleen, in the obesity-triggered immune response. (Submitted)

Martinez-Micaelo, N., Gonzalez-Abuin, N., Pinent, M., Ardevol, A., Blay, M. (2014) Procyanidins and docosahexaenoic acid suppress cafeteria diet-triggered inflammation and boost immune system to counteract obesity-induced immunosuppression. (Submitted)

Martinez-Micaelo N., González-Abuín N., Ardévol A., Pinent M., Behmoaras J., Petretto E., Blay M. (2014) Leptin signalling underlies the differential response of LEW and WKY rats to diet-induced obesity. (In preparation)

González-Abuín N., **Martínez-Micaelo N.,** Margalef M., Blay M., Arola-Arnal A., Muguerza B., Ardévol A., Pinent M. (2014) Grape-seed derived procyanidins inhibit dipeptidyl-peptidase 4 and increase plasma glucagon-like peptide-1. (Submitted)

González-Abuín N., **Martínez-Micaelo N.**, Blay M., Ardévol A., Pinent M. (2014) Grape-seed procyanidins prevent the cafeteria diet-induced decrease of glucagon-like peptide-1 production. Journal of Agricultural and Food Chemistry 62(5), 1066-1072.

González-Abuín N., **Martínez-Micaelo N.**, Blay M., Green B.D., Pinent M., Ardévol A. (2013) Grape-seed procyanidins modulate cellular membrane potential and enteroendocrine hormone secretion. American Journal of Physiology-Cell Physiology 306(5), C485-92.

González-Abuín N., **Martínez-Micaelo N.**, Blay M., Pujadas G., García-Vallvé S., Pinent M., Ardévol A. (2012) Grape-seed derived procyanidins decrease dipeptidyl-peptidase 4 activity and expression Journal of Agricultural and Food Chemistry 60, 9055-9061.

Martinez-Micaelo, N., Gonzalez-Abuin, N., Pinent, M., Ardevol, A., Blay, M. (2012) Procyanidins and inflammation: Molecular targets and health implications. Biofactors 38(4), 257-265.

Martinez-Micaelo, N., Gonzalez-Abuin, N., Tera, X., Richart, C., Pinent, M., Ardevol, A., Blay, M. (2012) Omega-3 docosahexaenoic acid and procyanidins inhibit cyclooxygenase activity and attenuate NF-κB activation through a p105/p50 regulatory mechanism in macrophage inflammation. Biochemical Journal **441**, 653-663.

Abstracts

Martinez-Micaelo, N., Gonzalez-Abuin, N., Pinent, M., Ardevol, A., Blay, M. (2012) Docosahexaenoic acid (DHA) and procyanidins dimers (B_1 and B_2) and trimer (C_1) are strong and selective inhibitors of Cyclooxygenase-1 and 2. FEBS Journal. **279**, 398. Presented at 22nd IUBMB and 37th FEBS Congress, Sevilla, Spain.

Gonzalez-Abuin, N., **Martinez-Micaelo, N.**, Blay, M., Pinent, M. and Ardevol, A. (2012) Grape-seed derived procyanidins decrease intestinal dipeptidyl-peptidase 4 (DPP4) activity and improve glycemia. FEBS Journal. **279**, 398. Presented at 22nd IUBMB and 37th FEBS Congress, Sevilla, Spain.

Martinez-Micaelo, N., Gonzalez-Abuin, N., Pinent, M., Ardevol, A., Blay, M. (2011) Procyanidin dimer B_2 and DHA decrease cytokine-mediated inflammatory response in *ex-vivo* LPS-stimulated PBMC. Presented at NuGOweek 2011 Congress, International Nutrigenomics Organization, Wageningen, The Netherlands.

Martinez-Micaelo, N., Gonzalez-Abuin, N., Pinent, M., Ardevol, A., Blay, M. (2011) Anti-inflammatory effects of procyanidins and docosahexaenoic acid in human macrophages THP-1. Inflammation Research. **60**, 258-259. Presented at 10th World Congress on Inflammation, Paris, French.

Gonzalez-Abuin, N., **Martinez-Micaelo, N.**, Blay, M., Pinent, M. and Ardevol, A. (2011) Grape-seed derived procyanidins decrease intestinal dipeptidyl peptidase 4 (DPP4) activity and expression. FEBS Journal. **278**, 192-192. Presented at 36th FEBS Congress, Torino, Italy.

Gonzalez-Abuin, N., **Martinez-Micaelo, N.**, Blay, M., Ardevol, A. and Pinent, M. (2010) Grape-seed derived procyanidins inhibit dipeptidyl peptidase 4 (DPP4) activity. FEBS Journal. **277**, 62-63. Presented at 35th FEBS Congress, Gothenburg, Sweden.

Martinez-Micaelo, N., Gonzalez-Abuin, N., Pinent, M., Ardevol, A., Blay, M. (2010) Procyanidins and Docosahexaenoic acid modulates inflammatory response by in THP-1 macrophages. Presented at Macrophages and inflammation Barcelona BioMed Conferences.

Martinez-Micaelo, N., Gonzalez-Abuin, N., Pinent, M., Ardevol, A., Blay, M. (2010) Role of procyanidins and Docosahexaenoic acid in macrophage inflammation. Presented at Inflammatory Diseases and Immune Response, FEBS & EFIS Workshop, Vienna, Austria.

Other publications

Galceran, J., Chito, D., **Martinez-Micaelo, N.**, Companys, E., David, C. and Puy, J. (2010) The impact of high Zn degrees concentrations on the application of AGNES to determine free Zn(II) concentration. Journal of Electroanalytical Chemistry. **638**, 131-142.

Companys, E., Naval-Sanchez, M., **Martinez-Micaelo, N.**, Puy, J. and Galceran, J. (2008) Measurement of free zinc concentration in wine with AGNES. Journal of Agricultural and Food Chemistry. **56**, 8296-8302.