PRECLINICAL EVALUATION OF THE IMMUNOMETABOLIC EFFECTS OF OCTAPEPTIDE GPETAFLR

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CERTIFICAN:

Que la Tesis Doctoral titulada "**PRECLINICAL EVALUATION OF THE IMMUNOMETABOLIC EFFECTS OF OCTAPEPTIDE GPETAFLR**" realizada por Dª. Ana Lemus Conejo para optar al grado de Doctor, ha sido llevada a cabo bajo nuestra dirección.

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V.º B.º
El Tutor

Dr. Víctor Sánchez Margalet

A mis padres,
A mis hermanos,
A mis amigos

“Todo momento es un regalo de la vida”

Thich Nhat Hanh

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CHAPTER 01

General Introduction & Thesis Outline

HISTORICAL BACKGROUND

There is a strong and growing interest in legumes as a protein source that can replace animal protein, both because of its lower production cost and its lower impact on environmental pollution. The legume family (or legumes) are those plants that produce a pod with seeds inside [1,2]. The term "legumes" is defined by the Food and Agriculture Organization of the United Nations to mean those crops harvested solely for the purpose of obtaining the dry seed of legumes. This excludes pulses used to obtain oils such as soya and peanuts and those harvested green for food such as green beans, peas and sprouts considered as vegetables [3]**.** Among these legumes we could find the genus Lupinus, which in addition to its nutritional interest it could be presented as an ingredient in the nutraceutical industry [4,5]. Legumes, which have been considered over the years as food for the poor, are re-emerging as a healthy food staple in rich societies. It has been suggested that combining and supplementing diets based on cereals, roots and tubers with legumes may be one of the solutions to malnutrition in poor and developing countries. In addition, in developed countries, regular consumption of legumes would increase the biodiversity of the diet [3]**.** Legumes have been considered an inexpensive dietary source of protein and their protein content (especially in Lupines) is higher than that of most other plant foods [3].

Lupinus angustifolius L. belongs to the Fabaceae or Leguminosae family, and within this to the Faboideae subfamily and specifically to the Genisteae tribe which soya, chickpeas and beans also belong [6]. The **Table 1** shows the taxonomy of the specie *Lupinus angustifolius* L.,

specifying the kingdom from which it comes, as well as its division, family and finally the species to which it belongs.

Table 1. Taxonomy of the specie *Lupinus angustifolius* L. (USDA-ARS GRIN Taxonomy)

Kingdom	Plantae
Subkingdom	Viridiplantae
Infrakingdom	Streptophyta
Superdivision	Embryophyta
Division	Tracheophyta
Subdivision	Spermatophytina
Class	Magnoliopsida
Superorder	Rosanae
Order	Fabales
Family	Fabaceae/ Leguminosae
Genus	Lupinus L.
Species	*Lupinus angustifolius* L.

The sweet varieties of this legume have been cultivated for a long time in various parts of the world such as Europe, South America, and Australia [5,7]. There are about 200 varieties of Lupinus that are classified as "Old World" species that are spread between the Mediterranean and North and East Africa and "New World" species in South and North America [8]**.** Only four of these numerous species are today cultivated for human or animal consumption. These are: *Lupinus albus* L., known as white lupine*; Lupinus angustifolius* L., known as blue or narrow-leaved lupin, *Lupinus luteus* L., known as yellow lupine, and *Lupinus mutabilis* L., known as Andean lupine. The by-name sweet refers to the lower number of toxic alkaloids, compounds that are secondary

metabolites, compared to the bitter varieties [9,10]**.** *L. albus* is mainly grown in Europe, *L. angustifolius* is largely produced in Australia, *L. luteus* is widely distributed in the Mediterranean region and *L. mutabilis* is cultivated in South America [8,11,12]**.** The different species of Lupinus have been domesticated throughout history. *L. mutabilis* was domesticated in the Andean states between 700-600 BC, *L. luteus* and *L. angustifolius* were domesticated in the Baltic countries in the 1860s. Lupines are not native to Australia, as numerous species of lupines were introduced to Australia by botanists in the mid-19th century, so it was not a food consumed by indigenous Australians [13]. **Figures 1** and **2** show the different seeds and plants of the comestible Lupinus species

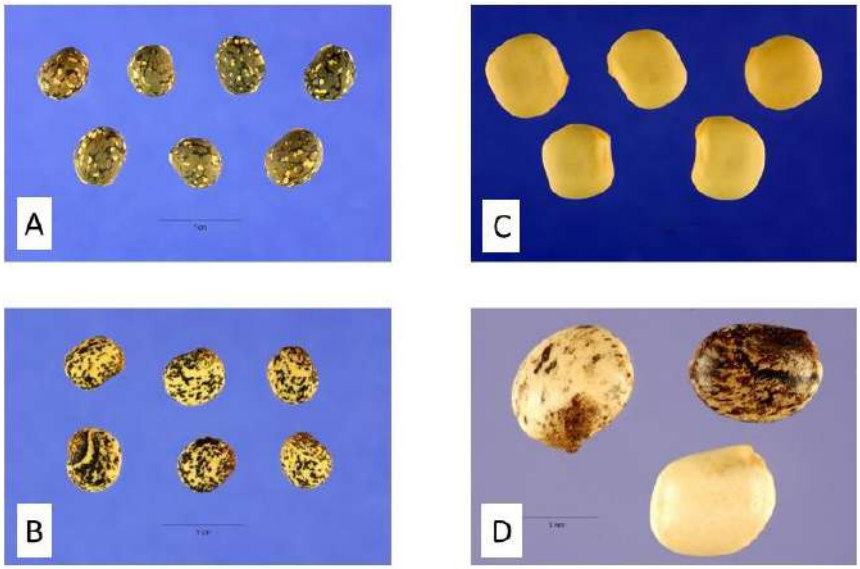

Figure 1. Picture of the seeds of the four comestible Lupinus, also known as sweet Lupinus. (A) *Lupinus angustifolius* L.; (B*) Lupinus luteus* L.; (C) *Lupinus albus* L.; (D) *Lupinus mutabilis* L. The PLANTS Database (https://plants.usda.gov, 17 February 2021) National Plant Data Team, Greensboro, NC 27401-4901 USA.

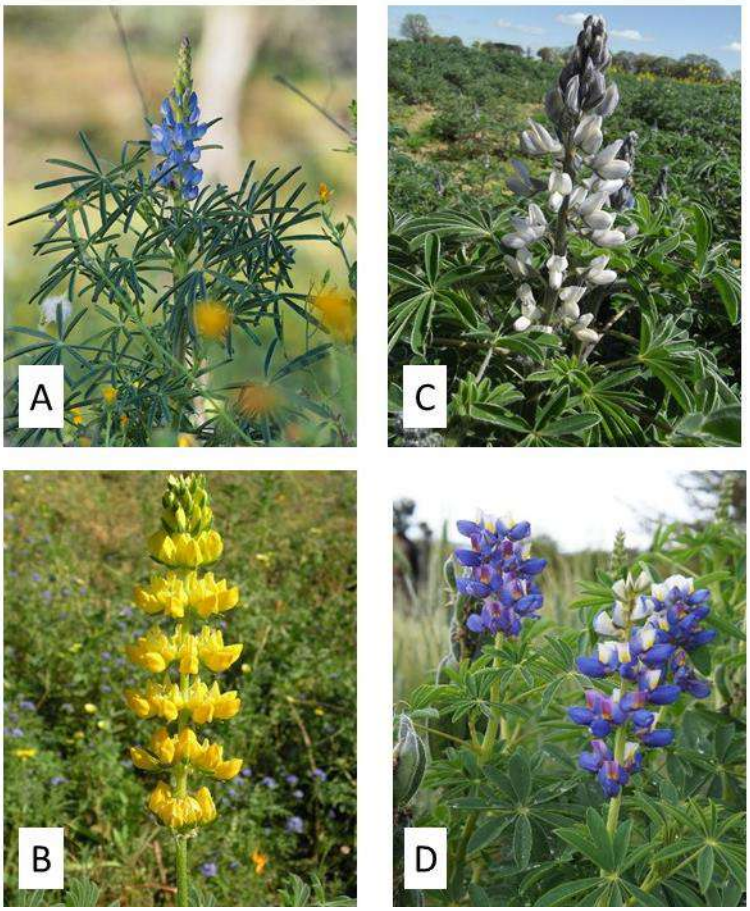

Figure 2. Picture of the plants of the four comestible Lupinus, also known as sweet Lupinus. (A) *Lupinus angustifolius* L.; (B*) Lupinus luteus* L.; (C) *Lupinus albus* L.; (D) *Lupinus mutabilis* L. The PLANTS Database (https://plants.usda.gov, 17 February 2021) National Plant Data Team, Greensboro, NC 27401-4901 USA.

Their main use is in food, both for humans and animals, although they have recently been found to be an excellent candidate for the development of functional foods. Lupines have highly desirable characteristics for cultivation due to their ability to fix nitrogen and to grow in infertile soils, and its value in crop season rotations with cereal grains, hay and oil seeds [14]. World producers of Lupinus include Australia, which is the largest producer with 64% of production, and

Europe with 29% of production [3]. *Lupinus angustifolius* L. is the most widely grown seed in Australia and Northern Europe while *L. albus* is the most widely grown in Southern Europe. It should be noted that the domestication and cultivation of lupine species has been largely hampered by their alkaloid content [6,15,16]**.** Returning to a diet rich in vegetables as our ancestors did or as some cultures do today will provide good nutrition with less environmental impact, as well as producing food at a lower economic cost. Today we know the role of many plant foods in reducing the risk of chronic and systemic diseases such as cardiovascular disease, cancer, familial hypercholesterolemia, obesity, and diabetes and hypertension [2,17]**.** This benefit is due to the bioactive components contained in these foods of plant origin. These components include proteins and peptides, whose beneficial effects on health have been widely studied [18,19]**.** *Lupinus angustifolius* L. is becoming a legume with quite interesting nutritional and bioactive properties, with a very positive potential for human nutrition [20,21]**.**

The industrial uses of sweet lupine are varied and its use as a food ingredient is increasing every day. It is used in bakery products such as bread, biscuits or pasta products improving the nutritional value of these foods by reducing the content of refined carbohydrates and doubling the content of protein and dietary fibre. It has also been converted into milk and is even used in the production of ice cream. In addition, lupine grains in flakes are starting to be used in recipes as sauces or falafels [3,13]**.**

NUTRITIONAL COMPOSITION OF *LUPINUS ANGUSTIFOLIUS* L.

Lupine is a seed with a high protein content, over 35% of its dry weight, low fat content (6%), no starch (with a glycaemic index very low), rich in dietary fibre (28%), as well as containing micronutrients such as iron (Fe), zinc (Zn), copper (Cu), calcium (Ca) and phosphorus (P), and bioactive compounds such as polyphenols, phytosterols and peptides. In addition, Lupinus is cholesterol-free and high in essential amino acids. All these values depend on the environmental or genetic conditions of the seeds [14,18,22-24]**.** Lupine seeds have a high protein content, similar to that of soya, as well as being remarkably high in total dietary fibre at ~40 g/100 g dry basis (db). The average seed protein content (g/100 g db) reported in the literature was *L. albus*, 38.2*; L. angustifolius*, 33.9*; L. luteus*, 42.2; and *L. mutabilis*, 43.3 [14]**.** Lupine is a good source of arginine (3.6 g/100 g) but contains lower levels of sulphur-containing amino acids such as cysteine (0.4 g/100) [18]**.** The seed contains between 37.5 and 40.2 g/100 g db of total dietary fibre, of which 74% is insoluble dietary fibre and 26% is soluble dietary fibre and 5-9% hemicellulose [3,23]**.** In addition, oligosaccharides are also present in the seed, notably the raffinose family [14]. The hull fraction concentrates most of the phytochemicals while the protein fraction is mainly concentrated in the cotyledons [25]**. Table 2** shows chemical composition of the four domesticated Lupine species and **Table 3** shows the specific chemical composition of *Lupinus angustifolius* L.

Table 2. Chemical composition of four Lupine species [6].

	L.angustifolius	*L.luteus*	*L.mutabilis*	*L.albus*
Crude Protein (% of DM*)	31-37	37-38	32-52	33-47
Crude fiber (% of DM)	15-17	12-15	10	13-16
Metabolized energy (MJ/ Kg DM)	12-13	10	na	13-16
Oil (%)	6-7	5-9	13-24	6-13
Total oligosaccharides (% of DM)	8-9	na	na	7-8
Non-starch polysaccharides (%)	47-51	na	na	18

*DM: Dry Matter

Table 3. Chemical composition of *Lupinus angustifolius* L. [23]**.**

	L. angustifolius **(g/100 g DM*)**
Dry matter	90.6
Proteins	33.0
Ashes	3.7
Crude fat	6.8
Crude fibre	14.0
Alkaloids	0.06

***DM: Dry matter

Lupine contains about 6-7% of fat, where the lipids are mostly in the form of triglycerides. The lipid content is 19% in saturated fatty acids, 33% in monounsaturated and 48% in polyunsaturated, where omega-3 linolenic acid represents 6% of the total lipids [14]. The fatty acid profile of Lupinus (**Table 4**) is similar to that of other oils such as peanut and rapeseed, and is rich in oleic (C 18:1), linoleic (C 18:2) and linolenic (C 18:3), palmitic (C 16:0) and stearic (C 18:0) fatty acids [11,26]**.** It is important to note that the omega-3/omega-6 ratio is 1:3, which means that it is a very favourable proportion [3]**.**

Table 4. Fatty acid profile of *Lupinus angustifolius* L. [27].

Fatty acid	L. angustifolius (%)
16:0	11.0
18:0	3.8
18:1	38.2
18:2 (ω-6)	37.1
18:3 (ω-3)	5.3
20:0 & 20:1	1.2
22:0 & 22:1	1.9
ω-6/ω-3	7.0

An extremely important nutritional component in legumes is protein, with a significant content of lysine and other essential amino acids, but deficient in sulphur amino acids [28]. Proteins in legumes can

be separated by differential solubility into albumen, globulin, prolamin and glutelin, with globulin being the most abundant and prolamin and glutelin being very scarce or even absent. Globulins are storage proteins, which serve as sources of nitrogen and carbon skeleton for the emerging plant, which can be separated by ultracentrifugation into Leguminosae (11S) and vicillins (7S) and additional minor fractions [27-29]**.** Albumin is a water-soluble protein with enzymatic, regulatory and plant defence functions in the case of lectin or storage functions [30]**.** Legume proteins have a composition of amino acids that are complementary to cereals, so the combination of both food sources provides a complete protein [25]**.**

As previously mentioned, 58%, 85% and 60% of the total protein of *L. mutabilis, L. albus* and *L. angustifoliu*s, respectively, is associated with two Osborne fractions: albumin and globulin. From these data, it is possible to understand the extractability of the protein using traditional isolation techniques [9]**.** According to Osborne's classification, Lupinus can be fractionated into water-soluble albumin, salt-soluble globulins, alcohol-soluble prolamins and acid/alkali-soluble glutelins. The main globulin fractions are α-conglutin (35-37 g/100 g total protein), β-conglutin (44-45 g/100 g total protein), γ-conglutin (4-5 g/100 g total protein), δ-conglutin (10-12 g/100 g total protein) [8].

The grain of *Lupinus angustifolius* L. contains an important source of vegetable protein, which is low in starch and free of gluten. The largest fraction of proteins are globulins or conglutins, with albumin constituting the rest. There are four types or subfamilies of globulins in lupine seeds. Among the proteins, the conglutins have four subfamilies called α, β, γ, and δ-conglutin. Approximately 80% of the protein is α-

conglutin (11s Leguminosae-like globulins) and β-conglutin (7s vicilin-like globulins) [5,20]**.** Two of these subfamilies are storage proteins: α-conglutin and β-conglutin [30]**.** β-conglutins are the most highly expressed family of conglutins in *Lupinus angustifolius* L., and constitute 44% w/w of the total conglutin transcription content in the seeds. Its molecular weight is in the range of 143-260 kDa, and consists of precursor and proteolytically divided polypeptides of molecular weights around 15-72 kDa. The second most abundant globulin is α-conglutin, which represents 33% w/w of the total conglutins. It is a hexamer with a native molecular weight of 300-430 kDa, formed by subunits that vary between 53 and 74 kDa and which consist of acid and basic chains linked together by a disulphide bond. The third conglutin is δ-conglutin and represents 12% w/w of the total conglutins. The minor globulin of the seed is γ-conglutin and represents 4-5% w/w of the total globulins. It has a monomeric molecular weight approximately of 47-48 kDa, and consists of two polypeptide chains of 27-30 kDa and 16-18 kDa respectively [8,23]**.** The main limiting amino acids of lupine are the sulphur amino acids (methionine and cysteine), valine, and tryptophan (**Table 5**) [11,14,31]**.**

Table 5. Amino acid composition of lupine seeds [32].

Amino acid	*L. albus*	*L.angustifolius*	*L.mutabilis*	*L.luteus*
	(g/100 g of Crude Protein)			
Aspartic acid	10.52	10.35	10.36	n. a
Threonine	3.65	3.76	3.61	3.0
Serine	4.63	4.05	4.04	n. a
Glutamic acid	21.66	21.20	22.45	n. a
Proline	4.38	4.43	4.23	n. a
Glycine	4.07	4.65	4.25	n. a
Alanine	3.45	3.89	3.73	n. a
Cysteine	1.74	1.77	1.46	2.4
Valine	4.35	4.56	4.32	3.4
Met+Cys	2.50	2.58	2.20	3.0
Methionine	0.76	0.80	0.73	0.6
Isoleucine	4.71	4.56	4.82	3.6
Leucine	7.74	7.52	6.87	7.8
Tyrosine	4.32	3.59	4.20	2.9
Phe+Tyr	8.39	7.81	8.17	6.6
Phenylalanine	4.07	4.22	3.97	3.7
Lysine	5.02	5.62	5.93	4.5
Histidine	2.38	2.91	2.95	3.1
Arginine	10.86	10.56	10.67	9.1
Tryptophan	0.76	1.06	0.97	0.9

*n.a: not available

In terms of anti-nutrients, sweet Lupinus contains very low levels of phytochemicals such as alkaloids, saponins, lectins, and phytates compared to its bitter analogues and other legumes [24]. In addition, they have negligible amounts of trypsin and chymotrypsin

inhibitors (<0.1 mg/kg), compounds that interfere with digestion and are present in other legumes [3]. Narrow-leaved lupin (NLL) has a low content of anti-nutritional factors among which saponins stand out with a content of 570 mg/kg of seed (0.057%) approximately. Tannins, which can act as metal ion chelators inhibiting the absorption of non-haem iron and interfere with the absorption of proteins, are present in a low proportion with a content of between 0.013-0.077 mg/g [14]. The sweet lupine must have low alkaloid levels of <200 mg/kg according to food standards. In contrast, bitter varieties are ten thousand times higher in alkaloids with an amount of 15.000-22.000 mg/kg [3,14].

As regards phytochemicals, Lupinus is rich in polyphenols, phytosterols, and squalene. The most numerous phenolic compounds present in *L. angustifolius* seeds are, flavones, phenolic acids and isoflavones that represents 76%, 19% and 4% of the total identified phenols, respectively [10,19,21,30,33]. The main anti-nutritional factors (ANFs) in Lupinus are alkaloids (quinolizidine, sparteine and lupanine), phytates, protease inhibitors and lectins. Lupine has a high content of non-starch polysaccharides (NSP) whose main component is galactan, which consists of different amounts of arabinose monosaccharides and galactose [28,34]. The concentration of alkaloids in *L. angustifolius* is 200 mg/kg, but these alkaloids have a low toxicity [18]. In addition, lupine contains beneficial phytochemicals in its composition such as carotenoids, composed of 134 μg/g of zeaxanthin and 50.43 μg/g of β-carotene, which gives the flour its unique yellow colour, and also contains various phenolic compounds, such as catechins [3].

PROTEIN ISOLATES AND HYDROLYSATES FROM *LUPINUS ANGUSTIFOLIUS* L.

Legumes are considered an ideal food for the preparation of protein ingredients, concentrates and isolates, due to their low cost and high protein content, as well as their wide acceptance as the second most popular food source after cereals. It should be noted that proteins from legumes are a low-cost alternative and have bioactive characteristics that are beneficial to health [25]**.** They also have very interesting techno-functional properties that can be used by the food industry, such as foaming and emulsification, water and fat absorption and gelification. The functional properties of proteins differ according to the proportion of albumin to globulins and legumes to vicillins, secondary structure, composition of amino acids, molecular weight, hydrophobicity and surface charge [25]**.** In general, lupine proteins have good protein solubility, good emulsion and moderate gelling properties compared to soy proteins [11]**.**

One of the most interesting components of Lupinus are the proteins and in particular the peptides that compose it. These can be obtained by hydrolysis, which can be enzymatic by proteolytic enzymes. Obtaining these hydrolysates involves protein foods with much lower allergenicity and easier to digest, in addition to the biological activities contained in these compounds [15]**.** Furthermore, the amino acid concentration changes depending on whether it is seed, flour or protein hydrolysate (**Table 6**) and the chemical composition varies between the seed and the hydrolysate**,** (**Table 7**).

Table 6. Amino acid profile of Lupin seed, Lupin Flour and Lupin isolate [35,36].

	Lupin seed	Lupin Flour	Lupin Protein Isolate	FAO*
		(g/100 g of Crude Protein)		
Aspartic acid	8.95	11.6	6.9	
Glutamic acid	22.74	25.6	19.9	1.9
Serine	4.39	7.1	7.7	
Histidine	3.75	2.7	2.7	3.4
Glycine	3.98	4.6	5.3	
Threonine	3.27	4.9	6.4	
Arginine	12.17	11.5	13.5	
Alanine	3.03	3.8	3.0	
Proline	5.58	4.6	8.8	
Tyrosine	3.47	5.9	7.5	6.3
Valine	3.57	3.9	4.8	3.5
Methionine	0.53	1.3	0.7	2.5
Cysteine	1.38	3.5	2.9	
Isoleucine	3.54	5.5	5.9	2.8
Leucine	5.90	8.7	9.4	6.6
Phenylalanine	3.48	5.2	6.1	
Lysine	5.26	5.4	4.3	5.8
Tryptophan		0.6	0.6	

*FAO/WHO/ONU. Energy and protein requirement, 1985.

Table 7. Chemical composition of *Lupinus angustifolius* L. [35].

	Lupin Flour	Lupin Protein Isolate
	(g/100 g DM*)	
Lipids	13.6 ±2.0	3.2±0.5
Moisture	7.9 ±0.6	3.4±1.5
Ash	2.1±0.1	0.7±0.0
Protein content	33.8±6.9	87.4±0.2
Polyphenols	>0.1	>0.1
Fibre	39.9±6.1	4.0±0.3
Soluble sugars	2.7±0.1	1.3±0.1

***DM: Dry matter

Various techniques have been used to produce lupine protein concentrates/isolates, including alkaline/neutral extraction followed by isoelectric precipitation (IEP) or ultrafiltration (UF), salt extraction/micellization and air classification but the most common is extraction in an alkaline medium and subsequent precipitation by isoelectric point (pI) or recovery by ultrafiltration [5].

Plant protein isolates are prepared from plant materials containing more than 90% protein in their composition. In the process of obtaining protein isolates, non-protein components are eliminated or reduced through solubilization of proteins and recovery of the solubilized protein to achieve a final product with 80-90% protein [14,37]. They are used both to improve the nutritional composition and to improve the functional characteristics of the food. There is a wide variety of techniques for the extraction and fractionation of proteins and peptides based on their physicochemical and structural characteristics, such as solubility, hydrophobicity, molecular weight and pI [37]. The removal of lipids, nucleic acids, phenolic compounds, carbohydrates, proteolytic and oxidative enzymes, and pigments is crucial to obtain protein isolates [38].

From these protein isolates, which have low solubility and allergenicity potential, protein hydrolysates are developed that solve both problems. In protein hydrolysates, peptide bonds are broken, releasing smaller peptides and free amino acids. The degree of hydrolysis is the fundamental property of a hydrolysate and determines the percentage of broken peptide bonds in relation to the original protein. Bioactive peptides are small sequences of amino acids that are inactive in the original protein but when released by proteolysis, either chemical or enzymatic, they exert a certain biological action such as antioxidant, anti-inflammatory or antibacterial activity among others [39].

Hydrolysis can be done by chemical or enzymatic methods. In the first one, acids or bases are used producing a chemical hydrolysis that can have harmful effects on the quality of the hydrolysate since they can destroy L-amino acids and form toxic compounds such as lysinoalanine. In the enzymatic field, enzymes such as protease break the peptide bonds under milder pH and temperature conditions, thus reducing the formation of undesirable compounds. In addition, the nutritional value of the original protein is maintained, since no degradation of the hydrolysed components occurs [39]. The Lupine protein hydrolysates (LPH) was obtained by the hydrolysis of lupine protein isolate (LPI) with Izyme and Alcalase, two food grade proteases produced by Novozymes. The enzyme has similar activity to trypsin, while Alcalase is a non-specific endoprotease [38]. The LPH was fractionated through an ultrafiltration membrane with a molecular

weight cut-off of 10 kDa. The fraction exhibiting the highest anti-inflammatory activity after ultrafiltration was further purified in a 3mL RPC Resource column. The sequence of the purified peptide was identified by sequence analysis using a nanoHPLC coupled to a Polaris Q ion trap mass spectrometer. Then, it was analysed by mass spectrometry and a peptide larger than 889.6 Da was identified whose sequence was Gly-Pro-Glu-Thr-Ala-Phe-Leu-Arg [39]. The whole process is schematized in **Figure 4.**

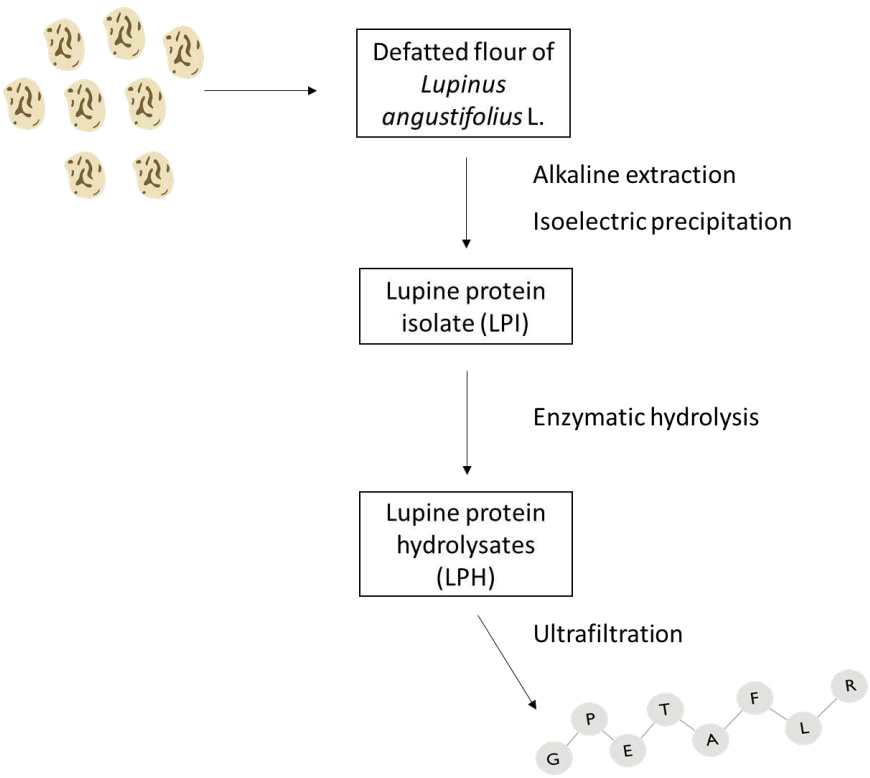

Figure 4. Diagram of the physicochemical processes to obtain the GPETAFLR peptide.

In *Lupinus angustifolius* L. the molecular weight range of peptides within β-conglutin was 17-100 kDa. The electrophoretic analysis α-conglutin isolated from *L. angustifolius* seeds shows the presence of peptides with molecular weights from 50 to 100 kDa, while under reducing conditions, bands with lower molecular masses (37-60 kDa) were observed [30].

BIOACTIVE COMPOUNDS AND BIOLOGICAL PROPERTIES

The different bioactive compounds in Lupinus seeds such as phenolic compounds, fibre and proteins have shown various positive effects on human health in different diseases and syndromes such as type 2 diabetes mellitus, dyslipemias, hypertension, neurodegenerative diseases or cancer [31,40-42]. The beneficial effect of these components will depend on the concentration, exposure time and bioavailability of these compounds [7,30]. Phenolic compounds, secondary metabolites that have protective functions in plants, have anti-inflammatory, cardio-protective and antibacterial effects, and a very high antioxidant power [23,43,44]. Phenolic compounds contain at least one aromatic ring (C6) and one or more hydroxyl groups. The different phenols are distinguished by the number of constituent carbon atoms in addition to this basic phenolic skeleton [45].

The health benefits of *Lupinus angustifolius* L. include the following effects: hypoglycaemic, hypotensive, cholesterol-lowering, anti-cancer and anti-inflammatory, in addition to protection against the symptoms of menopause and osteoporosis [11,46]. The phytochemicals present in Lupinus, including bioactive peptides, alkaloids and phenolic

compounds, have been shown to play a key role in preventing various chronic inflammation-related diseases such as diabetes or hypertension because of the antioxidant, antihyperlipidemic and anti-inflammatory activities they possess [21]. Consumption of *Lupinus angustifolius* L. proteins could have preventive and protective effects against various diseases related to chronic inflammation such as type 2 diabetes mellitus, metabolic syndrome, familial hypercholesterolemia, hypertension and cardiovascular disease, mainly because of their anti-inflammatory role [4].

It has been described in the literature possible interactions between proteins and phenolic compounds that influence the solubility and thermal stability of proteins [7,30]. It has been proven that seed fermentation of Lupinus increases its total phenolic content, thus increasing its antioxidant capacity and DPPH radical scavenging capacity [31].

Anti-oxidant properties

The antioxidant property of *Lupinus angustifolius* L. is due to its content of bioactive peptides and phenolic compounds. Protection against phenolic diseases can be associated with the powerful antioxidant and free radical scavenging properties of these compounds. In addition, this antioxidant activity can also be determined by the presence of tocopherols and carotenoids present in lupine seeds [21,40]. The main bioactive function of phenolic compounds is their high antioxidant power, which acts against the harmful effects of reactive oxygen species and free radicals generated by oxidation processes. This

antioxidant power depends on the structure where the number and position of the hydroxyl groups is crucial. The content of phenolic compounds in *Lupinus angustifolius* L. was found to be between 535.1 and 578.4 mg/100 g of seeds [45]. Lupines peptides showed a higher antioxidant activity and greater protection against H_2O_2-induced oxidative damage in HepG2 cells than those with larger size [43].

Cardiovascular Disease Protective Effects

Cardiovascular diseases (CVD) include abnormalities of lipoproteins, high blood pressure, diabetes, smoking and overweight [27]. It was observed that the consumption of vegetable protein is associated with a decrease in blood pressure [18]. The phenolic content and composition of *L. angustifolius* may have positive implications for reducing the risk of cardiovascular disease due to its protective effect on blood vessel health [1]. Adding lupine fibre to a range of foods for 28 days was studied significantly reduced LDL cholesterol levels in healthy men. Consumption of lupine protein isolate (25g per day) for four weeks was also reported to reduce LDL cholesterol beneficially compared to those taking milk protein isolate. Other studies determined that the addition of Lupinus meal to foods such as bread, biscuits or pasta can result in a beneficial reduction in blood pressure compared to a diet with the equivalent grain foods without lupin meal [14].

Antibacterial and fungicidal properties

Antimicrobial activity has been detected in an extract of *Lupinus angustifolius* with a high content of alkaloids and phenolic compounds

[7,10]. They found that the saponins in the methanolic extract of *L. angustifolius* seed exhibited antifungal activity against *Candida albicans* [21]. The antifungal activity of a multifunctional glycooligomer with 210 kDa, composed mainly of BLAD (*Lupinus albus* 12 band), a 20kDa polypeptide, a stable intermediate product of β-conglutin catabolism, was recently demonstrated and found to accumulate exclusively in the cotyledons of lupine species [20].

Anti-diabetic properties

In a similar population of overweight men and women, a diet enriched with lupine flour was found to significantly reduce fasting insulin concentrations by 16-21% 4 months after its introduction [3].

The effect of Lupinus proteins on blood sugar has been studied. Lupine protein enriched with γ-conglutin reduced blood glucose levels in both humans and animals and this may be due to the insulinotropic effects of this compound [10]. It has been shown that intact multimeric protein is capable of entering and being released through a monolayer of CaCo-2 cells, as well as crossing the intestinal barrier in an *ex vivo* intestinal model without changing its covalent continuity. It is therefore reasonable to argue that peptides derived from γ-conglutin could be absorbed through the intestinal cell wall and that these are responsible for their bioactive capacity in the regulation of glucose levels [3,8]. It is possible that some di- and tripeptides in combination with some amino acids have synergistic effects on insulin secretion stimulated by glucose. It was also found that protein hydrolysates have a higher bioavailability than the protein isolate. In conclusion, Lupinus protein hydrolysates

improve glucose homeostasis in patients with Type 2 Diabetes Mellitus (T2DM) or who are intolerant to glucose as they increase insulin secretion due to the activation of the Gαq subunit of GPCR which stimulates signalling through the PLC/PKC pathway which, when activated, increases glucose uptake. In addition, closure of K-ATP channels is promoted which triggers depolarisation of the cell membrane, influence of extracellular Ca^{2+} and exocytosis of insulin. The direct or indirect increase in intracellular Ca^{2+} promotes the fusion of insulin granules with the plasma membrane in β-cells [47].

The β-conglutin of *L. angustifolius* have been studied to increase the levels of mRNA and proteins involved in the insulin signalling pathway in peripheral blood mononuclear cells of patients with T2DM that could be used for the treatment and prevention of Type 2 Diabetes [8,10,42].

Hypocholesterolaemia properties

Several investigations focused on the lipid-lowering activity of different lupine constituents such as fibre, proteins and flour. Proteins were found to reduce the expression of genes related to cholesterol synthesis [18]. The potential mechanism responsible for these health-promoting properties of lupine seed proteins is associated with increased activity of low-density lipoprotein receptors [15].

Several studies have studied the effects of Lupinus consumption, both whole seed and dietary fibre or isolated protein, on cardio-metabolic parameters. One study compared the effect of lupine protein consumption on plasma lipoprotein levels in individuals with

high cholesterol levels to those who consumed casein and found that at week 6 individuals who consumed the vegetable protein had a greater improvement in the ratio between LDL and HDL cholesterol than the group who consumed casein. In another study, it was observed that the incorporation of 25 g/d of lupine protein in different food products resulted in a decrease in total cholesterol and LDL, triglycerides and uric acid in those individuals with hypercholesterolemia. And in a similar study, but this time using lupine dietary fibre (25 g/d), it was observed that after 4 weeks of supplementation, the supplemented individuals improved total and LDL cholesterol levels compared to individuals in the control group, with the impact of fibre on the formation of short-chain fatty acids possibly causing this effect [3]**.** It has also recently been shown that lupine protein hydrolysates have high ACE inhibitory activity, most notably in reducing blood pressure [3]**.** A study that was conducted by involving consumption of *L. angustifolius* protein hydrolysate with a pea protein isolate and combinations of both with oat soluble fibre or apple pectin. It was determined that within four weeks lupine protein is more effective than pea protein in reducing cholesterol and that the combination of soluble fibre with vegetable protein may have interesting cholesterol-lowering applications [27]**.**

LPH have shown bile acid binding activity, having potential applications for cholesterol reduction in patients with hypercholesterolemia [22,48]**.** The role of protein hydrolysates from *L. angustifolius* in reducing plasma lipids was studied in several studies. A purified and enriched vicilin fraction from *Lupinus angustifolius* L. was found to lower cholesterol levels compared to total protein extract. The Treatment with this compound significantly reduced the total

cholesterol level compared to casein, which was the control group with no difference between triglyceride and HDL cholesterol levels. In an animal model of rats fed with Lupinus, it was found a decrease in the levels of mRNA of SREBP-2, transcriptional regulator of the levels of intracellular cholesterol and CYP7A1, enzyme that limits the rate of biosynthesis of bile acids [27,48]**.** It was observed in an animal model of hyperlipidaemic pigs that the amount of total and LDL cholesterol was significantly reduced, this being due to an increase in cholesterol in the faeces, i.e., not absorbed into the blood. In conclusion, the diet based on the protein of *L. angustifolius* showed marked hypocholesterolaemic properties compared to the casein diet [22]**.**

Anti-inflammatory properties

The β-conglutins of NFL seed protein hydrolysates have possible anti-inflammatory properties as they appear to inhibit to some extent the enzymes involved in the inflammation pathway such as phospholipase A2 and cyclooxygenase-2 [38].

The β-conglutin protein obtained from *Lupinus angustifolius* L. had positive effects in *ex vivo* assays (blood samples from patients diagnosed with T2DM) stimulated with LPS and pancreatic PANC-1 cell culture assays. This protein fraction promoted the attenuation of mRNA expression in pro-inflammatory mediators that damage β cells in type 2 diabetes mellitus, including IL-1β, CCL5 MCP-1, ICAM-1, TNF-α, INF-γ and the NF-kβ mediator. In addition, protein production decreased significantly for the pro-inflammatory cytokines TNF- α, INF-γ, IL-1β, IL-2, IL-6, IL-8, IL-12, and IL-17. The β-conglutin inhibited NO production

and iNOS mRNA and protein levels [2,4]**.** In pancreatic culture cell trials, β-conglutin (β1, β3 and β6) treatments had the following effects: β-conglutin increased mRNA expression in key insulin-signalling pathway-activating mediators such as IRS-1, GLUT4 as well as increased protein synthesis of GLUT-4 and p85-PI3K. Activation of the intracellular IRS-1/PI-3 kinase pathway that regulates glucose homeostasis and stimulation of protein synthesis was also observed, increasing the level of phosphorylation (active forms) of the insulin p-IRS-1 pathway activator and subsequent mediators such as p-Akt, p-Cbl and p-caveolin proteins that play an important role in vesicular transport of the GLUT-4 transporter to the cell surface [41,42]**.**

Anti-inflammatory properties of *L. angustifolius* protein hydrolysates were investigated on *in vitro* models using a macrophage model derived from THP-1. LPH was obtained by enzymatic hydrolysis of lupine protein isolate (LPI) using two proteases: Izyme and Alcalase. Diminished gene expression of proinflammatory cytokines TNF-α, IL-6 and IL-1β was determined and while expression of anti-inflammatory cytokines (chemokine (C-C motif) ligand 18) CCL18 was increased [21,49]**.** In addition, it was observed that bioactive peptides generated from enzymatic proteolysis modulated the expression of pro- and anti-inflammatory mediators and attenuated the migratory response of the cells. The sequence of one of these peptides could be characterized as GPETAFLR [2,39]**.**

Prebiotic properties

Like other legumes, Lupinus acts as a prebiotic in the human gut that can play an important role in modifying the microbiome by improving the health of individuals. Studies have shown that consumption of 17-30 g/d of lupine fibre for 28 days modified the faecal microbiota in humans, increasing levels of Bifidobacterium spp. and decreasing levels of *C. ramosum* and *C. spiroforme*. In another investigation with L. angustifolius it was observed that the supplementation of 25 g/d of lupin fibre produced a significant increase in the formation of short chain fatty acids and a 16% reduction in the faecal concentrations of total and secondary bile acids, in addition to increasing primary bile excretion and decreasing the faecal pH [3].

CONCLUSIONS

In recent years, dietary proteins from seeds have begun to occupy a key place in human nutrition, due to their nutritional function and the number of bioactive effects they can exert on the human body, such as hypolipidemic, hypoglycaemic, hypotensive, anticarcinogenic and anti-diabetic activities, among others. In addition, plant proteins can play important techno-functional roles, and can be used as food ingredients that improve the nutritional, technological and health profiles of foods [29]. The increased interest in legume grains is due to an improvement in the separation techniques available in the industry, which can separate proteins, fibres and minor components [29].

The properties of the seeds of the genus Lupinus are associated with the high content of protein and dietary fibre that help reduce blood

pressure and the risk of cardiovascular disease, as well as the prevention and treatment of type 2 diabetes [42]**.** *L. angustifolius* is a legume with the highest amount of protein and dietary fibre and the lowest levels of anti-nutritional factors, making the protein and its nutrients more bioavailable, and there is a growing body of evidence to support its health benefits [3]. Today the inclusion of *L. angustifolius* as an ingredient is still low and it is mainly used for livestock feed [14]**,** but the bioactive compounds, mainly phytochemicals and bioactive peptides, seems to be a promising functional food that can be used as a high value-added ingredient [14,42].

The discovery of the novel peptide GPETAFLR and its proven biological activity has opened a very interesting field for its development in the field of nutraceuticals.

HYPOTHESIS AND THESIS OUTLINE

We hypothesize that the bioactive octapeptide GPETAFLR could be used as a nutraceutical in several pathologies related to inflammation and oxidation. In this Thesis, various approaches are used to evaluate the health benefits of this peptide isolated from lupine (*Lupinus angustifolius* L.) seed both in a high-fat diet-induced obese mice model and *in vitro* studies in different cell lines (**overall purpose**).

In **Chapter 01**, a review is given in order to understand the historical background, the nutritional composition, the biological properties of *Lupinus angustifolius* L., and the isolation of the octapeptide GPETAFLR.

This Thesis was also aimed (**specific purposes**):

To investigate the role of GPETAFLR on the inflammatory response by modulating the activation and plasticity of macrophages derived from monocytes of healthy men **(Chapter 02).**

To investigate the role of GPETAFLR on hepatic metabolism and inflammation associated to non-alcoholic fatty liver disease in a high-fat diet-induced obese mice model **(Chapter 03)**.

To investigate the role of GPETAFLR on the neuroprotection in a high-fat diet-induced obese mice model and the anti-inflammatory effects on microglia using the BV2 cell line. **(Chapter 04).**

Finally, **Chapter 05** discusses the most important findings of this Thesis and puts them into a broader perspective of the current status of the field.

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CHAPTER 02

GPETAFLR, an octapeptide isolated from *Lupinus angustifolius* L. protein hydrolysate, promotes the skewing to the M2 phenotype in human primary monocytes

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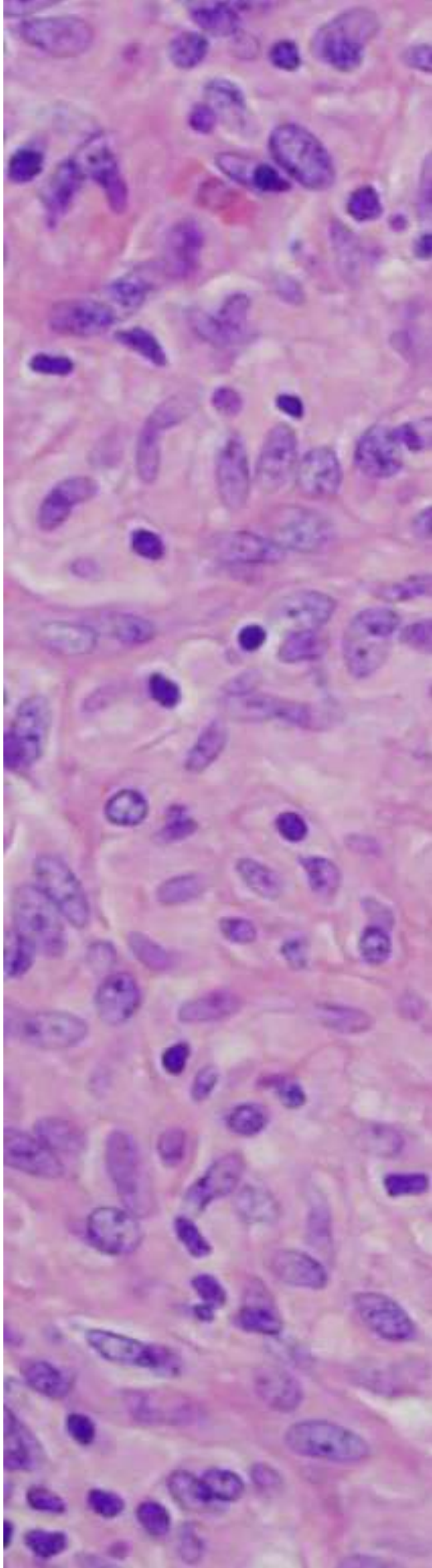

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GPETAFLR, an octapeptide isolated from *Lupinus angustifolius* L. protein hydrolysate, promotes the skewing to the M2 phenotype in human primary monocytes

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The present study aimed to test the mechanisms by which GPETAFLR, released from the enzymatic hydrolysis of lupine protein, may modulate the inflammatory response and plasticity in human primary monocytes. Human circulating monocytes and mature macrophages were used to analyze the effects of GPETAFLR on plasticity and inflammatory response using biochemical, flow cytometry, quantitative real-time PCR, and ELISA assays. GPETAFLR skewed the monocyte plasticity towards the anti-inflammatory non-classical $CD14^{+}CD16^{++}$ monocyte subset and reduced the inflammatory competence of LPS-treated human monocytes diminishing IL-1β, IL-6, and TNF-α and increasing IL-10 production and gene expression. Results showed that GPETAFLR decreased the frequency of the LPS-induced activated monocyte population ($CD14^{++}CD16^{-}$), diminished monocyte activation involved down-regulation of CCR2 mRNA expression and protein expression, and decreased gene expression of the LPS-induced chemoattractant mediator CCL2. Our findings imply a new understanding of the mechanisms by which GPETAFLR favor a continuous and gradual plasticity process in the human monocyte/macrophage system and offer novel benefits derived from the consumption of *Lupinus angustifolius* L. in the prevention of inflammatory-related diseases.

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Introduction

The word nutraceutical is the association of the terms *nutrition* and *pharmaceutical* and is considered as a food or food product that can offer medical and health benefits for the prevention and treatment of diseases. Most nutraceutical peptides are isolated from protein precursors by digestive enzymes, during food processing, storage, or by the *in vitro* hydrolysis of several proteolytic enzymes.[1] Although milk proteins are the main source of bioactive peptides studied so far, there are many other peptides of animal and plant origin. In fact, more than 3000 peptides with 44 different biological activities have been identified and recently collected in the "Biopep" database.[2] In this sense, the study of plant proteins has emerged as an alternative to produce bioactive peptides,[3] small sequences of amino acids that are inactive within the intact protein but can be released by gastrointestinal enzymatic hydrolysis reaching the bloodstream as active molecules.[4]

The immune system is responsible for responding to any type of aggression in the body. It has its origin in the lymphoid organs (lymph nodes, spleen, thymus, and bone marrow) and in its action many cellular subtypes (immunocompetent cells), including monocytes and neutrophils, participate. Monocytes express several receptors that control and detect environmental changes, are highly plastic and heterogeneous, and change their functional phenotype in response to environmental stimulation.[5,6] Inflammation is one of the complex biological responses of an organism's immune system. Evidence from murine and human studies has suggested that monocytosis may be an indicator of several inflammatory diseases.[7] Therefore, the use of primary monocytes as a model of inflammation is widespread and is used in numerous studies to test the anti-inflammatory activity of various compounds.[8,9] The secretion of pro-inflammatory mediators works as an inflammatory beacon for leukocytes, which contribute to all stages of several inflammatory disorders, therefore representing an important therapeutic target.[10]

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Nutraceutical is the association of the terms nutrition and pharmaceutical and is considered as a food or food product that can offer medical and health benefits for the prevention and treatment of diseases. Most nutraceutical peptides are isolated from protein precursors by digestive enzymes, during food processing, storage, or by *in vitro* hydrolysis of several proteolytic enzymes [1]. Although milk proteins are the main source of bioactive peptides studied so far, there are many other peptides obtained from different sources of animal and plant origin. In fact, more than 3000 peptides with 44 different biological activities have been identified and recently collected in the "Biopep" database [2]. In this sense, the study of plant proteins has emerged as an alternative to produce bioactive peptides [3], small sequences of amino acids that are inactive within the intact protein but can be released by hydrolysis enzymatic gastrointestinal reaching the bloodstream as active molecules [4].

The immune system is responsible for responding to any type of aggression in the body. It has its origin in the lymphoid organs (lymph nodes, spleen, thymus, and bone marrow) and in its action many cellular subtypes (immunocompetent cells), including monocytes and neutrophils, participate. Monocytes express several receptors that control and detect environmental changes, are highly plastic and heterogeneous, and change their functional phenotype in response to environmental stimulation [5,6]. Inflammation is one of the complex biological responses of an organism's immune system. Evidence from murine and human studies has suggested that monocytosis may be an

indicator of several inflammatory diseases [7]. Therefore, the use of primary monocytes as a model of inflammation is widespread and is used in numerous studies to test the anti-inflammatory activity of various compounds [8,9]. The secretion of pro-inflammatory mediators works as an inflammatory beacon for leukocytes, which contribute to all stages of several inflammatory disorders, therefore representing an important therapeutic target [10].

Human monocytes are classified into three subsets; $CD14^{++}CD16^{-}$ (classical monocytes), intermediate $CD14^{++}CD16^{+}$ (intermediate monocytes), and $CD14^{+}CD16^{++}$ (non-classical monocytes) [11]. So far, classical monocytes represent the major fraction (about 85% of total monocytes) and highly express CCR2, they are professional phagocytes giving rise to M1 macrophages, which secrete cytokines (TNF-α, IL-1β, and IL-6) in response to LPS during infection or inflammation [12]. Intermediate monocytes display highest levels of CCR5, TLR4, CD163, and HLA-DR during activation and also secrete pro-inflammatory cytokines as IL-10 [13]. Non-classical monocytes are less granular and smaller in size, with lower expression of CCR2 than classical or intermediate subsets [14,15]. These monocytes rich in CD16 are functionally involved in tissue repairing, patrolling, and wound healing, and have the tendency to be polarized into M2 macrophages with an anti-inflammatory phenotype in response to a variety of stimuli, including IL-4 [16].

We have previously shown the presence of a new peptide isolated from a protein hydrolysate of *Lupinus angustifolius* L. [17]. The aim of the present study was to investigate the effects of GPETAFLR peptide on human primary monocytes and human mature macrophage

activation as hallmarks of inflammatory disorders.

MATERIALS AND METHODS

Synthesis of the peptide

Seeds of lupine (*Lupinus angustifolius* L.) were a gift from Koipesol Semillas, S.A. (Sevilla, Spain). Alcalase 2.4 L was provided by Novozymes (Bagsvaerd, Denmark). Lupine protein isolate (LPI) was obtained using a previously described method at pilot plant scale (Plant Proteins Group, Instituto de la Grasa, Seville, Spain). The chemical composition of LPI in dry matter was: proteins 86.83%; dietary fibre 5.97%; oil 5.08%; ash 0.78%, carbohydrates 1.34%. Hydrolysis was carried out and Lupine protein isolate (LPI) was suspended in distilled water (10% w/v), and hydrolysis with Alcalase was performed under the following conditions: pH 8, 50 °C, E/S=0.3 AU/g protein, and a hydrolysis time of 15 min. The mixture was heated at 85 °C for 15 min to inactivate the enzyme, centrifuged at 6500g for 15 min, and the supernatant constituted the Lupine protein hydrolysate (LPH). LPH was purified by ultrafiltration and chromatographic techniques. The sequence of the purified peptide was identified through sequence analysis by nanoHPLC coupled to a Polaris Q ion trap mass spectrometer (Mass Spectrometry Unit, Instituto de la Grasa). Finally, the peptide was synthesized by Fmoc solid phase method by the Barcelona Scientific Park Foundation (Barcelona, Spain) at 95% purity, measured by HPLC-UV at 220 nm.

Blood collection and isolation of human monocytes

This study was conducted according to Good Clinical Practice Guidelines and in line with the principles outlined in the Helsinki Declaration of the World Medical Association. Informed consent for the study was obtained from healthy male blood donors (age <35 years) at the University Hospital Virgen del Rocio (UHVR, Seville). Ethics approval was obtained from the Human Clinical Research and Ethics Committee of the UHVR (AGL2012-40247-C02-01). Participants declared that they were non-smokers and were not taking any medication. Peripheral blood samples were drawn from a large antecubital vein and collected into K_3EDTA-containing tubes (Becton Dickinson, NJ, USA). Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood samples by centrifugation over a Ficoll-Histopaque (Sigma-Aldrich, Madrid, Spain) gradient. Monocytes were isolated from PBMCs using CD14 microbeads and LS columns on a midiMACS system (Miltenyi Biotec, Madrid, Spain) according to the manufacturer's instructions. The purity for CD14 monocyte isolations was routinely >95% by flow cytometry (FACScanto II flow cytometer and FACSDiva software, BD). Following isolation, monocytes were suspended in a RPMI 1640 medium supplemented with L-glutamine, penicillin, streptomycin and 10% heat-inactivated foetal bovine serum. For treatments, 5×10^5 of purified monocytes, after *in vitro* stimulation with or without LPS (100 ng/mL), were exposed to GPETAFLR at 50-100 µg/mL (P2 and P1, respectively) for 24 h.

Monocyte differentiation and polarization into M1 and M2 macrophages

Monocytes were induced to differentiate for 6 days in the presence of recombinant human M-CSF (25 ng/mL) to obtain M0 macrophages. These cells were then cultured in RPMI 1640 supplemented with L-glutamine, penicillin, streptomycin, and 10% heat-inactivated FBS. For M1 and M2 polarization, M0 macrophages were exposed to LPS (100 ng/mL) plus IFNγ (20 ng/mL) and to IL-4 (20ng/mL), respectively, for additional 24h. To evaluate the effect of GPETAFLR peptide on macrophage polarization, M0 macrophages were exposed to GPETAFLR at 50-100 µg/mL for 24 h.

Cell viability assay (MTT)

Monocytes were incubated with different concentrations of GPETAFLR peptide in 96-well plates (1x104 cells/well) during 24 h. Afterward, the MTT solution (Sigma) was incubated in the well until a purple precipitate was visible. MTT-formazan crystals were solubilized with DMSO (Sigma), and then measured with a microplate reader at 570 nm corrected to 650 nm [18]. Cell survival was expressed as the percentage of absorbance compared with that obtained in control, non-treated cells.

Immunostaining of circulating monocytes by FACS

Circulating monocyte membrane expression of CD16 (PE anti-

human CD16, Miltenyi), CD14 (APC-Cy7 anti-human CD14, Miltenyi), and CCR2 (APC anti-human CCR2, Vitro) on monocytes was analysed by flow cytometry. According to the manufacturer's instructions, 5×10^5 of purified monocytes, after *in vitro* stimulation with or without LPS (100 ng/mL), were exposed to GPETAFLR at 50-100 µg/mL for 24 h. Thereafter, cells were incubated with antibodies at room temperature and in the dark for 15 min; erythrocytes were removed with FACS lysing solution (BD). Mean fluorescence intensity (MFI) was measured by using a FACSCanto II flow cytometer (BD) and calibrated by using a FACSDiva software (BD). MFI of 10^4 counted cells was assessed for each sample. Monocytes were gated as forward scatter^{high} (FSC^{high})-side scatter^{high} (SSC^{high}) cells. Expression levels are presented as MFI corrected for nonspecific binding of isotope control antibodies.

RNA isolation and qRT-PCR analysis

Total RNA was extracted by using Trisure Reagent (Bioline), as instructed by the manufacturer. A260/A280 ratio in a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Madrid, Spain) was used to determinate RNA quality. Momentarily, RNA (1 µg) was subjected to reverse transcription (iScript, Bio-Rad, Madrid, Spain). An amount of 10 ng of the resulting cDNA was used as a template for real-time PCR amplifications. The mRNA levels for specific genes were determined in a CFX96 system (Bio-Rad). For each PCR reaction, cDNA template was added to Brilliant SYBR green QPCR Supermix (Bio-Rad) containing the primer pairs for either gene or for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyltransferase 1 (HPRT) as housekeeping genes (**Table 8**). All amplification reactions

were performed in triplicate and average threshold cycle (Ct) numbers of the triplicates were used to calculate the relative mRNA expression of candidate genes. The magnitude of change of mRNA expression for candidate genes was calculated by using the standard $2^{-(\Delta\Delta Ct)}$ method. All data were normalized to endogenous reference (GAPDH and HPRT) gene content and expressed as percentage of controls.

Table 8. Sequences of RT-qPCR primers for gene expression analysis

Target	GenBank accession number	Direction	Sequence (5'→3')
TNF-α	NM_000594	Forward	TCCTTCAGACACCCTCAACC
		Reverse	AGGCCCCAGTTTGAATTCTT
IL-1β	NM_000576	Forward	GGGCCTCAAGGAAAAGAATC
		Reverse	TTCTGCTTGAGAGGTGCTGA
IL-6	NM_000600	Forward	TACCCCCAGGAGAAGATTCC
		Reverse	TTTTCTGCCAGTGCCTCTTT
GAPDH	NM_001289746	Forward	CACATGGCCTCCAAGGAGTAAG
		Reverse	CCAGCAGTGAGGGTCTCTCT
HPRT	NM_000194	Forward	ACCCCACGAAGTGTTGGATA
		Reverse	AAGCAGATGGCCACAGAACT
CD80	NM_005191.3	Forward	GGGAAAGTGTACGCCCTGTA
		Reverse	GCTACTTCTGTGCCCACCAT
CD200R	NM_138940.2	Forward	GTTGCCCTCCTATCGCATTA
		Reverse	TGGAAATTCCCATCAGGTGT

Cytokine quantification

The levels of TNF-α IL-1β, IL-6, and IL-10 in culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA), following the indications of the manufacturer (Diaclone, Besancon, France). The cytokine concentrations were expressed in pg per mL, as calculated from the calibration curves from serial dilution of human recombinant standards in each assay.

Statistical analysis

All values are expressed as arithmetic means ± standard deviations (SD). Data were evaluated with Graph Pad Prism Version 5.01 software (San Diego, CA, USA). The statistical significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance (ANOVA), following Tukey multiple comparisons test as *post hoc* test. *P* values less than 0,05 were considered statistically significant.

RESULTS

Effect of GPETAFLR on classical, non-classical, and intermediate monocyte subsets

FACS analysis of monocyte surface markers CD14 and CD16 showed different results with the different treatments on monocyte subsets (**Figure 5A**). Classical monocytes ($CD14^{++}CD16^{-}$) were increased in LPS-treated monocytes; however, the treatment with GPETAFLR decreased this population (**Figure 5B**). Intermediate monocytes did not

show significant differences when it was expose with GPETAFLR (**Figure 5C**). In contrast, non-classical monocytes ($CD14^{+}CD16^{++}$) were decreased with LPS. However, the treatment with GPETAFLR at 100 µg/mL increased the non-classical monocytes subset (**Figure 5D**).

Effect of GPETAFLR on CCR2/CCL2 axis in monocyte subsets

CCR2/CCL2 axis were studied (**Figure 6**). CCR2 protein expression was analysed in monocyte subsets by FACS analysis (**Figure 6A**). GPETAFLR decreased significantly CCR2 expression primarily on classical monocyte subsets compared to LPS (**Figure 6B**), although no significantly differences were found on CCR2 MFI in non-classical or intermediate monocytes, there was a reduction tendency. In addition, mRNA expression of CCL2 and its receptor, CCR2 was analysed. LPS-treated monocytes showed a CCR2 up-regulation (**Figure 6C**). However, those that were treated with GPETAFLR peptide had a lower increased in CCR2 mRNA levels than those that were treated with LPS. Regarding to CCL2 mRNA levels, LPS treatment up-regulated its expression, whereas that GPETAFLR treatment down-regulated CCL2 expression compared to these treated with LPS (**Figure 6D**).

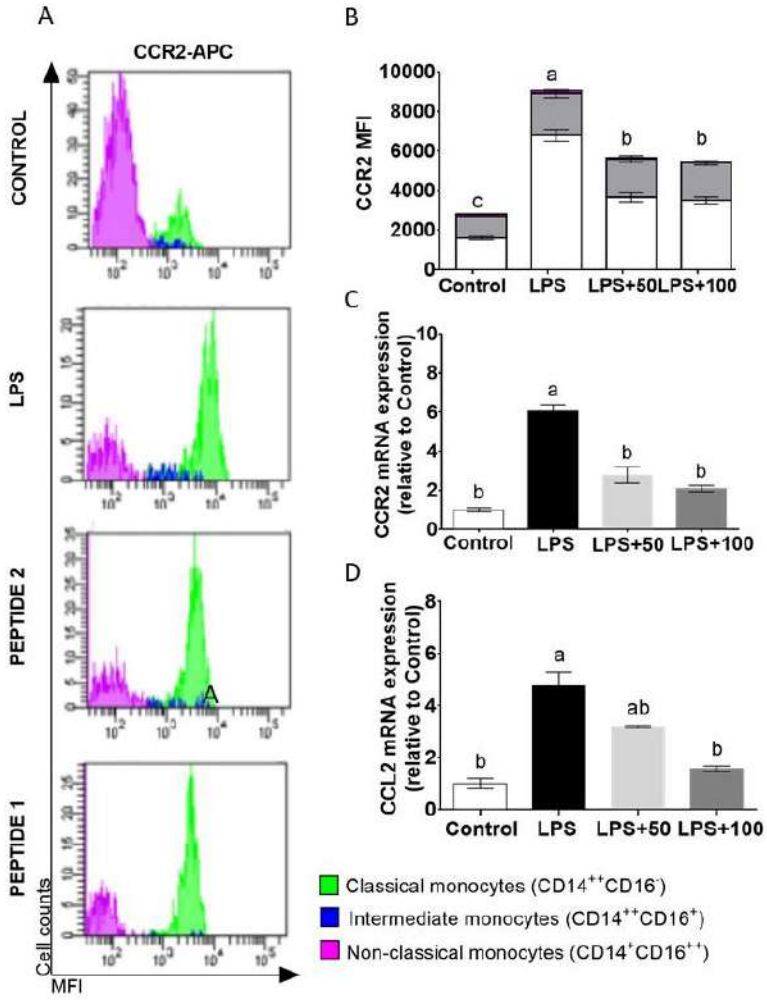

Figure 5. Effect of GPETAFLR on monocytes subsets. (**A**) FACS analysis (MFI) of monocyte surface markers CD14 and CD16 after 24 h incubation with or without LPS (100 ng/mL) and peptide at 50-100 µg/mL (Peptide 2 and Peptide 1, respectively). (**B**) Classical $CD14^{++}CD16^{-}$ monocytes, (**C**) intermediate $CD14^{++}CD16^{+}$ monocytes, and (**D**) non-classical $CD14^{+}CD16^{++}$ monocytes. Values are presented as means ± SD (n = 3) and those marked with different letters are significantly different ($P< 0.05$).

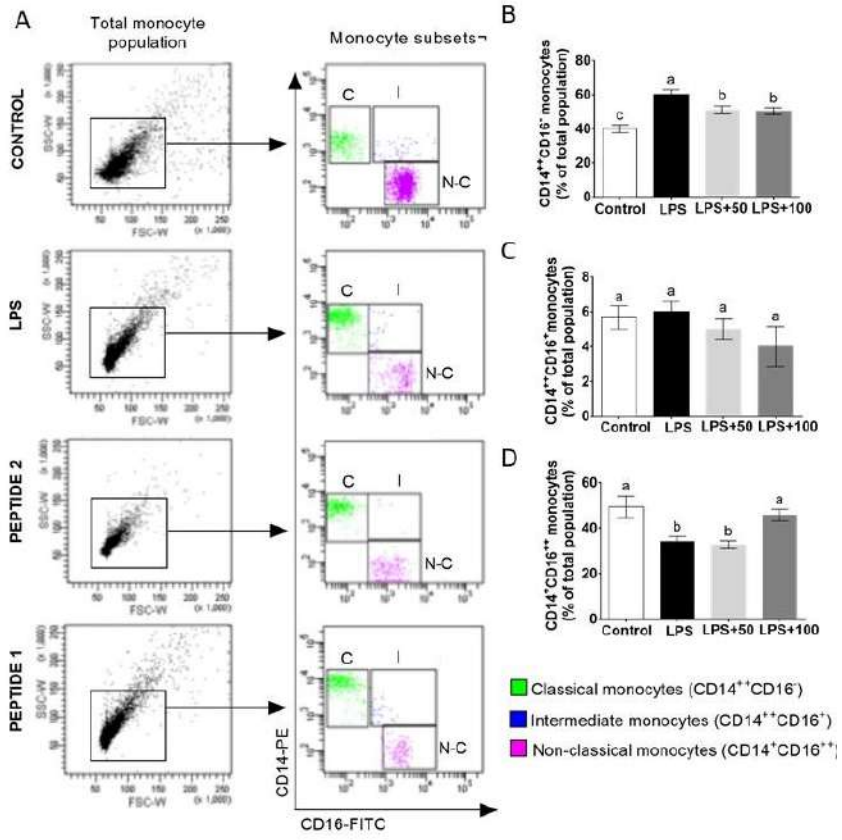

Figure 6. Effect of GPETAFLR on CCR2/CCL2 axis. (**A**) Representative overlay of MFI as a marker for CCR2 in monocyte subsets after 24 h incubation with or without LPS (100 ng/mL) and peptide at 50-100 µg/mL (Peptide 2 and Peptide 1, respectively). (**B**) FACS analysis of monocyte CCR2 expression in subsets by means of CD14 and CD16 surface marker expression. (**C, D**) Relative expression of CCR2 and CCL2 genes were measured by RT-qPCR. Values are presented as means ± SD (n = 3) and those marked with different letters are significantly different ($P < 0.05$).

Effect of GPETAFLR on cytokine expression and release in primary human monocytes

In addition to monocyte subsets, we observed that LPS-induced release of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and expression of genes encoding these cytokines were diminished by GPETAFLR in LPS-treated human monocytes (**Figure 7A-7F**). In all this cases, GPETAFLR effects were dose-dependent. Secretion and gene expression of the anti-inflammatory IL-10 was also promoted by GPETAFLR (**Figure 7G** and **7H).**

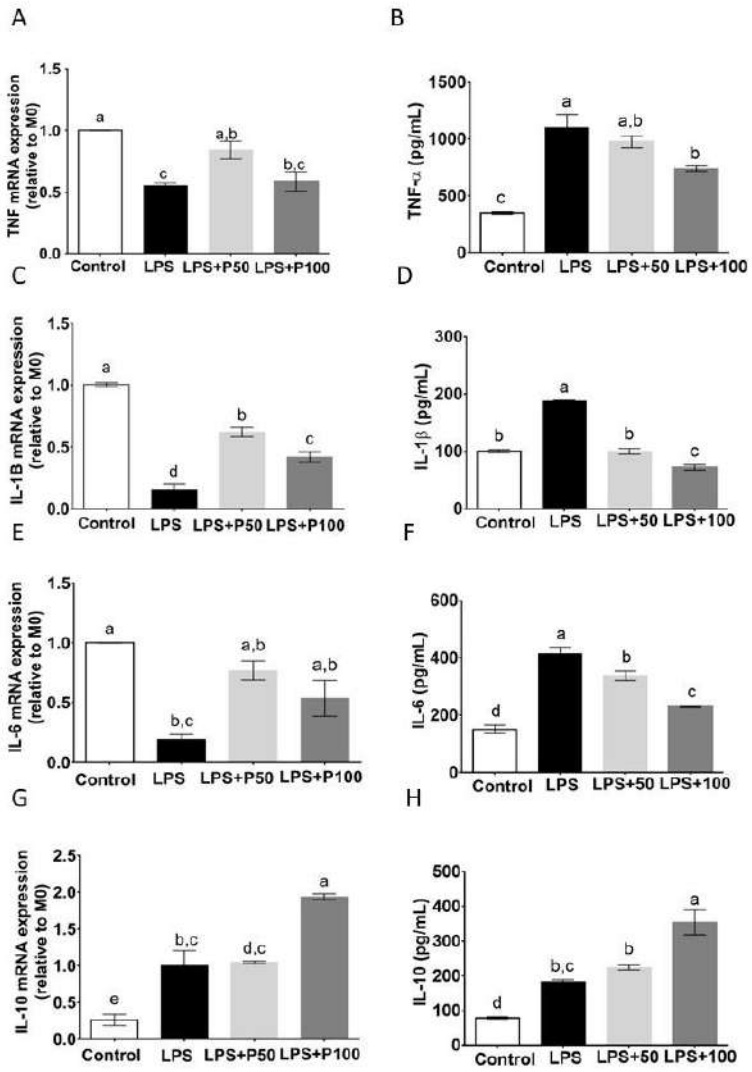

Figure 7. Effect of GPETAFLR on pro-inflammatory cytokine release in primary human monocytes. (**A, C, E, G**) Gene expression and (**B, D, F, H**) and secretion of TNFα, IL-1β, IL-6, and IL-10 in primary human monocytes after 24 h incubation with or without LPS (100 ng/mL) and peptide at 50-100 µg/mL (Peptide 2 and Peptide 1, respectively). Values are presented as means ± SD (n = 3) and those marked with different letters are significantly different (P< 0.05).

Effect of GPETAFLR on modulation of M1/M2 macrophage polarisation

Macrophage polarization depends on different microenvironment and signals that promote specific phenotypes. These phenotypes correspond to classically (M1) and alternatively (M2) polarized macrophages. Relative expression of CD80, CD64, CD200R and MRC-1 were measured by RT-qPCR (**Figure 8**).

M0 macrophages were incubated with LPS plus IFNγ (M1), IL-4 (M2) or LPS plus IFNγ plus GPETAFLR peptide (M1+P) to study the phenotype plasticity. M0 macrophages treated with LPS plus IFNγ showed an increased in CD80 and CD64 mRNA levels (**Figure 8A and 8B,** respectively) and a decreased in CD200R and MRC-1 mRNA levels (**Figure 8C and 8D**, respectively). M0 macrophages treated with LPS plus IFNγ plus GPETAFLR peptide down-regulated CD80 and CD64 mRNA levels compared to M1 macrophages, whereas CD200R and MRC-1 mRNA levels were up-regulated compared to M1 macrophages. The results at 100 µg/mL were more remarkable than with 50 µg/mL.

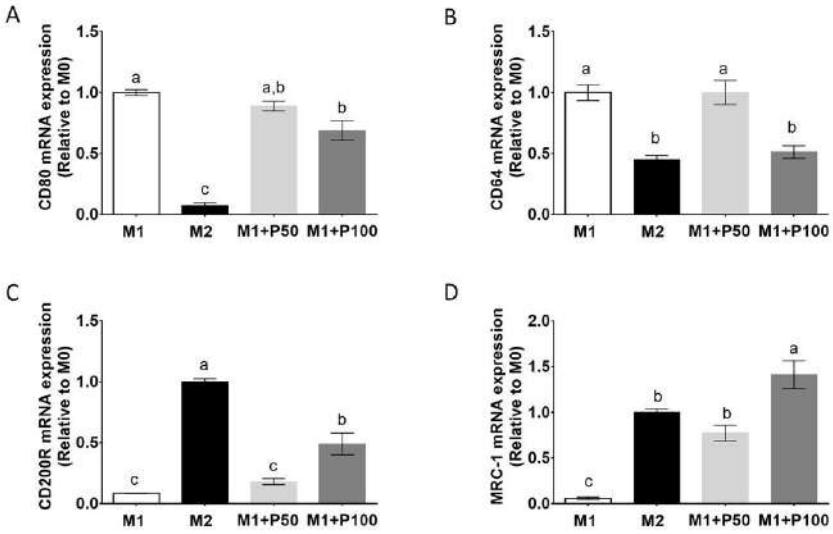

Figure 8. Effect of GPETAFLR on M1/M2 macrophage polarisation. M0 macrophages were incubated with LPS plus IFNγ (M1 control), IL-4 (M2 control), or with LPS plus IFNγ plus GPETAFLR (50-100 μg/mL, Peptide 2 and Peptide 1, respectively) for additional 24 h. Relative expression of (**A**) CD80, (**B**) CD64, (**C**) CD200R, and (**D**) MRC-1 genes were measured by RT-qPCR. Values are presented as means ± SD (n = 3) and those marked with different letters are significantly different (P< 0.05).

In addition, cytokine production in macrophage phenotype was also studied (**Figure 9**). TNF-α, IL-1β, and IL-6 cytokine levels were increased in M1 macrophages (**Figure 9A, 9B, and 9C**, respectively). M0 macrophages treated with LPS plus IFNγ plus GPETAFLR peptide decreased pro-inflammatory activity of M1 phenotype, whereas IL-10 cytokine levels were increased compared to M1 macrophages (**Figure 9D**).

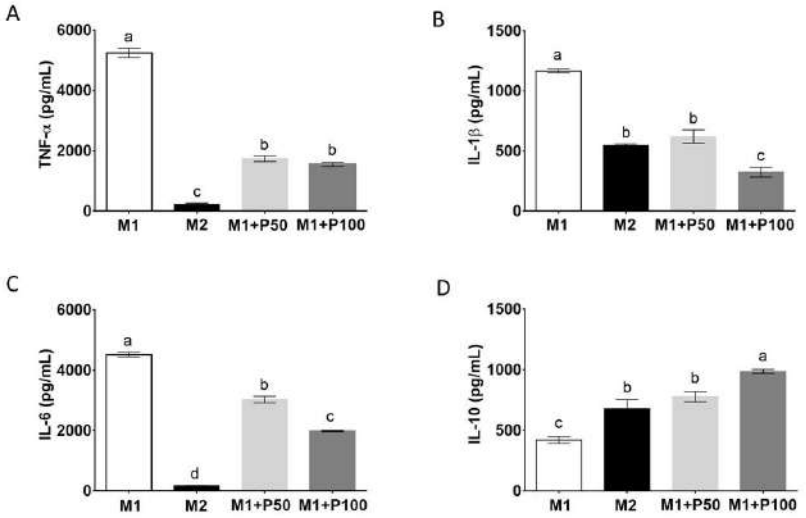

Figure 9. Effect of GPETAFLR on expression cytokines in M1 macrophages. M0 macrophages were incubated with LPS plus IFNγ (M1 control), IL-4 (M2 control), or with LPS plus IFNγ plus GPETAFLR (50-100 μg/mL, Peptide 2 and Peptide 1, respectively) for additional 24 h. Relative expression of (A) TNF-α, (B) IL-1β, (C) IL-6, and (D) IL-10 genes were measured by RT-qPCR. Values are presented as means ± SD (n = 3) and those marked with different letters are significantly different (P< 0.05).

DISCUSSION

In a world where resources are scarce, food plant processing residues are an alternative source to find proteins or peptides with bioactive properties [19]. Plant-derived bioactive peptides are very interesting compounds that can be used with potential pharmaceutical and nutraceutical benefit [20].

In the literature, it possible to find large number of examples where plant-derived biopeptides are used as anti-inflammatory or antioxidant compounds. One of them is 1,2,3,4,6 penta-*O*-galloyl-β-D-glucose, a naturally polyphenolic compound present in some medicinal

herbs as Rhuschinensis Mill [21] *Fagopyrum tataricum*, commonly known as buckwheat, is another example of bioactive plant. Researchers found that buckwheat extracts may inhibit adipogenesis and inflammatory response during adipocyte differentiation of 3T3-L1 cells [22]. Brazilian red propolis (*Apis mellifera*), Copaifera oleoresins, flavonoid fraction of Bergamot Juice (*Citrus bergamia*), effusanin C (*Isodon japonicus*), oligomeric proanthocyanidins (*crataegus oxyacantha*) are others isolated compounds with anti-inflammatory actions in activated monocytes and macrophages [23-27].

Lupinus angustifolius L. is a legume that contents a high level of plant protein [17]. GPETAFLR peptide was isolated from a lupine protein hydrolysate. This biopeptide showed anti-inflammatory effect in different line cells as osteoclasts [28] and THP-1-derived macrophages [17,29]. However, it is the first time that the anti-inflammatory activity of GPETAFLR was shown on primary monocytes.

Macrophage polarisation depends of different microenvironment and signals to promote specific phenotypes. These phenotypes correspond to classically (M1) and alternatively (M2) polarized macrophages that exhibit pro-inflammatory and anti-inflammatory functions, respectively [30-32]. In the present study, it was observed a modulation capacity of GPETAFLR on macrophage polarization. This could be explained by the fact that GPETAFLR peptide increase anti-inflammatory cytokines as IL-10 while decrease pro-inflammatory as IL-6 or IL-1β. These cytokines regulate and modulate macrophages phenotype, stimulating M1 phenotype in the case of IL-1β or M2 phenotype in the case of IL-10 [33,34].

GPETAFLR could also modulate monocyte subsets. Under LPS stimulation, classical monocytes (monocytes that promote inflammatory reactions) increased their population while non-classical suffer a decrease with respect to monocytes treated with the peptide. This mean that GPETAFLR can modulate monocyte subset ratio, stimulating those monocytes that participate in anti-inflammatory process and decreasing classical monocytes [35,36].

In conclusion, GPETAFLR peptide showed an anti-inflammatory effect in monocyte cells. Our findings imply a new understanding of the mechanisms by which GPETAFLR favour a continuous and gradual plasticity process in the human monocyte/macrophage system and offer novel benefits derived from the consumption of *Lupinus angustifolius* L. in the prevention of inflammatory-related diseases.

Acknowledgments

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CHAPTER 03

Lupine (*Lupinus angustifolius* L.) peptide prevents non-alcoholic fatty liver disease in high-fat diet-induced obese mice

Lemus-Conejo A, Grao-Cruces E, Toscano R, Varela LM, Claro C, Pedroche J, Millan F, Millan-Linares MC, Montserrat-de la Paz S

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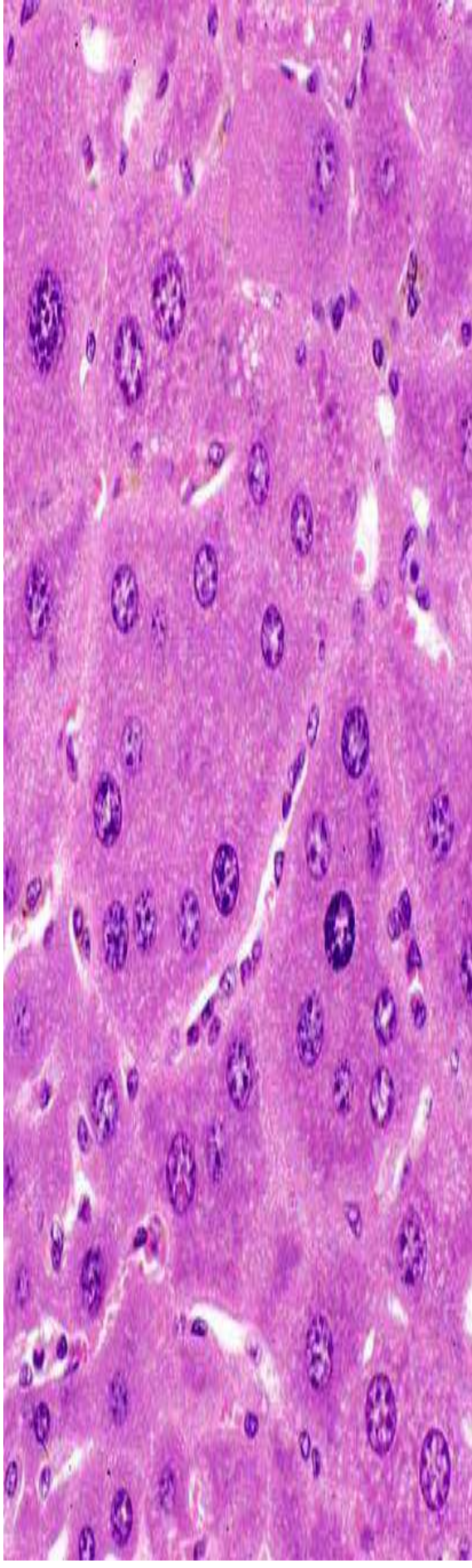

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A lupine (*Lupinus angustifolious* L.) peptide prevents non-alcoholic fatty liver disease in high-fat-diet-induced obese mice†

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Bioactive peptides are related to the prevention and treatment of many diseases. GPETAFLR is an octapeptide that has been isolated from lupine (*Lupinus angustifolius* L.) and shows anti-inflammatory properties. The aim of this study was to evaluate the potential activity of GPETAFLR to prevent non-alcoholic fatty liver disease (NAFLD) in high-fat-diet (HFD)-induced obese mice. C57BL/6J mice were fed a standard diet or HFD. Two of the groups fed the HFD diet were treated with GPETAFLR in drinking water at 0.5 mg kg^{-1} day^{-1} or 1 mg kg^{-1} day^{-1}. To determine the ability of GPETAFLR to improve the onset and progression of non-alcoholic fatty liver disease, histological studies, hepatic enzyme profiles, inflammatory cytokine and lipid metabolism-related genes and proteins were analysed. Our results suggested that HFD-induced inflammatory metabolic disorders were alleviated by treatment with GPETAFLR. In conclusion, dietary lupine consumption can repair HFD-induced hepatic damage possibly *via* modifications of liver's lipid signalling pathways.

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Introduction

Nowadays, the population is growing exponentially and meanwhile, natural sources are decreasing due to their overexploitation and climate change. For these reasons, it is necessary to discover new sources of nutritional compounds that have nutritional value and can be easy to obtain and process. This research opens a novel area that takes advantage of agroindustry by-products to further propose adaptive supply regarding the specific demand of nutrients and bioactive compounds.[1,2]

Vegetables are an important source of nutrients. The isolation and purification of peptides are relatively easy; moreover, their growth procedure is cheap and simple. Therefore, peptides are an ideal target for obtaining bioactive compounds. The main sources of peptides are soy, eggs, milk, and fish.[3] The main ways by which the bioactive peptides act on human health are as follows: decreased inflammation, lower blood pressure, anti-obesity, and prevention and improvement in the symptoms related to diabetes.[4] In addition, the diseases that are on the rise in developed countries such as obesity, dyslipidemias, type II diabetes, metabolic syndrome, and non-alcoholic fatty liver disease (NAFLD) have pharmacological treatment that is not fully effective and also has adverse side effects.[5] This is why many scientists are looking for substances of plant origin that can alleviate or prevent these diseases.[6]

Bioactive peptides contain a short sequence of amino acids with different biological activities. Initially, they are found in protein precursors in the organism of origin and they are generally not active.[7,8] Peptides are obtained from plant proteins as a result of enzymatic hydrolysis during digestion *via* fermentation by micro-organisms or by *in vitro* chemicals or enzymatic hydrolysis processes.[7]

Lupinus angustifolius L. is one of the targets for the research of such peptides because it has a high amount of proteins and low fat content.[9,10] GPETAFLR is an octapeptide isolated from *Lupinus angustifolius* L. with the sequence glycine-proline-glutamic acid-threonine-phenylalanine-leucine-arginine. This peptide features a three-dimensional secondary structure of

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ABSTRACT

Bioactive peptides are related to the prevention and treatment of many diseases. GPETAFLR is an octapeptide that has been isolated from lupine (*Lupinus angustifolius* L.) and shows anti-inflammatory properties. The aim of this study was to evaluate the potential activity of GPETAFLR to prevent non-alcoholic fatty liver disease (NAFLD) in high-fat-diet (HFD)-induced obese mice. C57BL/6J mice were fed a standard diet or HFD. Two of the groups fed the HFD diet were treated with GPETAFLR in drinking water at 0.5 mg kg/d or 1 mg kg/d. To determine the ability of GPETAFLR to improve the onset and progression of non-alcoholic fatty liver disease, histological studies, hepatic enzyme profiles, inflammatory cytokine and lipid metabolism-related genes and proteins were analysed. Our results suggested that HFD-induced inflammatory metabolic disorders were alleviated by treatment with GPETAFLR. In conclusion, dietary lupine consumption can repair HFD-induced hepatic damage possibly via modifications of liver's lipid signalling pathways.

INTRODUCTION

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Lupinus angustifolius L. is one of the targets for the research of such peptides because it has a high amount of proteins and low-fat content [9,10]. GPETAFLR is an octapeptide isolated from *Lupinus angustifolius* L. with the sequence glycine-proline-glutamic acid-threonine-phenylalanine-leucine-arginine. This peptide features a three-dimensional secondary structure of α-helix (**Figure 10**). Its immunomodulatory and anti-inflammatory capacities have been demonstrated [11]. Its effects have been previously investigated in macrophage THP-1-line derivatives [11], osteoclasts derived from human primary monocytes [12], and primary human monocytes [13]. Hence, it is an interesting compound to treat or prevent diseases characterized by inflammation.

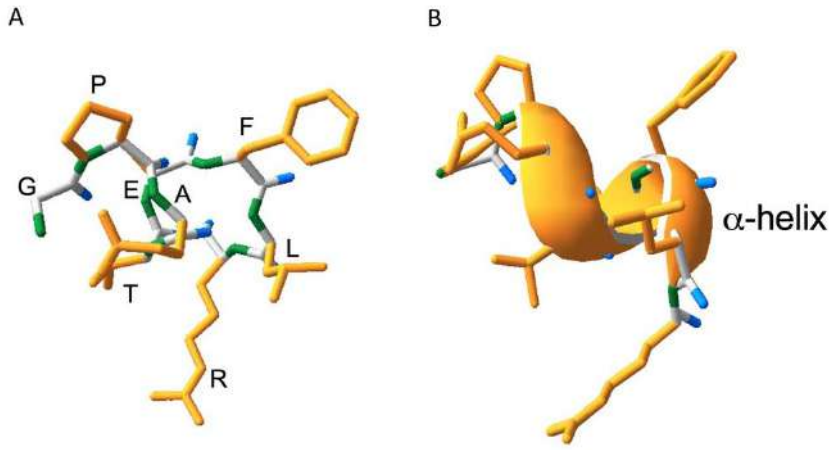

Figure 10. Chemical (a) and secondary three-dimensional (b) structure of GPETAFLR peptide, an octapeptide isolated from Lupinus angustifolius L., which amino acid sequence is identified as: Glycine (G), Proline (P), Glutamate (E), Threonine (T), Alanine (A), Phenylalanine (F), Leucine (L), and Arginine (R). Yellow colour was used for side chain, green colour was used for amino group, and blue colour was used for carboxyl group.

Non-alcoholic fatty liver disease (NAFLD) is a health problem that affects a large percentage of the world's population with a global prevalence of 25% [14]. The prevalence of this disease is very common in developed countries due to the fact that it typically occurs in people with obesity, type 2 diabetes, and metabolic syndrome among other pathologies related to lifestyle [15–18]. NAFLD consists of the accumulation of fat in the liver tissue, which is derived from a high amount of free fatty acids (FFAs) and triglycerides (TGs) in the circulating blood. These levels may increase after higher caloric and fat intakes. For this reason, fatty liver disease is often associated with obesity and

dyslipemias [15–18]. The best models for the study of this disease are animal models of induced obesity; specifically, mice and rat models present pathogenesis more similar to humans [19]. NAFLD has 4 phases: non-alcoholic fatty liver (NAFL), accumulation of fat in the liver; non-alcoholic steatohepatitis (NASH), liver fat accumulation with inflammation; fibrosis, accumulation of fat and inflammation resulting in tissue damage that drifts into fibrosis; and cirrhosis, most of the cells are replaced by fibrosis, thus considerably damaging the cellular hepatic function. This state leads to hepatic dysfunctionality, and the development of NAFLD may culminate in the development of liver cancer [18]. Specifically, cirrhosis (last stage of NAFLD) is the major risk factor for developing hepatocellular carcinoma, the most common liver cancer. Hepatic steatosis is also related to the occurrence of hepatocellular carcinoma [20]. Thus, NAFLD, even in its early stages, promotes the development of the most common type of liver cancer. Herein, the peptide GPETAFLR was used to investigate its role in the development of NAFLD in the hepatic tissues of mice with HFD-induced obesity.

MATERIALS AND METHODS

Isolation, purification, and synthesis of GPETAFLR

Seeds of sweet lupine (*Lupinus angustifolius* L.) were a gift from Koipesol Semillas, S.A. (Seville, Spain). Alcalase 2.4 L was provided by Novozymes (Bagsvaerd, Denmark). Lupine protein isolate was obtained using a previously described method at the pilot plant scale (Plant

Protein Unit, Instituto de la Grasa, Seville, Spain) [21]. The chemical composition of lupine protein isolate in dry matter was as follows: protein 86.83%, dietary fiber 5.97%, fat 5.08%, ash 0.78%, and carbohydrate 1.34%. Lupine protein isolate was suspended in distilled water (10% w/v), and hydrolysis with Alcalase was performed under the following conditions: pH 8, 50 °C, E/S = 0.3 AU g−1 protein, and a hydrolysis time of 15 min. The mixture was then heated at 85 °C for 15 min to inactivate the enzyme and centrifuged at 6500g for 15 min, and the supernatant constituted LPH [11]. This fraction was purified by ultrafiltration and chromatographic techniques [22]. The sequence of the purified peptide was identified through sequence analysis using nano-HPLC coupled with a Polaris Q ion-trap mass spectrometer (Mass Spectrometry Unit, Instituto de la Grasa, Seville, Spain). Finally, the peptide was synthesized by an Fmoc solid-phase method (Barcelona Scientific Park Foundation, Barcelona, Spain) at 95% purity measured using HPLC-UV at 220 nm.

Animal Diets and Experimental Design

In order to keep the experimental number of mice as low as possible, forty male mice C57BL/6J were used in the study. They were obtained from the Animal Production and were divided into four groups (n = 10 per group), in which 3 of them were fed HFD and the last group was fed a standard chow diet (CD) and was used as a control group (210 SAFE, Augy, France). Mice were fed for 8 weeks. The HFD, which contained 60% energy as fat, was prepared by Panlab Laboratories (HF 260 SAFE) and presented as pellets to the animals (**Table 9**). Two of the experimental animal groups fed HFD were also treated with the

synthesized GPETAFLR peptide, which was added into drinking water and administered at a dose of 0.5 or 1 mg/kg/d. Mice were anesthetized by an intraperitoneal injection of ketamine (100 mg/kg) and diazepam (5 mg/kg) and then euthanized by cervical dislocation. Hepatic tissues were immediately removed and then frozen at −80 °C until processing. Homogenization was done with TRIsure (Bioline, Memphis, TN, USA).

Table 9. Composition of standard chow and high-fat diets

Macronutrients		**Kcal/kg**	**% Kcal**
Standard chow diet (amount per Kg)			
Protein	205.9 g	823.8	23.3
Fat	55.1 g	496.4	14
Carbohydrates	554.2 g	2216.8	62.7
Total	815.2 g	3537	100
High-fat diet (amount per Kg)			
Protein	200.2 g	800.9	1.6
Fat	359.4 g	3235.2	58.8
Carbohydrates	374.1 g	1496.3	26.7
Total	933.7 g	5505.4	100

Dosage information

Doses of 0.5 and 1 mg/kg/d of the synthesized GPETAFLR peptide were established according to previous studies [23–28] and the expected water consumption of the C57BL/6J strain [29]. These selected doses for animals were comparable to human equivalent doses (HED) of 40 and 80 µg/kg [30].

RNA isolation and real-time quantitative PCR analysis

RNA from hepatic tissues was isolated to quantify gene expression by RT-qPCR. Total RNA was extracted by using TRIsure Reagent (Bioline). RNA quality was assessed by the A_{260}/A_{280} ratio using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Madrid, Spain). Briefly, RNA (250 ng) was subjected to reverse transcription (iScript, Bio-Rad). An amount of 40 ng of the resulting cDNA was used as a template for RT-qPCR amplifications. The mRNA levels for specific genes were determined in a CFX96 system (Bio-Rad). For each PCR reaction, a cDNA template was added to Brilliant SYBR green QPCR Supermix (Bio-Rad) containing the primer pairs for either gene or hypoxanthine phosphoribosyltransferase (HPRT) as a housekeeping gene. All amplification reactions were performed in triplicate and the average threshold cycle (Ct) numbers of the triplicates were used to calculate the relative mRNA expression of the candidate genes. The magnitude of the change in the mRNA expression for candidate genes was calculated by using the standard $2^{-(\Delta\Delta Ct)}$ method. All data were normalized to endogenous reference (HPRT) gene. content and expressed as relative fold-change of control. The sequences of the designed oligonucleotides are shown in **Table 10.**

Table 10. Sequence and GenBank accession number of oligonucleotides used in qRT-PCR.

Target	GenBank accession number	Direction	Sequence (5'→3')
FAS	NM_001146708.1	Forward	TGCAGACATGCTGTGGATCT
		Reverse	GGGCTGAATTTCTGATGGTC
HGPRT	NM_013556.2	Forward	TGCTCGAGATGTCATGAAGG
		Reverse	TATGTCCCCCGTTGACTGAT
IL-1β	NM_008361	Forward	GCCCATCCTCTGTGACTCAT
		Reverse	AGGCCACAGGTATTTTGTCG
IL-6	NM_031168	Forward	AGTTGCCTTCTTGGGACTGA
		Reverse	TCCACGATTTCCCAGAGAAC
IL-10	NM_010548.2	Forward	CCAGGGAGATCCTTTGATGA
		Reverse	CATTCCCAGAGGAATTGCAT
OB-R	NM_010704.2	Forward	AACTGCAGTCTTCGGGGATG
		Reverse	AAGGGTGGATCGGGTTTCAC
PPARα	NM_001113418.1	Forward	GCCAGGTTTGACTGAACCAT
		Reverse	GAGAAATGTGGCCAGGACAT
TNF-α	NM_001278601	Forward	AGCCCCCAGTCTGTATCCTT
		Reverse	CTCCCTTTGCAGAACTCAGG
UCP1	NM_009463.3	Forward	GGGCCCTTGTAAACAACAAA
		Reverse	GTCGGTCCTTCCTTGGTGTA

Histological analysis

Dissected liver sections were fixed overnight with 4% paraformaldehyde, cryoprotected with 15%–30% sucrose and embedded in Tissue-Tek OCT embedding medium (Sakura Finetek Europe, The Netherlands). Cryostat sections (8–10 microns) were rinsed with PBS and stained with a 0.3% solution of Oil Red O for 10 minutes at room temperature. After washing in PBS, the sections were counterstained with haematoxylin and eosin for 1 minute and washed with water [31]. The percentage of the stained area was determined using Image J software.

Biochemical analysis

The levels of leptin in supernatants were measured by ELISA following the indications of the manufacturer (ThermoFisher). The adipokine concentrations were expressed in ng per mL, as calculated from the calibration curves from a serial dilution of mice recombinant standards. In addition, the TG content in serum and liver tissues was determined by colorimetric enzyme assays (Bio-Science-Medical). The enzyme activities of liver enzymes, alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) were measured using a commercial kit (Bio-Science-Medical) following the instructions. Absorbance was read using a microplate reader.

Statistical analysis

All values in the figures and text are expressed as the arithmetic mean ± SD. Experiments were carried out in triplicate. Data were evaluated with GraphPad Prism Version 7.0 software (San Diego, CA, USA). The statistical significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance (ANOVA) using Tukey's test for multiple comparison analyses. P values < 0.05 were considered statistically significant.

RESULTS

GPETAFLR decreases body and liver weight gain in HFD-induced obese mice

After 8 weeks of diet, the animals showed body weight gain (**Figure 11A**), and the increase in the body weight of the groups fed HFD was higher than that for the control group (**Figure 11B**). Weight gain during the 8 weeks with HFD and GPETAFLR treatment at 1 mg/kg/d was significantly lower (1.3-fold lower for HFD-GPETAFLR (1 mg/kg/ d) than HFD) than that for animals fed HFD not containing the peptide. Liver weight was also significantly higher in the obese control (animals fed only HFD) (1.3-fold lower for HFD-GPETAFLR (1 mg/kg/d) than HFD) with respect to that for the animals treated with GPETAFLR in drinking water (**Figure 11C**).

In line with the results described above, the level of TGs in the blood was significantly higher in the obese control group (2.1-fold higher for HFD than HFD-GPETAFLR (1 mg/kg/d)) with respect to that in the

animals that, in addition to HFD, were treated with GPETAFLR in their drinking water (**Figure 11D**). In this case, similar to that observed for liver weight, the effect of GPETAFLR was dose-dependent.

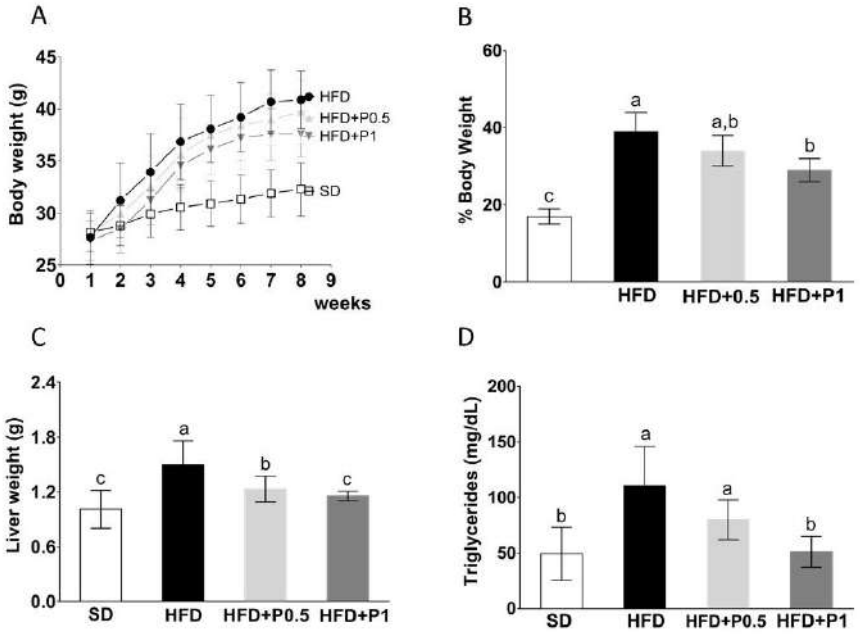

Figure 11. Effect of GPETAFLR on the evolution of body weight (**A**), percentage of body weight gain (**B**), liver weight (**C**) and blood triglyceride levels (**D**) in mice fed a standard chow diet (CD), an HFD or an HFD + GPETAFLR at a dose of 0.5 mg/kg/d and 1 mg/kg/d in drinking water. Values are presented as means ± SD (n = 10) and those marked with different letters are significantly different ($P < 0.05$).

GPETAFLR decreases hepatic leptin resistance in HFD-induced obese mice

In obesity, the blood levels of leptin increase due to the lack of sensitivity between leptin and its receptor (OB-R), which is similar to that observed in insulin resistance. After 8 weeks of HFD, mice presented higher serum levels of leptin than the animals fed the standard diet (4.2-fold higher for HFD than CD) (**Figure 12A**). The treatment with GPETAFLR significantly decreased these levels (2.7-fold lower for HFD-GPETAFLR (1 mg/kg/d) than HFD). Similarly, the OB-R mRNA levels were significantly higher in the animals fed HFD in contrast to that in the animals that did not receive GPETAFLR through drinking water (3.9-fold lower for HFD-GPETAFLR (1 mg/kg/d) than HFD, **Figure 12B**).

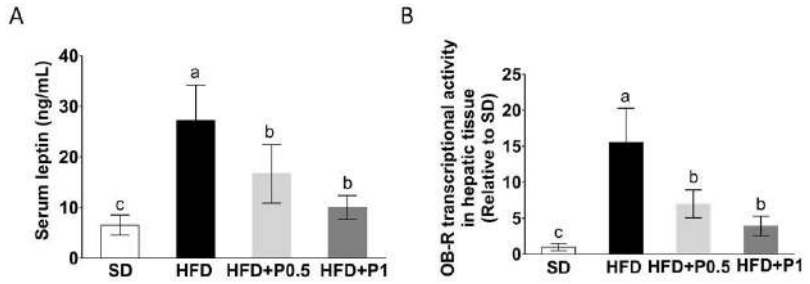

Figure 12. Effect of GPETAFLR on leptin resistance. Serum leptin levels (**A**) and gene expression in leptin receptor hepatic tissue (OB-R) (**B**) in mice fed a standard chow diet (CD), an HFD or an HFD + GPETAFLR at a dose of 0.5 mg/kg/d and 1 mg/kg/d in drinking water. Values are presented as means ± SD (n = 10) and those marked with different letters are significantly different ($P < 0.05$).

GPETAFLR decreases hepatic steatosis in HFD-induced obese mice

Figure 13 shows the results of the histological study carried out with ORO and H&E staining for the determination of the lipid content in hepatocytes. **Figure 13A** shows the representative staining images of the histological slices of livers obtained from each experimental group. After 8 weeks of HFD, mice presented higher levels (3.2-fold higher for HFD than CD) of hepatic lipids than the animals fed a standard diet (**Figure 13B**). The mice treated with GPETAFLR exhibited significant decrease in the hepatic steatosis induced by HFD (1.9-fold lower for HFD-GPETAFLR (1 mg/kg/d) than HFD).

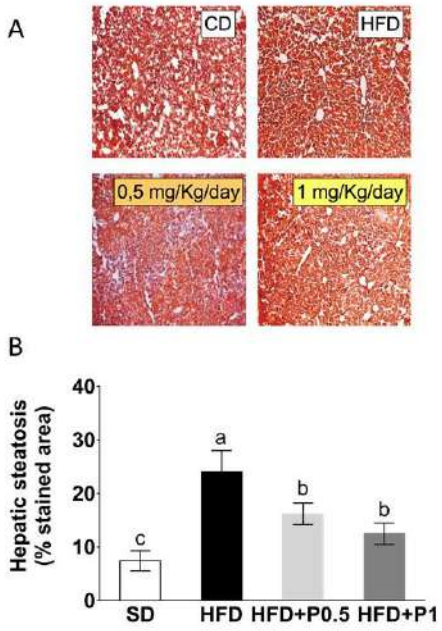

Figure 13. Effect of GPETAFLR on hepatic steatosis. Representative images (10×) of stained histological slices with Oil Red O (ORO) and haematoxylin–eosin (H&E) (**A**) and quantification of the stained area with ORO from each experimental group (**B**) in mice fed a standard chow diet (CD), an HFD or an HFD + GPETAFLR at a dose of 0.5 mg/kg/d and 1 mg/kg/d in drinking water. Values are presented as means ± SD (n = 10) and those marked with different letters are significantly different ($P < 0.05$).

GPETAFLR modulates hepatic lipid metabolism-related genes in HFD-induced obese mice

After 8 weeks of HFD, the levels of TGs in liver tissues were higher compared to that for CD (5.2-fold higher for HFD than CD, **Figure 14A**). However, the TG levels were lower in the animals that were fed HFD and received GPETAFLR treatment (2.7-fold lower for HFD-GPETAFLR (1 mg/kg/d) than HFD). Significantly, GPETAFLR at 1 mg/kg/d

restored the values of CD. In line with these results, the expression of the fatty acid synthase (FAS) enzyme (**Figure 14B**) and peroxisome proliferator-activated receptor α (PPARα) were determined (**Figure 14C**). The FAS mRNA levels in the animals fed HFD were significantly higher than those in the animals fed CD (33.6-fold higher for HFD than CD) or treated with GPETAFLR (18.6-fold higher for HFD than CD). In addition, the transcriptional activity of PPARα increased in the groups that received the octapeptide (6.6-fold higher for HFD-GPETAFLR (1 mg/kg/d) than HFD). Finally, the mRNA levels of mitochondrial decoupling protein 1 (UCP1) were determined (**Figure 14D**). The treatment with GPETAFLR in drinking water significantly increased the hepatic transcriptional activity of UCP1 in comparison to those groups that did not receive the peptide (4.9-fold higher for HFD-GPETAFLR (1 mg/kg/d) than HFD).

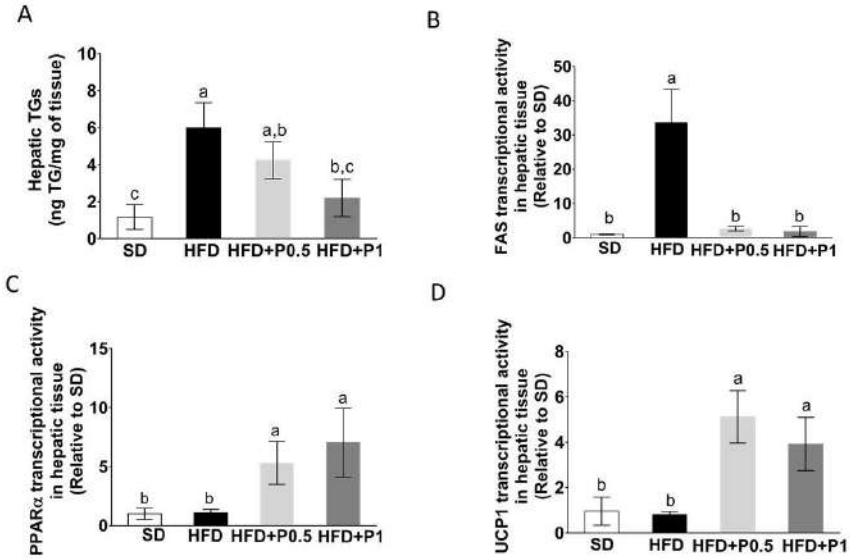

Figure 14. Effect of GPETAFLR on the TG levels in the liver tissue (**A**) and the expression of genes related to lipid and energy metabolism, such as the fatty acid synthase (FAS) (**B**), peroxisome proliferator-activated receptor α (PPARα) (**C**) and uncoupling protein 1 (UCP1) (**D**) in mice fed a standard chow diet (CD), an HFD or an HFD + GPETAFLR at a dose of 0.5 mg/kg/d and 1 mg/kg/d in drinking water. Values are presented as means ± SD (n = 10) and those marked with different letters are significantly different ($P < 0.05$).

GPETAFLR decreases hepatic inflammation in HFD-induced obese mice

After 8 weeks of HFD, the gene expressions of proinflammatory cytokines on hepatic tissues such as tumour necrosis factor α (TNF-α) (**Figure 15A**), interleukin 1β (IL-1β) (**Figure 15B**), and interleukin 6 (IL-6) (**Figure 15C**) were significantly higher in the animals fed exclusively with HFD. In both doses, GPETAFLR significantly decreased the mRNA levels of the pro-inflammatory cytokines. The gene expression of the anti-inflammatory cytokine interleukin 10 (IL-10) significantly increased in

the mice that received GPETAFLR in drinking water compared to that in the groups that did not receive the peptide (5.1-fold higher for HFD-GPETAFLR (1 mg mg/kg/d) than HFD, **Figure 15D**).

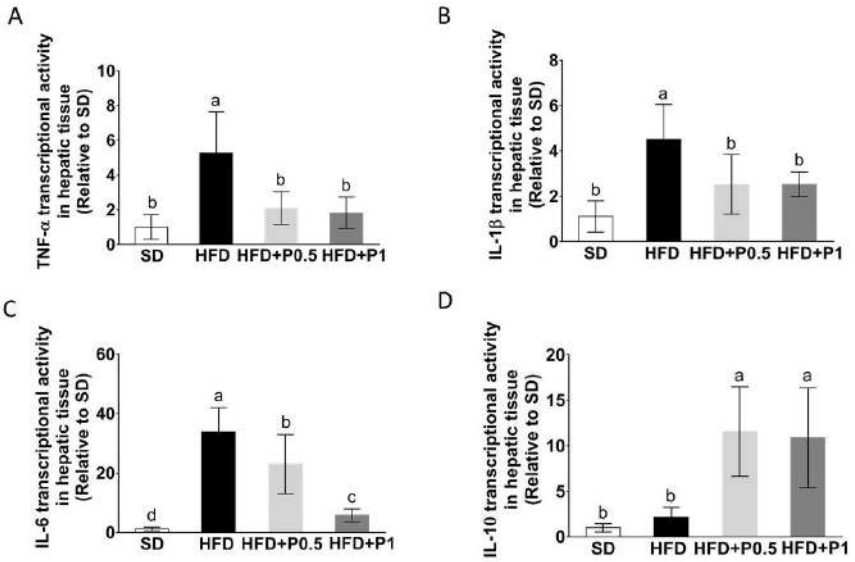

Figure 15. Effect of GPETAFLR on the expression of pro-inflammatory and anti-inflammatory cytokine genes in mice fed a standard chow diet (CD), an HFD or an HFD + GPETAFLR at a dose of 0.5 mg/kg/d and 1 mg/kg/d in drinking water. The mRNA levels of TNF-α (**A**), IL-1β (**B**), IL-6 (**C**) and IL-10 (**D**) were measured in the liver tissue. Values are presented as means ± SD (n = 10) and those marked with different letters are significantly different ($P < 0.05$).

GPETAFLR decreases blood markers of liver damage in HFD-induced obese mice

After 8 weeks of HFD, mice showed higher serum activity for AST (5.1-fold higher for HFD than HFD-GPETAFLR (1 mg/kg/d), **Figure 16A**) and ALT (8.2-fold higher for HFD than HFD-GPETAFLR (1 mg/kg/d), **Figure 16B**) than those animals that received the GPETAFLR peptide in drinking water. GPETAFLR restored the values of transaminases to the levels in the animals fed CD. The activity of serum ALP was significantly higher in the obese control (1.4-fold higher for HFD than HFD-GPETAFLR (1 mg/kg/d), **Figure 16C**). GPETAFLR at 1 mg/kg/d restored the values of this marker to similar levels to the animals fed CD. On the other hand, the values for the serum activity of the enzyme LDH were higher in the obese control (2.8-fold higher for HFD than HFD-GPETAFLR (1 mg/kg/d), **Figure 16D**). GPETAFLR at 1 mg/kg/d significantly decreased the values of this enzyme compared to that for the mice fed HFD without the GPETAFLR treatment.

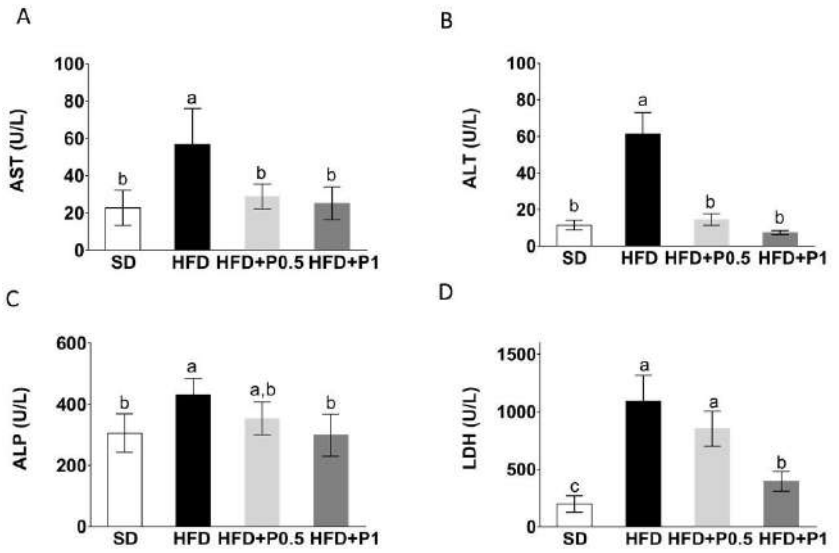

Figure 16. Effect of GPETAFLR on serum liver damage markers such as aspartate aminotransferase (AST) **(A)**, alanine aminotransferase (ALT) **(B)**, alkaline phosphatase (ALP) **(C)** and lactate dehydrogenase (LDH) **(D)** in mice fed a standard chow diet (CD), an HFD or an HFD + GPETAFLR at a dose of 0.5 mg/kg/d and 1 mg/kg/d in drinking water. CD, chow diet, was used as a control group. Values are presented as means ± SD (n = 10) and those marked with different letters are significantly different ($P < 0.05$).

DISCUSSION

The use of bioactive peptides obtained from food is increasingly gaining importance because of their health benefits and use in preventing chronic diseases, whose incidences are increasing worldwide [4]. Many chronic diseases are characterized by a marked inflammatory process. Therefore, GPETAFLR, a novel anti-inflammatory biopeptide obtained from *Lupinus angustifolius* L., can be a potent molecule for the prevention of these types of diseases. Its anti-inflammatory activity has been demonstrated. Specifically, both the peptide and the extracts of hydrolyzed lupin proteins have shown the ability to decrease the

expression of pro-inflammatory cytokines and increase the expression of anti-inflammatory cytokines [11–13].

The liver is the main metabolic organ of the body. NAFLD affects its functionality, endangering the homeostasis metabolic system. This disease is typically associated with obesity [15–18], where a substance that has the effect of reducing body weight and obesity rates will greatly alleviate the symptoms and development of NAFLD. The octapeptide GPETAFLR prevents the weight gain resulting from a diet rich in fat in contrast to other substances such as isoliquiritigenin with hepatoprotective properties that do not have the capacity to prevent weight gain [32]. It can be said that GPETAFLR interferes by preventing the progression of the main cause of NAFLD: obesity.

On the other hand, the obtained data corroborated that the HFD-induced obese mice presented NAFLD pathophysiology similar to humans. The pathophysiology of this disease is characterized by increased body weight and liver weight, high levels of TGs in the blood, fat accumulation in adipose tissues, hepatic inflammation and elevated markers of liver damage [33]. In our study, the obese control group showed a greater liver weight derived from a greater accumulation of fat in the liver. The accumulation may be due to high levels of TGs in the blood and the increase in the FAS gene expression. In addition, this group presented a pro-inflammatory state provoked by high levels of the expressions of pro-inflammatory cytokines IL-6, IL-1β, and TNF-α since the Kupffer cells increase their expression in response to hepatic fat accumulation [34]. Finally, it presented elevated markers of liver damage since the activities of transaminases, LDH, and ALP in serum were significantly higher. These values were also obtained in other

similar studies on HFD-induced obese animals [19,32,35–37].

All these parameters of the pathology were palliated with the treatment with GPETAFLR owing to the fact that liver weight and liver fat accumulation were lower in the animals treated with the octapeptide. The factors that can influence the minor accumulation of hepatic fat in these individuals are a decreased gene expression of FAS and an increased expression of the enzymes that increase energy metabolism, such as UCP1 and PPARα. In addition, there is less circulation of TGs in the blood and the availability for its accumulation in different tissues including the liver. GPETAFLR could mediate the accumulation of hepatic fat by influencing the signaling pathways involved in the regulation of the gene expression or the activity of lipid metabolism proteins, such as the kinase pathway activated by AMP (AMPK). AMPK regulates the activity and expression of many important proteins related to metabolism; one of them is mTOR, another metabolic regulator. AMPK decreases the accumulation of fat (down-regulating FAS, between other proteins) and other metabolites and also down-regulates mTOR, the protein that increases the accumulation of fat and also has effects on the gene and protein expressions [38]. In previous studies, GPETAFLR has been demonstrated to present anti-inflammatory activity that is also exhibited by hepatocytes. The octapeptide decreased pro-inflammatory cytokine levels and increased the levels of the anti-inflammatory cytokine IL-10. IL-10 did not show a decrease in expression in the obese control compared to that in the standard control, but its expression greatly increased in the groups treated with GPETAFLR. These data are related to previous studies on other cell lines and primary cultures [11–13]. Pro-inflammatory

cytokines present several actions related to metabolism; for example, TNF-α decreases the degradation of fats and increases their accumulation, and IL-6 inhibits lipolysis and increases fat deposition [35,39]. For this reason, in addition to contributing to tissue damage from chronic inflammation, they contribute to the development of NAFLD by boosting fat accumulation in the liver. Chronic inflammation is primarily mediated by the nuclear factor κB (NF-κB) pathway, which stimulates the production of cytokines such as TNF-α [40]. For this reason, GPETAFLR may decrease inflammation by influencing the NF- κB pathway. The NF-κB pathway increases the expression of proinflammatory cytokines; this pathway can be activated by the interaction of free fatty acids with TLR4, a membrane receptor expressed by Kupffer cells, which produces a pro-inflammatory state [34]. The down-regulation of this pathway can be determined by the reduction in fat accumulation in the liver associated with the treatment with GPETAFLR.

Another parameter used as a marker of NAFLD pathogenesis in this study was the measurement of blood markers for liver damage, specifically transaminases, ALP, and LDH. The activity of these enzymes in serum was lower in both GPETAFLR treatments compared to that in the obese control group. This effect is probably due to the fact that the peptide decreases liver damage by reducing the accumulation of fat and inflammation (influenced by the lower accumulation of fat) and thus all the tissue damage associated with a chronic inflammatory process.

On the other hand, leptin is an adipokine produced by adipose tissues, and the levels of this hormone in the blood are indicative of the body's fat reserves because it is produced proportionally to the amount

of adipose tissues. Therefore, a higher amount of leptin results in a higher amount of fat reserve [41]. Leptin resistance is a characteristic of most obesity associated diseases. This syndrome is characterized by a rise in serum leptin due to the increase in adipose tissues, but there is a lack of its signaling because the tissues with receptors show resistance. A proof that leptin resistance is occurring is a high value of leptin in serum and the gene expression of its receptor in the tissues that express it, such as that observed in the animals of the obese control group. The liver is one of the organs that can present receptors for leptin and signaling by this molecule increases the catabolic metabolism, increases the degradation of fatty acids, decreases the synthesis of fatty acids and affects glucose metabolism, specifically hypoglycemia [41,42]. Leptin resistance in NAFLD can mediate an increase in the accumulation of hepatic fat in its direct form by a lack of signaling in the liver. In addition, leptin resistance may enhance the progression of NAFLD due to its role in intake and satiety. Leptin can cross the blood–brain barrier once inside the central nervous system (CNS), activate the production of anorexigenic peptides and inhibit the production of orexigenic peptides. This role translates into the inhibition of appetite and therefore into a decrease in caloric intake [41]. Supplementation with GPETAFLR produced a decrease in obesity-associated leptin resistance. These effects may be due to the fact that GPETAFLR decreases body weight or GPETAFLR decreases resistance to the leptin and therefore, there is a decrease in body weight. It may also be considered that both effects are produced. Other plant bioactive substances have been shown to have effects by decreasing the resistance to leptin without significantly affecting the body weight [32].

CONCLUSIONS

To conclude, GPETAFLR decreased body weight gain, liver weight and fat accumulation in the liver as well as TG levels in the blood and leptin resistance associated with obesity. GPETAFLR showed anti-inflammatory properties at the hepatic level, decreasing the mRNA levels of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-10 and increasing the expression of the anti-inflammatory cytokine IL-10 in liver tissues. The octapeptide also reduced blood markers related to liver damage such as AST, ALT, ALP, and LDH. Taken together, our findings suggest that the use of GPETAFLR is a potential treatment as well as an important preventive therapy for NAFLD. Thus, the dietary supplementation with *Lupinus angustifolius* L. would also contribute to a reduction in this pathology.

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CHAPTER 04

GPETAFLR, a peptide from *Lupinus angustifolius* L. prevents inflammation in microglial cells and confers neuroprotection in brain

Lemus-Conejo A, Millan-Linares MC, Toscano R, Millan F, Pedroche J, Muriana FJ, Montserrat-de la Paz S

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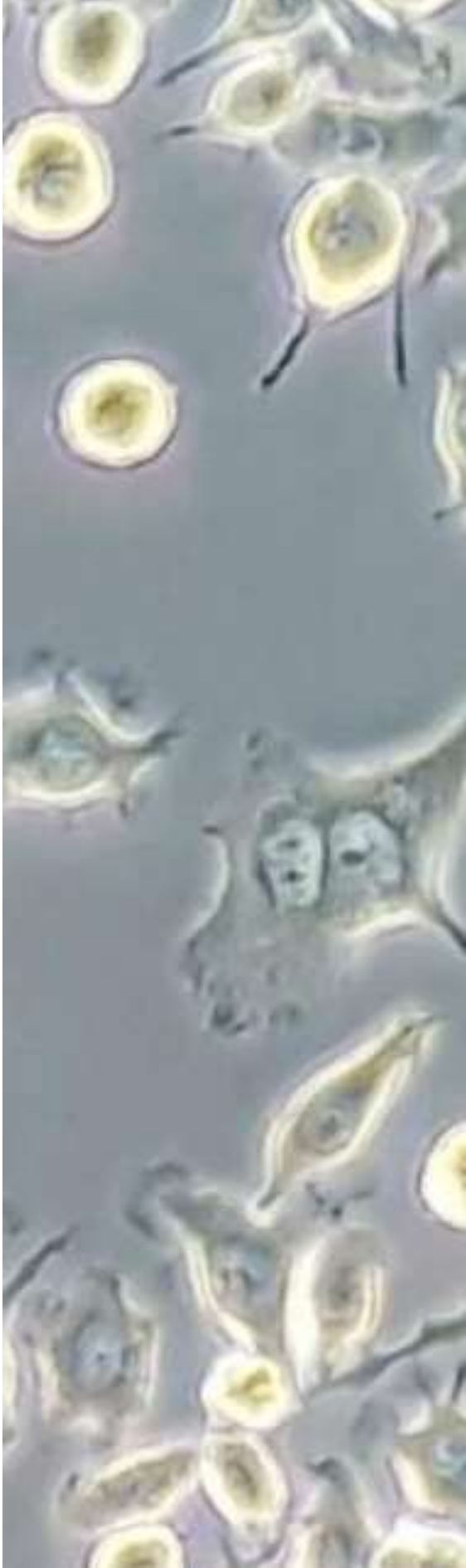

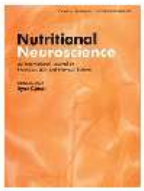

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GPETAFLR, a peptide from Lupinus *angustifolius* L. prevents inflammation in microglial cells and confers neuroprotection in brain

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ABSTRACT

Neuroinflammation is a complex inflammatory process in the central nervous system (CNS) where microglia may play a critical role. GPETAFLR is a peptide isolated from Lupinus angustifolius L. protein hydrolysates with functional activity in mononuclear phagocytes. However, it is unknown whether GPETAFLR has neuroprotective effects. We analysed the potential anti-neuroinflammatory activity of GPETAFLR by using two different models of neuroinflammation: BV-2 microglial cells and mice with high-fat diet (HFD)-induced obesity. GPETAFLR hampered LPS-induced upregulation of pro-inflammatory and M1 marker genes in BV-2 cells. This effect was accompanied by an unchanged expression of anti-inflammatory IL-10 gene and by an increased expression of M2 marker genes. GPETAFLR also increased the transcriptional activity of M2 marker genes, while the microglia population remained unchanged in number and M1/M2 status in brain of mice with high-fat diet (HFD)-induced obesity. Furthermore, GPETAFLR counteracted HFD-induced downregulation of IL-10 and upregulation of pro-inflammatory markers in the mouse brain, both at gene and protein levels. This is the first report describing that a peptide from plant origin robustly restrained the pro-inflammatory activation of microglial cells in cultures and in brain. Our data suggest that GPETAFLR might be instrumental in maintaining CNS homeostasis by inhibiting neuroinflammation.

INTRODUCTION

The substitution of animal proteins with plant proteins in the food industry or in our own diet is an important issue for health [1]. Within plant proteins, encrypted peptides of a small amino acid sequence become active when released from the target protein by enzymatic hydrolysis or chemical treatments [2–6]. In previous studies, lupine (*Lupinus angustifolius* L.) protein hydrolysates (LPH) [7] and GPETAFLR (**Figure 17**) from LPH [8] were shown to exhibit potent anti-inflammatory properties in phorbol ester-stimulated human monocytic THP-1 cells. More recently, GPETAFLR was reported to reduce osteoclastogenic markers during differentiation of human blood monocytes into osteoclasts [9].

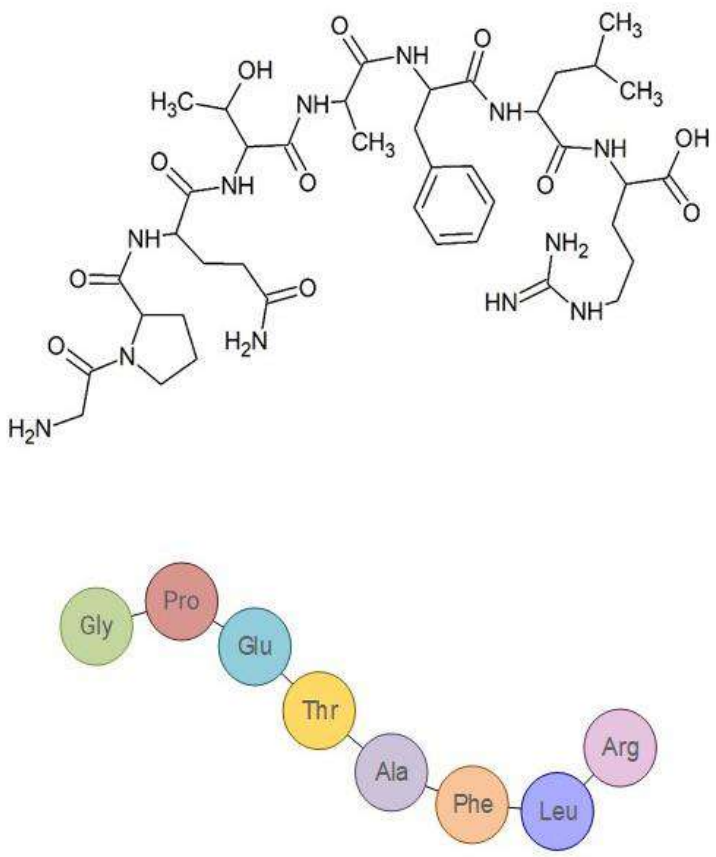

Figure 17. Chemical structure and amino acid sequence scheme of GPETAFLR. The sequence was identified as: Glycine, Proline, Glutamic acid, Threonine, Alanine, Phenylalanine, Leucine, and Arginine.

Microglial cells, which share a common myeloid origin with osteoclasts, are resident macrophages distributed in all regions of the adulthood brain and spinal cord, and represent between 10-15% of the total cells in the central nervous system (CNS) [10]. The principal function of microglia is to destroy and to clear foreign materials through phagocytic mechanisms [11]. Microglia also contributes to complex dynamics of inflammation and homeostasis by secreting a wide array of

cytokines and other signalling molecules, and has a role for antigen-presentation to T cells in the CNS [12]. As for the peripheral macrophage system, two phenotypes of activation in microglial cells *in vitro* have been proposed: classical M1 and alternative M2 microglial activation states [13, 14]. M1 phenotype may produce an excess of inflammatory mediators, including ROS, and pro-inflammatory cytokines and chemokines [15]. On the other hand, M2-activated microglia is believed to generate protective cytokines such as IL-10, IL-4, and IL-13, frequently involved in tissue repair and wound healing [16]. A sustained and not resolved inflammation in the CNS (termed neuroinflammation) is the primary pathological feature of neurodegenerative disorders including Alzheimer's disease, Parkinson's disease, Huntington's disease, tauopathies, age-related macular degeneration, and diseases of autoimmune origin such as multiple sclerosis [17]. Likewise, obesity is well known to be associated with multitude of risk factors that include local and systemic inflammation [18]. Chronic inflammation linked to obesity promotes alterations in circulating levels of cytokines and adipokines as a result of increased production by adipose tissue, liver, and cells of the immune system [19]. Such chronic low-grade inflammation also affects CNS and leads to neuroinflammation [20]. Previous studies have shown that chronic high-fat diet (HFD) may induce hypothalamic inflammation and production of pro-inflammatory cytokines, including tumour necrosis factor (TNF-α), IL-1β, and IL-6 [21], all of which expand inflammation in other brain areas and systemically [22]. However, the role of peptide GPETAFLR in the neuroinflammation field still remains to be elucidated.

Herein, the peptide GPETAFLR was used to investigate its anti-inflammatory activity in LPS-treated microglial BV-2 cells, as alternative model system for primary microglia cultures, and in the brain tissue of mice with HFD-induced obesity.

MATERIALS AND METHODS

All animal protocols received appropriate institutional approval (Animal Care and Use Committee of the University of Seville, AGL2012-40247) and were performed according to the official rules formulated in the Spanish law on the care and use of experimental animals (UE Directive of 2010: 2010/63/UE; RD 53/2013**).**

Isolation, Purification, and Synthesis of GPETAFLR

Seeds of lupine (*Lupinus angustifolius* L.) were a gift from Koipesol Semillas, S.A. (Seville, Spain). Alcalase 2.4L was provided by Novozymes (Bagsvaerd, Denmark). Lupine protein isolate was obtained using a previously described method [23] at pilot plant scale (Plant Protein Unit, Instituto de la Grasa, Seville, Spain). The chemical composition of lupine protein isolate in dry matter was: protein 86.83%, dietary fibre 5.97%, fat 5.08%, ash 0.78%, and carbohydrate 1.34%. Lupine protein isolate was suspended in distilled water (10% w/v), and hydrolysis with alcalase was performed under the following conditions: pH 8, 50 °C, E/S=0.3 AU/g protein, and a hydrolysis time of 15 min. The mixture was then heated at 85 °C for 15 min to inactivate the enzyme and centrifuged at 6500 g for 15 min, and the supernatant constituted the LPH [11]. This fraction was purified by ultrafiltration and chromatographic techniques [24]. The sequence of the purified peptide

was identified through sequence analysis by nanoHPLC coupled to a Polaris Q ion-trap mass spectrometer (Mass Spectrometry Unit, Instituto de la Grasa, Seville, Spain). Finally, the peptide was synthesized by Fmoc solid-phase method (Barcelona Scientific Park Foundation, Barcelona, Spain) at 95% purity, measured by HPLC-UV at 220 nm.

Animal Diets and Experimental Design

Male mice C57BL/6J were obtained from the Animal Production and Experimentation Centre at the University of Seville. At eight weeks old, mice (n = 40) were divided into four groups (10 animals per group) to receive for 8 weeks: standard chow diet, HFD, HFD plus GPETAFLR at a dose of 0.5 mg/kg/d, or HFD plus GPETAFLR at a dose of 1 mg/kg/d. GPETAFLR was added to the drinking water. HFD, which contained 60% energy as fat, was prepared by Panlab Laboratories (HF 260 SAFE) and presented as pellets to animals (**Table 11**).

Table 11. Composition of standard chow and high-fat diets

Macronutrients		**kcal/kg**	**% kcal**
Standard chow diet (amount per kg)			
Protein	205.9 g	823.8	23.3
Fat	55.1 g	496.4	14
Carbohydrates	554.2 g	2216.8	62.7
Total	815.2 g	3537.0	100
High-fat diet (amount per kg)			
Protein	200.2 g	800.9	1.6
Fat	359.4 g	3235.2	58.8
Carbohydrates	374.1 g	1496.3	26.7
Total	933.7 g	5505.4	100

Mice were anesthetized by an intraperitoneal injection of ketamine (100 mg/kg) and diazepam (5 mg/kg), and were underwent transcardial perfusion with 0.9% saline prior to harvesting the brain. Brain tissues were removed from the skull and frozen at -80 °C until processing. Homogenization was done with TRIsure (Bioline, Memphis, TN, USA) [25].

Dosage Information

Doses of 0.5 and 1 mg/kg/d for GPETAFLR were established according to previous studies [26-31] and to expected water consumption of the C57BL/6J strain [32]. These selected doses for animals were comparable to human equivalent doses (HED) of 40 and 80 µg/kg, respectively [33].

BV-2 Cell Culture and Treatments

BV-2 is a murine cell line used extensively in research related to neurodegenerative disorders [34, 35]. BV-2 microglial cells were obtained from the Cell Biology Unit at the Instituto de la Grasa (Seville, Spain). The cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum and 1% penicillin/streptomycin. They were maintained in 5% CO2 at 37 °C in a CO_2 incubator (Thermo Con Electron Corporation, Waltham, MA, USA). Experiments were done in twelve-well plates. BV-2 microglial cells were seeded at a density of 5 x 10^5 cells/well. Four different conditions were used: Untreated cells (negative control); cells exposed to LPS at 100 ng/mL (positive control); and cells exposed to LPS at 100 ng/mL + GPETAFLR at 50 or 100 μg/mL. LPS (*E. coli* 055: B5) (Sigma-Aldrich) was added for 1 h and then GPETAFLR. After incubation for an additional period of 24 h, cells were collected for RNA extraction.

Cell Viability Assay

BV-2 microglial cells were incubated in complete medium with different concentrations (10, 50, 100, 250, and 500 μg/mL) of GPETAFLR in 96-well plates (1 x 10^5 cells/well) during 24 h. Afterwards, the MTT reagent (Sigma-Aldrich) was added to each well until a purple precipitate was visible. MTT-formazan crystals were solubilized with DMSO (Sigma-Aldrich), and the absorbance was measured at 570 nm corrected to 650 nm with a microplate reader [36]. Cell survival was expressed as the percentage of absorbance compared with that

obtained in the negative control, untreated cells.

RNA Isolation and Quantitative Real-Time PCR Analysis

RNA from BV-2 microglial cells and from frozen brain samples was isolated to quantify gene expression by qRT-PCR. Total RNA was extracted by using TRIsure Reagent (Bioline). RNA quality was assessed by A_{260}/A_{280} ratio in a NanoDrop ND-1000 Spectrophotometer (ThermoFisher Scientific, Madrid, Spain). Briefly, RNA (250 ng) was subjected to reverse transcription (iScript, Bio-Rad). An amount of 40 ng of the resulting cDNA was used as template for qRT-PCR amplifications. The mRNA levels for specific genes were determined in a CFX96 system (Bio-Rad). For each PCR reaction, cDNA template was added to Brilliant SYBR green QPCR Supermix (Bio-Rad) containing the primer pairs for either gene or for hypoxanthine phosphoribosyltransferase (HPRT) as a housekeeping gene. All amplification reactions were performed in triplicate and average threshold cycle (Ct) numbers of the triplicates were used to calculate the relative mRNA expression of candidate genes. The magnitude of change of mRNA expression for candidate genes was calculated by using the standard $2^{-(\Delta\Delta Ct)}$ method. All data were normalized to endogenous reference (HPRT) gene content and expressed as relative fold-change of control. The sequences of the designed oligonucleotides are shown in **Table 12.**

Table 12. Sequence and GenBank accession number of oligonucleotides used in qRT-PCR.

Target	GenBank accession number	Direction	Sequence (5'→3')
TNF-α	NM_013693.3	Forward	GAGTTGGACCCTGAGCCATA
		Reverse	AGTCCGGGCAGGTCTACTTT
IL-1β	NM_008361.4	Forward	AGCTCATATGGGTCCGACAG
		Reverse	GACCTTCCAGGATGAGGACA
IL-6	NM_031168.2	Forward	TCCACGATTTCCCAGAGAAC
		Reverse	AGTTGCCTTCTTGGGACTGA
HPRT	NM_013556.2	Forward	TATGTCCCCCGTTGACTGAT
		Reverse	TGCTCGAGATGTCATGAAGG
IL-10	NM_010548.2	Forward	CATTCCCAGAGGAATTGCAT
		Reverse	CCAGGGAGATCCTTTGATGA
Ccr7	NM_007719.2	Forward	CCACGAAGCAGATGACAGAA
		Reverse	GTGTGCTTCTGCCAAGATGA
iNOS	NM_010927.4	Forward	GGTCAAACTCTTGGGGTTCA
		Reverse	CTCACTGGGACAGCACAGAA
Arg-1	NM_007482.3	Forward	ACAGACCGTGGGTTCTTCAC
		Reverse	CGCCTTTCTCAAAAGGACAG
Ym-1	NM_009892.3	Forward	AATGATTCCTGCTCCTGTGG
		Reverse	ACTTTGATGGCCTCAACCTG

Immunostaining Analysis by FACS

Brain cells were stained, in the dark and for 15 min, with fluorescent antibodies directed against CD45 (PE-Cy7 anti-mouse CD45, Beckton Dickinson, Madrid, Spain), CD11b (PerCP-Cy5.5 anti-mouse CD11b, BD), F4/80 (APC anti-mouse APC, Miltenyi Biotec, Madrid, Spain), CD80 (FITC anti-mouse FITC, Miltenyi Biotec), and CD200R (PE anti-mouse CD200R, Miltenyi Biotec). Mean fluorescence intensity (MFI) of 10^4 counted cells was measured in each sample with a flow cytometer FACSCanto II (BD, Palo Alto, CA, USA). Myeloid cells were gated on high side scatter (SSC^{high})-$CD45^+$ cells. The population of microglial cells was then gated as $CD11b^+F4/80^+$ [37]. Pro-inflammatory M1-like and anti-inflammatory M2-like subsets were further gated as $CD80^+$ and $CD200R^+$ microglial cells, respectively. Expression levels were presented as MFI corrected for non-specific binding of isotype control antibodies.

Measurement of Cytokine Release

The levels of IL-10 and IL-6 in brain tissue homogenate supernatants were measured by ELISA, following the indications of the manufacturer (Diaclone, Besancon, France). Cytokine concentrations were expressed as pg/mL, according to calibration curves from serial dilution of mice recombinant standards in each assay.

Statistical analysis

All values in the figures and text are expressed as the arithmetic mean ± SD. All experiments were carried out in triplicate and repeated at least three times independently, except for the whole animal

experiments. Data were evaluated with GraphPad Prism Version 7.0 software (San Diego, CA, USA). The statistical significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance (ANOVA), using Tukey's test for multiple comparison analysis.

RESULTS

GPETAFLR on BV-2 Microglial Cell Viability

BV-2 microglial cells were incubated with GPETAFLR at concentrations up to 500 µg/mL for 24 h. The cell viability evaluated by the MTT method was not affected by any GPETAFLR concentration (**Figure 18**).

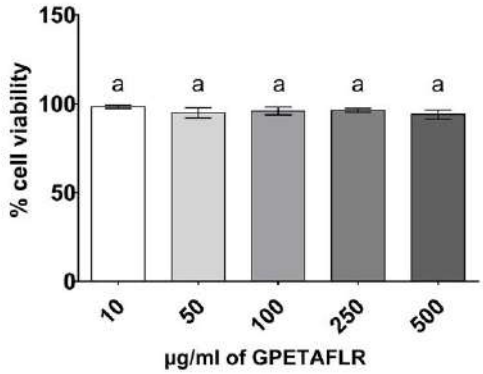

Figure 18. Effect of GPETAFLR on viability of BV-2 microglial cells. Cells were treated with GPETAFLR (10-500 µg/mL) in complete medium for 24 h. According to MTT assay, cell viability was expressed as percentage of absorbance relative to control (untreated) cells. Experiments were carried out in triplicate, at least three independent times. Values are presented as means ± SD and those marked with different letters are significantly different ($P < 0.05$), as determined through one-way ANOVA, followed by a post hoc Tukey test.

GPETAFLR on Cytokine Gene Expression in BV-2 Microglial Cells

Gene expression of cytokines TNF-α, IL-1β, IL-6, and IL-10 was measured by qRT-PCR in BV-2 microglial cells. As expected, LPS increased mRNA levels of TNF-α, IL-1β, and IL-6 genes (**Figures 19A, 19B, and 19C**). However, these LPS-induced changes were blocked in the presence of GPETAFLR at 50 or 100 µg/mL. Both doses of GPETAFLR were effective to keep the expression levels of IL-6 gene close to those found in untreated cells. GPETAFLR also showed a statistically significant dose-dependent effect on IL-1β gene expression. Furthermore, LPS induced a decrease in mRNA levels of IL-10 gene that was blocked by GPETAFLR (**Figure 19D**).

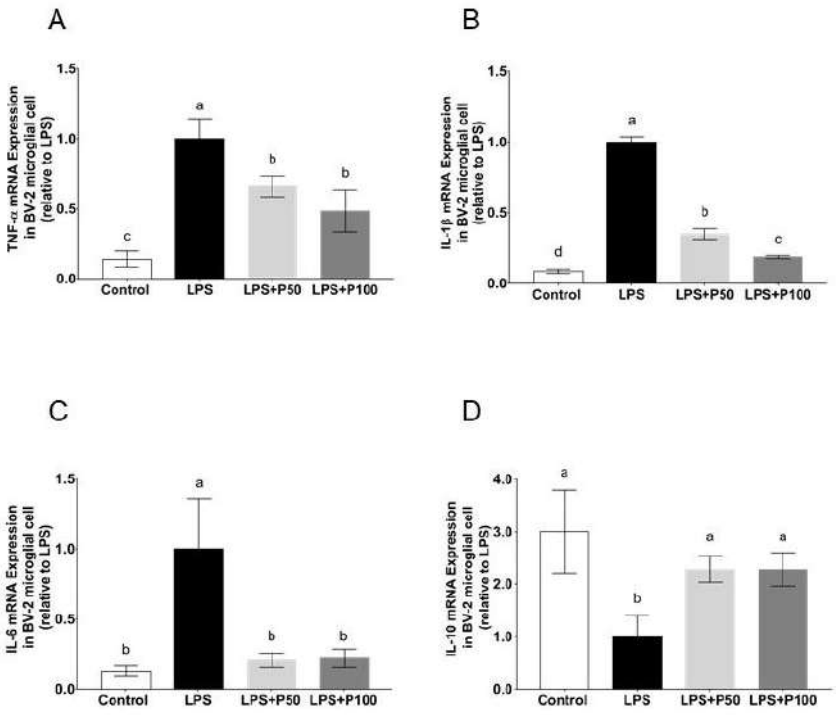

Figure 19. Effect of GPETAFLR on expression of pro-inflammatory and anti-inflammatory cytokine genes in LPS-treated BV-2 microglial cells. The mRNA levels of TNF-α (A), IL-1β (B), IL-6 (C), and IL-10 (D) genes were measured after the treatment of cells with LPS (100 ng/mL) in the absence or presence of GPETAFLR at 50 (P50) and 100 (P100) µg/mL. Control means untreated cells. Experiments were carried out in triplicate, at least three independent times. Values are presented as means ± SD and those marked with different letters are significantly different ($P < 0.05$), as determined through one-way ANOVA, followed by a post hoc Tukey test.

GPETAFLR on Plasticity in BV-2 Microglial Cells

Most tissue macrophages, including microglial cells, are highly plastic cells as they can polarize by exhibiting some of the characteristics of M1- and M2-like phenotypes upon appropriate stimulus *in vitro* [38-40]. To observe polarization of BV-2 microglial cells after LPS in the absence or presence of GPETAFLR at 50 µg/mL or 100 µg/mL, we extracted mRNA and performed qRT-PCR. LPS increased mRNA levels of M1-like gene markers (Ccr7, **Figure 20A** and iNOS, **Figure 20B**), but decreased mRNA levels of M2-like gene markers (Arg-1, **Figure 20C** and Ym-1, **Figure 20D**) in BV-2 microglial cells. Interestingly, the lowest dose of GPETAFLR completely suppressed the transcriptional activity of Ccr7 and iNOS genes. GPETAFLR also blocked LPS-induced decreased expression of Arg-1 and Ym-1 genes.

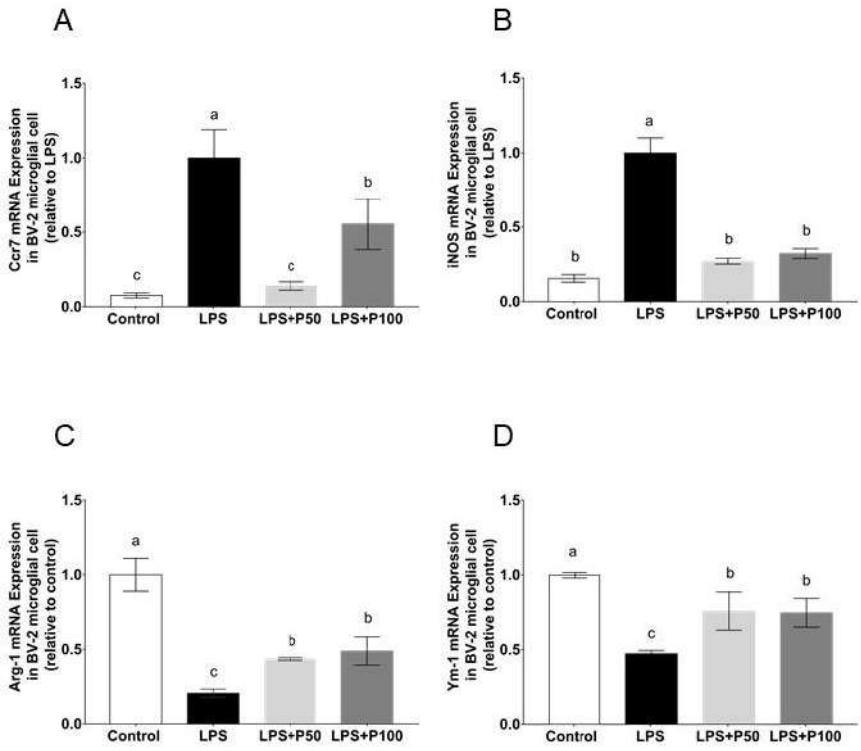

Figure 20. Effect of GPETAFLR on expression of M1 and M2 marker genes in LPS-treated BV-2 microglial cells. The mRNA levels of CCR7 (A), iNOS (B), Arg-1 (C), and Ym-1 (D) genes were measured after the treatment of cells with LPS (100 ng/mL) in the absence or presence of GPETAFLR at 50 (P50) and 100 (P100) µg/mL. Control means untreated cells. Experiments were carried out in triplicate, at least three independent times. Values are presented as means ± SD (n = 3) and those marked with different letters are significantly different ($P < 0.05$), as determined through one-way ANOVA, followed by a post hoc Tukey test.

GPETAFLR on Plasticity in Microglia of Brain from Mice with HFD-Induced Obesity

Mean daily food intake (standard chow diet, 2.92 ± 0.62; HFD, 2.35 ± 0.24; HFD plus GPETAFLR 0.5 mg/kg/d, 2.47 ± 0.25; HFD plus GPETAFLR 1 mg/kg/d, 2.37 ± 0.27 g/mouse) and water intake (standard chow diet, 3.00 ± 0.89; HFD, 2.95 ± 0.92; HFD plus GPETAFLR 0.5 mg/kg/d, 3.23 ± 0.78; HFD plus GPETAFLR 1 mg/kg/d, 4.02 ± 1.37 mL/mouse) did not statistically differ among the four diet groups. However, mean energy daily intake per mouse was lower ($P < 0.05$) with the standard chow diet (10.33 ± 2.19 kcal) than with HFD in the absence (12.94 ± 1.32 kcal) or presence of GPETAFLR at 0.5 mg/kg/d (13.60 ± 1.38 kcal) and at 1 mg/kg/d (13.05 ± 1.49 kcal).

To study plasticity of microglia as a consequence of an HFD-induced obesity in brain of mice during administration of GPETAFLR, we extracted mRNA and performed qRT-PCR as well as obtained primary microglia cells and used FACS to check their surface markers. As shown in **Figure 21A**, GPETAFLR at a dose of 1 mg/kg/d was able to decrease mRNA levels of Ccr7 gene as a marker for M1-like phenotype in the brain of mice on HFD, even to levels lower than those seen in mice fed with the standard chow diet. This effect was not evident for the iNOS gene (**Figure 21B**). It was noteworthy that GPETAFLR at a dose of 1 mg/kg/d also increased mRNA levels of Arg-1 (**Figure 21C**) and Ym-1 (**Figure 21D**) genes as markers for M2-like phenotype in the same brains. This effect of GPETAFLR on Ym-1 gene expression was dose-dependent.

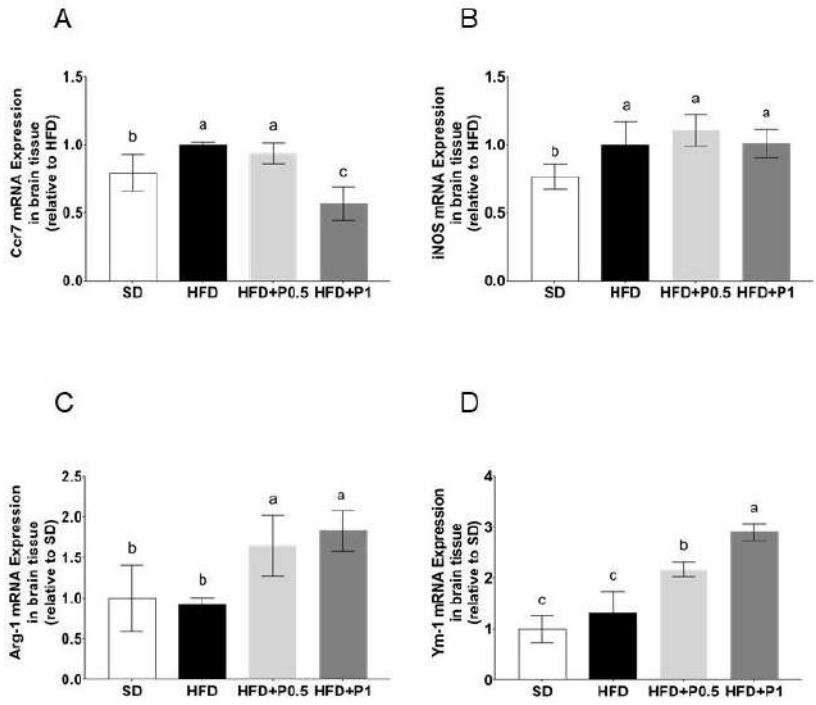

Figure 21. Effect of GPETAFLR on expression of M1 and M2 marker genes in brain of mice with HFD-induced obesity. The mRNA levels of CCR7 (A), iNOS (B), Arg-1 (C), and Ym-1 (D) genes were measured in brain of mice fed with a standard chow diet (SD), an HFD or an HFD + GPETAFLR at a dose of 0.5 mg/kg/d (HFD+P0.5) and 1 mg/kg/d (HFD+P1) in drinking water. Ten animals were used for analysis, and each sample was subjected to triplicate analyses. Values are presented as means ± SD and those marked with different letters are significantly different ($P < 0.05$), as determined through one-way ANOVA, followed by a post hoc Tukey test.

Using FACS, the microglia population from mouse brain was gated as $CD45^{high}CD11b^{+}F4/80^{+}$ cells (**Figure 22A**). The expression of microglial activation markers CD80 for M1-like phenotype and CD200R for M2-like phenotype was then determined. The HFD-induced increase

of total microglial population in the brain was completely abrogated by GPETAFLR (**Figure 22B**). No differences were observed on microglial expansion in animals fed with the HFD that received GPETAFLR at a dose of 0.5 mg/kg/d in drinking water compared to animals fed with the standard chow diet. Similar expression patterns were seen for CD80, the brain of mice on HFD doubling the number of $CD80^+$ microglial cells found in the brain of mice on HFD plus GPETAFLR or on standard chow diet (**Figure 22C**). The HFD-induced decrease of $CD200R^+$ in the brain was also completely abrogated by GPETAFLR (**Figure 22D**), with only the half of $CD200R^+$ microglial cells in the brain of mice fed on HFD regarding those found in the brain of mice on HFD plus GPETAFLR or on standard chow diet.

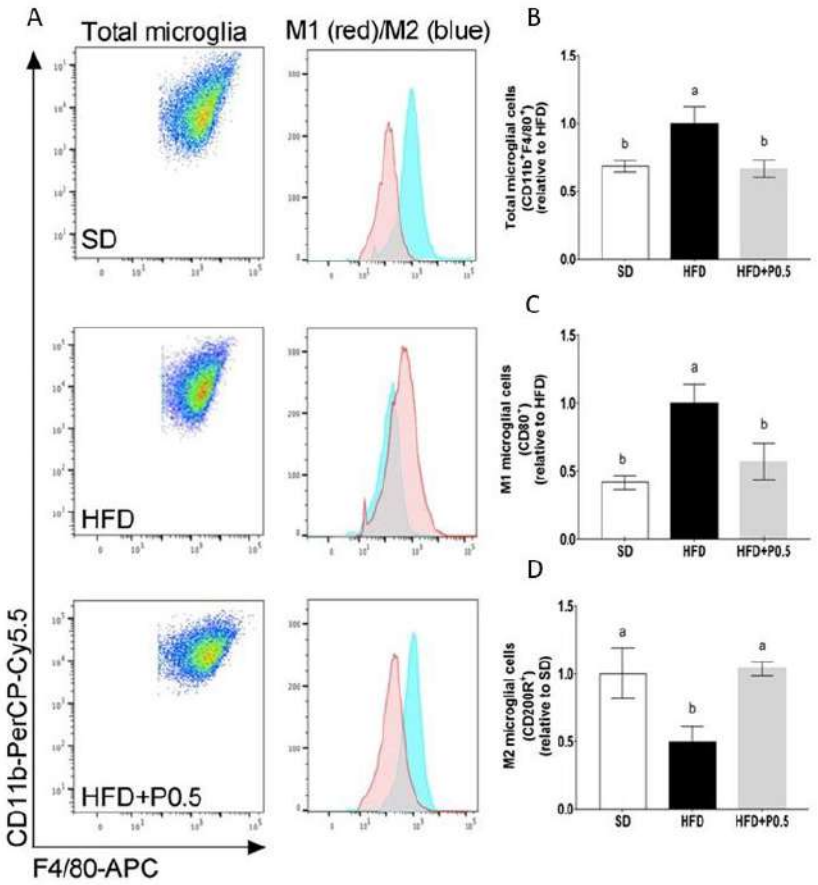

Figure 22. Representative dot plots showing the gating strategy for identification of microglia populations in brain tissue of mice with HFD-induced obesity. Brain cells of mice fed with a standard chow diet (SD), an HFD or an HFD + GPETAFLR at a dose of 0.5 mg/kg/d (HFD+P0.5) in drinking water were stained with multiple fluorescently conjugated antibodies directed to CD45, CD11b, F4/80, CD80, and CD200R. A region was created on a bivariate plot of CD45 vs SSC-A to select $CD45^{high}CD11b^{+}F4/80^{+}$ microglial cells (A). M1 and M2 subsets were quantified from histograms in total microglial cells (B) and in $CD80^{+}$ (C) and $CD200R^{+}$ (D) cells, respectively. Ten animals were used for analysis, and each sample was subjected to triplicate analyses. Values are presented as means ± SD and those marked with different letters are significantly different ($P < 0.05$), as determined through one-way ANOVA, followed by a post hoc Tukey test.

GPETAFLR on Cytokine Gene and Protein Expression in Brain from Mice with HFD-Induced Obesity

Gene expression of cytokines TNF-α, IL-1β, IL-6, and IL-10 was measured by qRT-PCR in brain of mice with HFD-induced obesity during administration of GPETAFLR. The protein level of IL-6 and IL-10 was also measured by ELISA in the supernatant of brain tissue homogenates. GPETAFLR protected in a dose-dependent fashion from the HFD-induced mRNA levels of TNF-α gene in brain (**Figure 23A**). GPETAFLR at a dose of 1 mg/kg/d also decreased the expression of IL-1β gene (**Figure 23B**). Furthermore, the expression of IL-6 gene was dose-dependently repressed by GPETAFLR, reaching levels below those detected in brain of mice on standard chow diet (**Figure 23C**). Despite HFD reduced the expression of IL-10 gene in brain, co-administration with GPETAFLR counteracted this effect (**Figure 23D**). In addition, GPETAFLR protected from the HFD-induced increase of IL-6 (**Figure 23E**) and reduction of IL-10 (**Figure 23F**) in brain tissue homogenates.

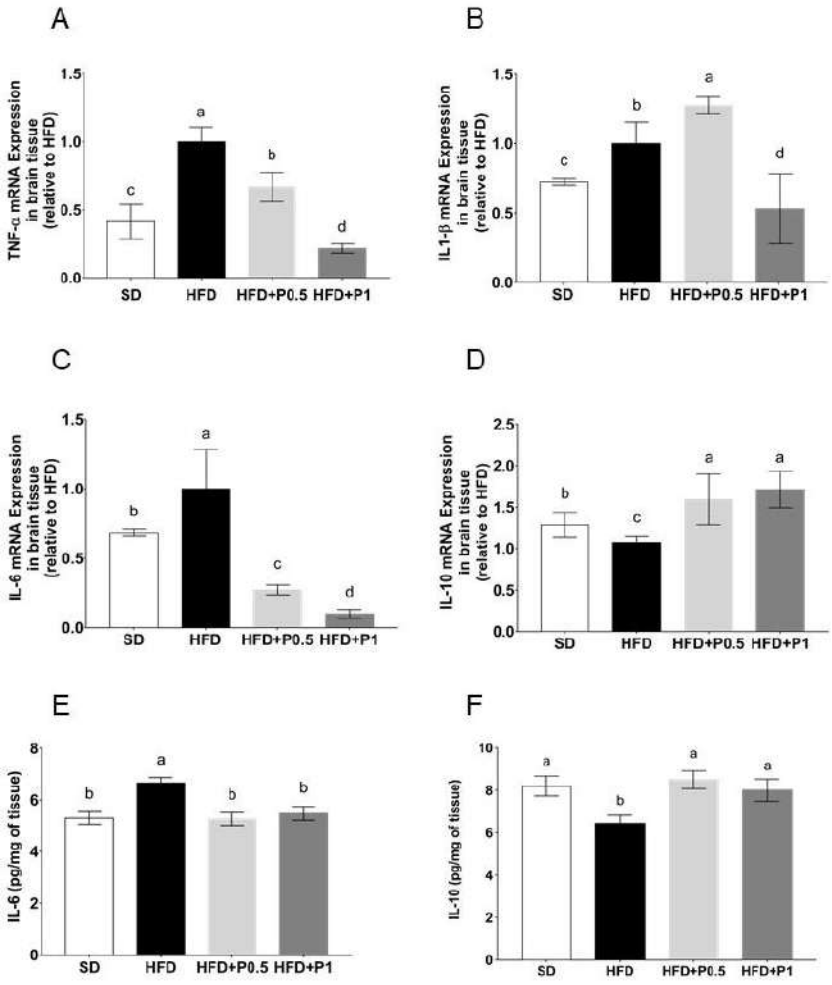

Figure 23. Effect of GPETAFLR on expression of pro-inflammatory and anti-inflammatory cytokine genes and proteins in brain of mice with HFD-induced obesity. The mRNA levels of TNF-α (A), IL-1β (B), IL-6 (C), and IL-10 (D) genes, and the amount of IL-6 (E) and IL-10 (F) were measured in brain of mice fed with a standard chow diet (SD), an HFD or an HFD + GPETAFLR at a dose of 0.5 mg/kg/d (HFD+P0.5) and 1 mg/kg/d (HFD+P1) in drinking water. Ten animals were used for analysis, and each sample was subjected to triplicate analyses. Values are presented as means ± SD and those marked with different letters are significantly different ($P < 0.05$), as determined through one-way ANOVA, followed by a post hoc Tukey test.

DISCUSSION

There is growing interest in obtaining biologically and functionally active peptide sequences that are encrypted inside native food proteins [41, 42]. In previous studies, we reported that LPH [7] and GPETAFLR derived therefrom [8] can inhibit LPS-induced inflammatory response in human monocytic THP-1 cells. GPETAFLR was also shown to exert anti-osteoclastogenic activity in human blood monocyte-derived osteoclasts [9]. Herein, we now report that GPETAFLR has the ability to prevent the pro-inflammatory activation of microglial cells in cultures and in brain of mice with HFD-induced obesity. Although it remains to be determined whether GPETAFLR is capable of reversing the inflammatory profile once obesity has been achieved.

Microglia, the set of professional phagocytes in the brain, usually exists in a quiescent or resting state, continuously monitoring their microenvironment, and can be activated in response to surrounding stimuli that could perturb the CNS homeostasis [43]. Upon a complex multistage activation, microglial cells become strong producers of pro-inflammatory mediators that include a broad spectrum of cytokines involved in degeneration or trauma of neuronal structures, dysfunction, and death [44]. Under prolonged microglial pro-inflammatory activation, the neuroinflammation turns into a driving force in the development of various neurodegenerative disorders [17, 37, 45]. In the process of developing new strategies to modulate the inflammatory response in the brain, a number of phytochemicals, mainly phenolic compounds, have been shown to target neuroinflammation in Alzheimer's disease due to their anti-oxidant and anti-inflammatory activities [46]; however, some of the benefits of

these natural products were further dependent on their poor bioavailability or activity when metabolically transformed into conjugates, apart from disease stage and treatment of duration. One promising attempt for an improved and safety therapy of neurodegenerative diseases by naturally occurring compounds has been recently reported with the soy-derived peptide lunasin [47]. Lunasin was able to rescue Aβ42 mediated neurodegeneration by blocking cell death in retinal neurons, and resulted in restoration of axonal targeting from retina to brain. Inspired in part by these studies and in our own findings on anti-inflammatory activity of GPETAFLR in mononuclear phagocytes, we envisioned that GPETAFLR might be an ideal candidate to test its efficacy in mitigating a neuroinflammatory environment by using both *in vitro* and *in vivo* models. To date there is little if any studies on the impact of a peptide from plant origin on microglial activation.

In the current study, we found that GPETAFLR has significant anti-inflammatory effects by markedly blocking the transcriptional activity of TNF-α, IL-1β, and IL-6 genes in LPS-treated BV-2 microglial cells. Importantly, IL-1β and IL-6 gene expression levels remained unchanged during LPS stimulation if GPETAFLR was added to the cultures. Given the robust relevance of these pro-inflammatory cytokines for microglia status [48] and of LPS as a potent trigger of neuroinflammation [49], our findings suggest that GPETAFLR may protect against pro-inflammatory activation of microglia, remaining intact its physiological capacity or resilience to an ex vivo LPS challenge. The improvement of the molecular repertoire to stop the inflammatory phase of activated microglial cells, which was supported by the abrogation of LPS-induced decrease in the expression of anti-

inflammatory IL-10 gene upon addition of GPETAFLR to the cultures, may also establish that GPETAFLR could have benefits on microglial function. Even though more research is needed to gain further insights into this possibility, IL-10 has been shown to be produced by brain cell types other than microglia, having the property to inhibit the release or the effects of pro-inflammatory cytokines and to promote survival of neurons and neuronal homeostasis [50]; other molecules enable the healing and functional recovery of neuron-glial interactions, such as resolvins, protectins, and maresins [51].

Microglial plasticity *in vitro* depends on different microenvironments and signals that promote specific phenotypes, which may correspond to a classically (M1) or an alternatively (M2) activation state [52]. However, this paradigm of microglial polarization may represent an oversimplified model of microglial states *in vivo* [53, 54] and should be carefully managed. M1-like activated microglia has been proposed to secrete pro-inflammatory cytokines, whereas M2-like activated microglia to be involved in reconstructing damaged neuron networks by the removal of toxic aggregates, the upregulation of neurotrophic factors and anti-inflammatory cytokines, and the downregulation of pro-inflammatory cytokines [55]. Therefore, in a context of sustained microglial M1 status, the M1-to-M2 microglial switch could protect the brain from inflammation [56]. We used two independent strategies to test if GPETAFLR was able to influence on microglial plasticity. Firstly, our study found that GPETAFLR suppresses the transcriptional activity of M1 genes and promotes that of M2 genes in LPS-treated BV-2 microglial cells. Secondly, we observed that GPETAFLR not only to some extent reduces the transcriptional activity

of genes but also markedly attenuates the expression of surface marker proteins corresponding to canonical M1-like activation in brain microglia of mice fed on HFD. Furthermore, the expression levels of M2 marker genes in brain and of M2 marker proteins in brain microglial cells are increased by GPETAFLR. Collectively, together with the analysis of the balance between brain gene and protein expression patterns of pro-inflammatory and anti-inflammatory cytokines, this study demonstrates both *in vitro* and *in vivo* that GPETAFLR may improve the subsets ratio of M1/M2 microglial cells under conditions of inflammatory insult. Obesity can induce neuroinflammation [57] and can produce a chronic damage in the CNS [58]. HFD feeding is also associated with the pro-inflammatory activation of microglial cells and the increase of inflammatory tone in the CNS [19, 59]. Hence, our findings are consistent with the notion that GPETAFLR could be a bioactive peptide against neuroinflammation and thereby in maintaining CNS homeostasis. Interestingly, our *in vivo* data showed that this activity remains following gastrointestinal transition of GPETAFLR. In a recent study, the dipeptide Gly-Arg (GR) from soybean protein hydrolysates was shown to induce neurotrophins in astrocyte cultures and neurogenesis in mouse brain after oral or intraperitoneal administration for a period of 2 weeks [60]. Strikingly, the amino acid sequence of GR is coincident with amino acids on ends of our peptide. Whether these positions occupied by Gly and Arg can represent structural requirements for anti-inflammatory activity of GPETAFLR in microglial cells should be further explored in the future.

To the best of our knowledge, this is the first report describing that a peptide from plant origin robustly restrained the pro-

inflammatory activation of microglial cells in cultures and in brain. Our data suggest that GPETAFLR might be instrumental in maintaining CNS homeostasis by inhibiting neuroinflammation.

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Ethics approval

All animal protocols received appropriate institutional approval (Animal Care and Use Committee of the University of Seville) and were performed according to the official rules formulated in the Spanish law on the care and use of experimental animals (UE Directive of 2010: 2010/63/UE; RD 53/2013).

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CHAPTER 05

General Discussion & Conclusions

GENERAL DISCUSSION

Plant proteins have emerged as an alternative for the production of bioactive peptides [1], which are small amino acid sequences that are inactive in the native protein but can be released by gastrointestinal enzymes and then reach systemic blood stream as active molecules [2]. Plant-derived bioactive peptides are very interesting compounds that can be used with potential pharmaceutical and nutraceutical benefit [3].

In previous studies, our research group has described the presence of a novel peptide isolated from *Lupinus angustifolius* L. protein hydrolysate [4]. The sequence was identified as Gly-Pro-Glu-Thr-Ala-Phe-Leu-Arg (GPETAFLR) and its anti-inflammatory activity was evaluated in a human monocytic cell line [4]. In this Thesis, we focused on several of hallmarks features of immunometabolism. The mechanisms by which GPETAFLR modulates inflammation and metabolism probably include multiple processes, but the understandings of these mechanisms are currently very unclear and a considerable effort in this area is required before we know how to manipulate for healing benefit the GPETAFLR intake as a therapeutic option for immunometabolic-related diseases.

In this Thesis, it is the first time that the anti-inflammatory activity of GPETAFLR was shown on primary human monocytes and macrophage polarization (**Chapter 2**). Taking into account that macrophage plasticity allows adapting to different environments and that one of the most aggressive metabolic challenges in immunometabolic-related diseases, an approximation (*in vitro* study) to the impact of GPETAFLR on differentiation a polarization of

macrophages derived from human monocytes were done. Macrophage polarisation depends of different microenvironment and signals to promote specific phenotypes. These phenotypes correspond to classically (M1) and alternatively (M2) polarized macrophages that exhibit pro-inflammatory and anti-inflammatory functions, respectively [5,6] In this Thesis, it was observed a modulation capacity of GPETAFLR on macrophage polarization. This could be explained by the fact that GPETAFLR peptide increase anti-inflammatory cytokines as IL-10 while decrease pro-inflammatory as IL-6 or IL-1β. These cytokines regulate and modulate macrophages phenotype, stimulating M1 phenotype in the case of IL-1β or M2 phenotype in the case of IL-10 [7,8]. In addition to macrophage plasticity, GPETAFLR could also modulate monocyte subsets. Under LPS stimulation, classical monocytes (monocytes that promote inflammatory reactions) increased their population while non-classical suffer a decrease with respect to monocytes treated with the peptide. This mean that GPETAFLR can modulate monocyte subset ratio, stimulating those monocytes that participate in anti-inflammatory process and decreasing classical monocytes [9,10]. GPETAFLR peptide showed an anti-inflammatory effect in primary monocytes. In chapter 2, our findings imply a new understanding of the mechanisms by which GPETAFLR favor a continuous and gradual plasticity process in the human monocyte/macrophage system and offer novel benefits derived from the consumption of *Lupinus angustifolius* L. in the prevention of inflammatory-related diseases.

The use of bioactive peptides obtained from food is increasingly gaining importance because of its health benefits and to prevent chronic diseases, whose incidence is increasing worldwide [11]. Many chronic

diseases are characterized by a marked inflammatory process. Therefore, GPETAFLR, a novel anti-inflammatory biopeptide obtained from *Lupinus angustifolius* L., can be a potent molecule for the prevention of these type of diseases.

The liver is the main metabolic organ of the body. NAFLD affects its functionality, endangering the homeostasis metabolic system. This disease is typically associated with obesity [12], wherefore a substance which has the effect of reducing body weight and obesity rates will greatly alleviate the symptoms and the development of NAFLD. In **Chapter 3**, the octapeptide GPETAFLR prevents the weight gain resulting from a diet rich in fat in contrast to other substances with hepatoprotective properties that do not have the capacity to prevent weight gain such as isoliquiritigenin [13].

On the other hand, the data obtained corroborate that HFD-induced obese mice, presented a NAFLD pathophysiology similar to humans. The pathophysiology of this disease is characterized by increased body weight and liver, high levels of TGs in the blood, fat accumulation in adipose tissue, hepatic inflammation and elevated markers of liver damage [14]. In this Thesis, the obese control group showed a greater hepatic weight derived from a greater accumulation of hepatic fat. The accumulation may be due to high levels of TGs in the blood and the increase in FAS gene expression. In addition, this group also presented a pro-inflammatory state, provoked by high levels of expression of pro-inflammatory cytokines as IL-6, IL-1β, and TNF-α, since the Kupffer cells increase their expression in response to hepatic fat accumulation [15]. Finally, it presented elevated markers of liver

damage, since the activity of transaminases, LDH, and ALP in serum were significantly higher.

All these parameters of the pathology were palliated with the treatment with GPETAFLR, owing to the fact that liver weight and liver fat accumulation were lower in the animals treated with the octapeptide. The factors that can influence the minor accumulation of hepatic fat in these individuals are a decreased gene expression of FAS and an increased expression of enzymes that increase energy metabolism, such as UCP1 and PPARα. In addition, there is less circulation of TGs in the blood and the availability for its accumulation in different tissues, including the liver. GPETAFLR could mediate the accumulation of hepatic fat by influencing the signaling pathways involved in the regulation of gene expression or the activity of lipid metabolism proteins, such as the kinase pathway activated by AMP (AMPK). AMPK regulates the activity and expression of many important proteins related to metabolism, one of them is mTOR, another metabolic regulator. AMPK decrease the accumulation of fat (down-regulating FAS, between other proteins) and other metabolites and also down-regulates mTOR, protein that increase the accumulation of fat and also have effects on the gene and protein expression [16]. GPETAFLR has been demonstrated to present anti-inflammatory activity that is also present on the hepatocytes. The octapeptide decreased pro-inflammatory cytokine levels and increased levels of the anti-inflammatory cytokine IL-10. IL-10 did not show a decrease inexpression in the obese control compared to the standard control, but it greatly increased its expression in the groups treated with GPETAFLR. Pro-inflammatory cytokines present several actions related to metabolism,

for example, TNF-α decreases the degradation of fats and increases their accumulation and IL-6 inhibits lipolysis and increases fat deposition [17]. For this reason, in addition to contributing to tissue damage from chronic inflammation, they contribute to the development of NAFLD by boosting fat accumulation in the liver. Chronic inflammation is primarily mediated by the nuclear factor κB (NF-κB) pathway, which stimulates the production of cytokines such as TNF-α [18]. For this reason, GPETAFLR may decrease inflammation by influencing the NF-κB pathway. The NF-kB pathway increases the expression of pro-inflammatory cytokines, this pathway can be activated by the interaction of free fatty acids with the TLR4, membrane receptor expressed by Kupffer cells, this produces a pro-inflammatory state [19]. The down-regulation of this pathway could be determined by the reduction of fat accumulation in liver associated to the treatment with GPETAFLR. Another parameter used as a marker of NAFLD pathogenesis in this study was the measurement of blood markers of liver damage, specifically transaminases, ALP, and LDH. The activity of these enzymes in serum was lower in both GPETAFLR treatments compared to the obese control group. This effect is due, probably, to the fact that peptide decreases liver damage by reducing the accumulation of fat and inflammation (influenced by the lower accumulation of fat) and thus all the tissue damage associated with a chronic inflammatory process. To conclude Chapter 3, GPETAFLR decreased body weight gain, liver weight and fat accumulation in liver, as well as TGs levels in the blood and leptin resistance associated with obesity. GPETAFLR showed anti-inflammatory properties at the hepatic level, decreasing the mRNA levels of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-10, and increasing the expression of anti-inflammatory cytokine IL-10 in liver

tissue. The octapeptide also reduced blood markers related to liver damage such as AST, ALT, ALP, and LDH. Taken together, our findings suggest that GPETAFLR is a potential treatment, as well as an important preventive therapy for NAFLD. Thus, the dietary supplementation with *Lupinus angustifolius* L. would also contribute to a reduction of this pathology.

In **Chapter 4**, we now report that GPETAFLR has the ability to prevent the pro-inflammatory activation of microglial cells in cultures and in brain of mice with HFD-induced obesity. Microglia are the resident macrophages in the CNS. These cells usually exist in a quiescent or resting state, continuously monitoring their microenvironment, and can be activated in response to surrounding stimuli that could perturb the CNS homeostasis [20]. Upon a complex multistage activation, microglial cells become strong producers of pro-inflammatory mediators that include a broad spectrum of cytokines involved in degeneration or trauma of neuronal structures, dysfunction, and death [21]. Under prolonged microglia pro-inflammatory activation, the neuroinflammation turns into a driving force in the development of various neurodegenerative disorders [22]. In the process of developing new strategies to modulate the inflammatory response in the brain, a number of phytochemicals, mainly phenolic compounds, have been shown to target neuroinflammation in Alzheimer's disease due to their anti-oxidant and anti-inflammatory activities [23]. However, some of the benefits of these natural products were further dependent on their poor bioavailability or activity when metabolically transformed into conjugates, apart from disease stage and treatment of duration, thereby make it difficult to draw any definitive conclusions. One promising

attempt for an improved and safety therapy of neurodegenerative diseases by naturally occurring compounds has been recently reported with the soy-derived peptide lunasin [24]. Lunasin was able to rescue Aβ42 mediated neurodegeneration by blocking cell death in retinal neurons, and resulted in restoration of axonal targeting from retina to brain. Inspired in part by these studies and in our own findings on anti-inflammatory activity of GPETAFLR in mononuclear phagocytes, we envisioned that GPETAFLR might be an ideal candidate to test its efficacy in mitigating a neuroinflammatory environment by using both in vitro and in vivo models. To date there is little if any studies on the impact of a peptide from plant origin on microglial activation. In the current Thesis, we found that GPETAFLR has significant anti-inflammatory effects by markedly blocking the transcriptional activity of TNF-α, IL-1β, and IL-6 genes in LPS-treated BV-2 microglial cells. Importantly, IL-1β and IL-6 gene expression levels remained unchanged during LPS stimulation if GPETAFLR was added to the cultures. Given the robust relevance of these pro-inflammatory cytokines for microglia status [25] and of LPS as a potent trigger of neuroinflammation [26], our findings suggest that GPETAFLR may protect against pro-inflammatory activation of microglia, remaining intact its physiological capacity or resilience to an *ex vivo* LPS challenge.

Microglial polarization depends on different microenvironment and signals that promote specific phenotypes, which may correspond to classically (M1) and alternatively (M2) microglia [27]. Similar to peripheral macrophages, M1 activated microglia secrete pro-inflammatory cytokines, whereas M2 activated microglia are involved in reconstructing damaged neuron networks by the removal of toxic

aggregates, the upregulation of neurotrophic factors and anti-inflammatory cytokines, and the downregulation of pro-inflammatory cytokines [28]. Therefore, in a context of sustained microglial M1 status, the M1-to-M2 microglial switch could protect the brain from inflammation [29]. We used two independent strategies to test if GPETAFLR was able to influence on microglial polarization. Firstly, our study found that GPETAFLR suppresses gene expression levels of M1 markers and promotes those of M2 markers in LPS-treated BV-2 microglial cells. Secondly, we observed that GPETAFLR not only to some extent reduces the transcriptional activity of genes but also markedly attenuates the expression of surface marker proteins corresponding to canonical M1 microglial activation in the brain of mice fed on an HFD. Furthermore, gene expression in brain and protein expression from brain microglia of M2 markers were increased by GPETAFLR. Collectively, together with the analysis of the balance between brain gene and protein expression patterns of pro-inflammatory and anti-inflammatory cytokines, this study demonstrates both in vitro and in vivo that GPETAFLR may improve the subsets ratio of M1/M2 microglial cells under conditions of inflammatory insult. Obesity can induce neuroinflammation [30] and can produce a chronic damage in the CNS [31]. HFD feeding is also associated with the pro-inflammatory activation of microglial cells and the increase of inflammatory tone in the CNS [32]. Therefore, our findings are consistent with the notion that GPETAFLR could be a bioactive peptide against neuroinflammation and thereby in maintaining CNS homeostasis. Interestingly, our in vivo data showed that this activity remains following gastrointestinal transition of GPETAFLR. In a recent study, the dipeptide Gly-Arg (GR) from soybean protein hydrolysates was shown to induce neurotrophins in astrocyte

cultures and neurogenesis in mouse brain after oral or intraperitoneal administration for a period of 2 weeks [33]. Strikingly, the amino acid sequence of GR is coincident with amino acids on ends of our peptide. Whether these positions occupied by Gly and Arg can represent structural requirements for anti-inflammatory activity of GPETAFLR in microglial cells should be further explored in the future. To the best of our knowledge, this is the first report describing that a peptide from plant origin robustly restrained the pro-inflammatory activation of microglial cells in cultures and in brain. Our data suggest that GPETAFLR might be instrumental in maintaining CNS homeostasis by inhibiting neuroinflammation.

CONCLUSSIONS

From this Thesis, it can be concluded that:

1.- GPETAFLR prevents the LPS-induced production and priming of circulating pro-inflammatory monocytes, systemic inflammation, and M1 macrophage polarization. Our findings imply a new understanding of the mechanisms by which GPETAFLR favor a continuous and gradual plasticity process in the human monocyte/macrophage system and offer novel benefits derived from the consumption of Lupinus angustifolius L. in the prevention of inflammatory-related diseases.

2.- The intake of GPETAFLR prevents the body weight gain, liver weight and fat accumulation in liver, as well as TGs levels in the blood and leptin resistance in HFD-induced obese mice. GPETAFLR showed anti-inflammatory properties at the hepatic level, decreasing the mRNA levels of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-10, and

increasing the expression of anti-inflammatory cytokine IL-10 in liver tissue.

3.- GPETAFLR restrained the pro-inflammatory activation of microglial cells in cultures and in brain of HFD-induced obese mice. Our findings imply that GPETAFLR might be instrumental in maintaining CNS homeostasis by inhibiting neuroinflammation.

Taken together, these conclusions suggest that the inclusion in the diet of GPETAFLR has benefits to prevent inflammation-mediated disorders such as NAFLD and neurodegenerative diseases. The findings in this Thesis are also indicative that the intake and metabolism of plant-based protein hydrolysates may prevent disturbances on immunometabolic-related diseases.

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LIST OF ABBREVIATIONS

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ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANFs	Anti-nutritional factors
AST	Aspartate aminotransferase
CCL	Chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CNS	Central nervous system
CVD	Cardiovascular diseases
DB	Dry basis
DM	Dry Matter
FA	Fatty acid
FAS	Fatty acid synthase
FBS	Fetal bovine serum
FFA	Free fatty acid
HDL	High-density lipoprotein
HFD	High fat diet
HPRT	Hypoxanthine phosphoribosyltransferase
ICAM	Intercellular adhesion molecule
IFN	Interferon
IEP	Isoelectric precipitation
IL	Interleukin
INF	Interferon
INOS	Inducible nitric oxide synthase

LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LPH	Lupine protein hydrolysates
LPI	Lupine protein isolate
LPS	Lipopolysaccharide
MCP	Monocyte chemoattractant protein
MFI	Mean fluorescence intensity
MRC	Mannose receptor C
NAFL	Non-alcoholic fatty liver
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NF-KB	Nuclear factor κB
NNL	Narrow-leaved lupin
NSP	Non-starch polysaccharides
OB-R	Leptin Receptor
PBMC	Peripheral blood mononuclear cell
PPAR	Peroxisome proliferator-activated receptor
ROS	Reactive oxygen species
SD	Standard chow diet
T2DM	Type 2 Diabetes Mellitus
TGS	Triglycerides
TLR	Toll-like receptor
TNF	Tumor necrosis factor
UCP	Uncoupling protein

This is a list of the most used abbreviations, but it has been decided to spell out each abbreviation the first time it is used in each chapter, followed by the abbreviation in parenthesis.

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1. Personal Information

Date and place of Birth: December 27th, 1991 (Seville, Spain)

2. Education

Expert course in R&D&i consultancy
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PhD in Molecular Biology, Biomedicine and Clinical Research
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Master´s degree in Food safety
University Camilo José Cela – Madrid, Spain.
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Master´s degree in Medical Research
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Bachelor's degree in Nutrition and Dietetics
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Erasmus Fellowship
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3. Complementary Training

Course on Handling of Experimental Animals (Certificate Category A, B, C). University of Seville – Seville, Spain (2018).

Course in Inflammation and Immunity: From Bench to clinic. University of Seville – Seville, Spain (2019).

Course in Food Safety Auditor. IFM Business School-Madrid, Spain (2019).

Course in Child Nutrition and Cooking. Stanford University Online course- Coursera, Online (2018).

Course in Biochemical Principles of Energy Metabolism. Korea Advanced Institute of Science and Technology (KAIST)- Coursera, Online (2020).

4. Skills

4.1. Languages

Spanish	Native speaker	
English	Advanced	C1

4.2. Knowledge areas

Areas: Inmunonutrition, Immunometabolism, Plant Peptide, Inflammation, Neuroinflammation, Functional Foods
Cellular and Molecular Biology: RT-qPCR, ELISA, Cell Cultures, Histology, Flow Cytometry.
Animal models: Obesity, Neuroinflammation
Chromatographic and Spectral Techniques: GC, HPLC, MS, FLC, CC
Statistical programs: Graphpad, SPSS, Statgraphics.

5. Professional and academic experience

5.1. Professional experience

Innovation Consultant
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Research fellow in technical and professional activities
Institute of Fat (CSIC)
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Young voluntary service
European Commission
Chisinau, Moldova
January 2017 – August 2017

5.2. Academic experience

Biochemistry and Biology Molecular Biology
Degree in Medicine, University of Seville
12 hours (course 2019-2020)

Molecular Biology and Biochemistry
Degree in Health Engineering, University of Seville
4 hours (course 2019-2020)

6. Scientific activity

6.2. Publications

Book chapters

1. Lopez S, Bermudez B, Montserrat-de la Paz S, Pacheco YM, Ortega-Gomez A, Varela LM, **Lemus-Conejo A**, Millan-Linares MC, Rosillo MA, Abia R, Muriana JGF. "*Oleic acid—the main component of olive oil on postprandial metabolic processes"* (Chapter 53) In: Olives and Olive Oil in Health and Disease Prevention. Academic Press. **2021**. ISBN: 978-0-12-819528-4.

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6.4. Conference participation

1. "*Neuroprotective Effects of Gly-Pro-Glu-Thr-Ala-Phe-Leu-Arg, a Peptide Isolate from Lupinus angustifolius L. Protein Hydrolysate".* IRC International Research Conference. Paris, France **2019** (Oral presentation).

2. *"Minor compounds from virgin olive oil promote M2 microglia polarization and neuroprotection".* 39TH Spanish Society of Pharmacology Meeting. Las Palmas de Gran Canarias, Spain **2019** (Poster).

3. "*Lupine (Lupinus angustifolius L.) peptide ameliorates the non-alcoholic steatohepatitis in mice".* 39TH Spanish Society of Pharmacology Meeting. Las Palmas de Gran Canarias, Spain **2019** (Poster).

4. *"Characterization and evaluation of hemp protein hydrolysates on neuroprotection".* 39TH Spanish Society of Pharmacology Meeting. Las Palmas de Gran Canarias, Spain **2019** (Oral presentation).

5. *"Anti-oxidant and anti-inflammatory effects of wheat protein hydrolysates in human primary monocytes".* XI Reunión de Jóvenes Farmacólogos de Andalucía. Málaga, Spain **2019** (Oral presentation).

6. *"Unsaponifiable and phenolic fractions from virgin olive oil prevent neuroinflammation skewing microglia polarization toward M2 phenotype".* XI Reunión de Jóvenes Farmacólogos de Andalucía. Málaga, Spain **2019** (Oral presentation).

7. *"Lupine (Lupinus angustifolius L.) peptide prevents non-alcoholic fatty liver disease in high-fat diet-induced obese mice".* XI Reunión de Jóvenes Farmacólogos de Andalucía. Málaga, Spain **2019** (Oral presentation).

8. *"GPETAFLR, a peptide from protein hydrolysate of Lupinus angustifolius L. has neuroprotective effects in BV-2 microglial cells and mouse brain tissue".* III Jornadas Internacionales en Investigación e Innovación en Ciencias de la Salud. Sevilla, Spain **2019** (Poster).

9. "Non-*alcoholic fatty liver disease in high-fat diet-induced obese mice is alleviated by lupine (Lupinus Angustifolius L.) peptide".* III Jornadas Internacionales en Investigación e Innovación en Ciencias de la Salud. Sevilla, Spain **2019** (Poster)

10. *"Neuroprotective effects of Chia protein hydrolysates".* IV Conferencia Internacional de la RED CHIA-LINK. Mérida, México **2018** (Oral presentation).

11. *"GPETAFLR, a novel bioactive peptide from Lupinus angustifolius L. protein hydrolysate, reduces osteoclastogenesis".* X Reunión de Jóvenes Farmacólogos de Andalucía. Granada, Spain **2018** (Oral presentation).

12. *"GPETAFLR, a novel bioactive peptide from Lupinus angustifolius L. protein hydrolysate, reduces osteoclastogenesis".* 38º Congreso de la Sociedad Española de Farmacología. Santiago de Compostela, Spain **2018** (Poster).

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GPETAFLR, an octapeptide isolated from *Lupinus angustifolius* L. protein hydrolysate, promotes the skewing to the M2 phenotype in human primary monocytes

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The present study aimed to test the mechanisms by which GPETAFLR, released from the enzymatic hydrolysis of lupine protein, may modulate the inflammatory response and plasticity in human primary monocytes. Human circulating monocytes and mature macrophages were used to analyze the effects of GPETAFLR on plasticity and inflammatory response using biochemical, flow cytometry, quantitative real-time PCR, and ELISA assays. GPETAFLR skewed the monocyte plasticity towards the anti-inflammatory non-classical $CD14^{+}CD16^{++}$ monocyte subset and reduced the inflammatory competence of LPS-treated human monocytes diminishing IL-1β, IL-6, and TNF-α and increasing IL-10 production and gene expression. Results showed that GPETAFLR decreased the frequency of the LPS-induced activated monocyte population ($CD14^{++}CD16^{-}$), diminished monocyte activation involved down-regulation of CCR2 mRNA expression and protein expression, and decreased gene expression of the LPS-induced chemoattractant mediator CCL2. Our findings imply a new understanding of the mechanisms by which GPETAFLR favor a continuous and gradual plasticity process in the human monocyte/macrophage system and offer novel benefits derived from the consumption of *Lupinus angustifolius* L. in the prevention of inflammatory-related diseases.

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Introduction

The word nutraceutical is the association of the terms *nutrition* and *pharmaceutical* and is considered as a food or food product that can offer medical and health benefits for the prevention and treatment of diseases. Most nutraceutical peptides are isolated from protein precursors by digestive enzymes, during food processing, storage, or by the *in vitro* hydrolysis of several proteolytic enzymes.[1] Although milk proteins are the main source of bioactive peptides studied so far, there are many other peptides of animal and plant origin. In fact, more than 3000 peptides with 44 different biological activities have been identified and recently collected in the "Biopep" database.[2] In this sense, the study of plant proteins has emerged as an alternative to produce bioactive peptides,[3] small sequences of amino acids that are inactive within the intact protein but can be released by gastrointestinal enzymatic hydrolysis reaching the bloodstream as active molecules.[4]

The immune system is responsible for responding to any type of aggression in the body. It has its origin in the lymphoid organs (lymph nodes, spleen, thymus, and bone marrow) and in its action many cellular subtypes (immunocompetent cells), including monocytes and neutrophils, participate. Monocytes express several receptors that control and detect environmental changes, are highly plastic and heterogeneous, and change their functional phenotype in response to environmental stimulation.[5,6] Inflammation is one of the complex biological responses of an organism's immune system. Evidence from murine and human studies has suggested that monocytosis may be an indicator of several inflammatory diseases.[7] Therefore, the use of primary monocytes as a model of inflammation is widespread and is used in numerous studies to test the anti-inflammatory activity of various compounds.[8,9] The secretion of pro-inflammatory mediators works as an inflammatory beacon for leukocytes, which contribute to all stages of several inflammatory disorders, therefore representing an important therapeutic target.[10]

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Human monocytes are classified into three subsets: $CD14^{++}CD16^{-}$ (classical monocytes), intermediate $CD14^{++}CD16^{+}$ (intermediate monocytes), and $CD14^{+}CD16^{++}$ (non-classical monocytes).[11] So far, classical monocytes represent the major fraction (about 85% of total monocytes) and highly express CCR2; they are professional phagocytes giving rise to M1 macrophages, which secrete cytokines (TNF-α, IL-1β, and IL-6) in response to LPS during infection or inflammation.[12] Intermediate monocytes display the highest levels of CCR5, TLR4, CD163, and HLA-DR during activation and also secrete pro-inflammatory cytokines such as IL-10.[13] Non-classical monocytes are less granular and smaller in size, with lower expression of CCR2 than classical or intermediate subsets.[14,15] These monocytes rich in CD16 are functionally involved in tissue repairing, patrolling, and wound healing, and have the tendency to be polarized into M2 macrophages with an anti-inflammatory phenotype in response to a variety of stimuli, including IL-4.[16]

We have previously shown the presence of a new peptide isolated from a protein hydrolysate of *Lupinus angustifolius* L.[17] The aim of the present study was to investigate the effects of the GPETAFLR peptide on human primary monocytes and human mature macrophage activation as hallmarks of inflammatory disorders.

Materials and methods

Synthesis of the peptide

Seeds of lupine (*Lupinus angustifolius* L.) were a gift from Koipesol Semillas, S.A. (Sevilla, Spain). Alcalase 2.4 L was provided by Novozymes (Bagsvaerd, Denmark). Lupine protein isolate (LPI) was obtained through the method of Yust, Pedroche, Millán-Linares, Alcaide-Hidalgo, and Millán (2010)[18] at the pilot plant scale (Plant Proteins Group, Instituto de la Grasa, Seville, Spain). The chemical composition of LPI in dry matter was: proteins 86.83%; dietary fibre 5.97%; oil 5.08%; ash 0.78%; and carbohydrates 1.34%. Hydrolysis was carried out according to Millan-Linares, Yust, Alcaide-Hidalgo, Millan, and Pedroche (2014).[19] Lupine protein isolate (LPI) was suspended in distilled water (10% w/v), and hydrolysis with alcalase was performed under the following conditions: pH 8, 50 °C, E/S = 0.3 AU g^{-1} protein, and a hydrolysis time of 15 min. The mixture was heated at 85 °C for 15 min to inactivate the enzyme, centrifuged at 6500*g* for 15 min, and the supernatant constituted the lupine protein hydrolysate (LPH). LPH was purified by ultrafiltration and chromatographic techniques according to Millán-Linares *et al.* (2015).[17] The sequence of the purified peptide was identified through sequence analysis by nanoHPLC coupled to a Polaris Q ion trap mass spectrometer (Mass Spectrometry Unit, Instituto de la Grasa). Finally, the peptide was synthesized by the Fmoc solid phase method by the Barcelona Scientific Park Foundation (Barcelona, Spain) at 95% purity, measured using HPLC-UV at 220 nm.

Blood collection and isolation of human monocytes

This study was conducted according to Good Clinical Practice Guidelines and is in line with the principles outlined in the Helsinki Declaration of the World Medical Association. Informed consent for the study was obtained from healthy male blood donors (age <35 years) at the University Hospital Virgen del Rocio (UHVR, Seville). Ethics approval was obtained from the Human Clinical Research and Ethics Committee of the UHVR (AGL2012-40247-C02-01). Participants declared that they were non-smokers and were not taking any medication. Peripheral blood samples were drawn from a large antecubital vein and collected into K_3EDTA-containing tubes (Becton Dickinson, NJ, USA). Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood samples by centrifugation over a Ficoll-Histopaque (Sigma-Aldrich, Madrid, Spain) gradient. Monocytes were isolated from PBMCs using CD14 microbeads and LS columns on a midiMACS system (Miltenyi Biotec, Madrid, Spain) according to the manufacturer's instructions. The purity for CD14 monocyte isolations was routinely >95% by flow cytometry (FACScanto II flow cytometer and FACSDiva software, BD). Following isolation, monocytes were suspended in an RPMI 1640 medium supplemented with L-glutamine, penicillin, streptomycin and 10% heat-inactivated foetal bovine serum. For treatments, 5×10^5 of purified monocytes, after *in vitro* stimulation with or without LPS (100 ng mL^{-1}), were exposed to GPETAFLR at 50–100 μg mL^{-1} (P2 and P1, respectively) for 24 h.

Monocyte differentiation and polarization into M1 and M2 macrophages

Monocytes were induced to differentiate for 6 days in the presence of recombinant human M-CSF (25 ng mL^{-1}) to obtain M0 macrophages. These cells were then cultured in RPMI 1640 supplemented with L-glutamine, penicillin, streptomycin, and 10% heat-inactivated FBS. For M1 and M2 polarization, M0 macrophages were exposed to LPS (100 ng mL^{-1}) plus IFNγ (20 ng mL^{-1}) and to IL-4 (20 ng mL^{-1}), respectively, for additional 24 h. To evaluate the effect of the GPETAFLR peptide on macrophage polarization, M0 macrophages were exposed to GPETAFLR at 50–100 μg mL^{-1} for 24 h.

Cell viability assay (MTT)

Monocytes were incubated with different concentrations of GPETAFLR peptide in a 96-well plates (1×10^4 cells per well) during 24 h. Afterward, the MTT solution (Sigma) was incubated in the well until a purple precipitate was visible. MTT-formazan crystals were solubilized with DMSO (Sigma), and then measured with a microplate reader at 570 nm corrected to 650 nm.[20] Cell survival was expressed as the percentage of absorbance compared with that obtained in control, non-treated cells.

Immunostaining of circulating monocytes by FACS

Circulating monocyte membrane expression of CD16 (PE anti-human CD16, Miltenyi), CD14 (APC-Cy7 anti-human CD14, Miltenyi), and CCR2 (APC anti-human CCR2, Vitro) on mono-

Table 1 Sequences of RT-PCR primers for gene expression analysis

Target	GenBank accession number	Direction	Sequence (5′ → 3′)
TNF-alpha	NM_000594	Forward	TCCTTCAGACACCCTCAACC
		Reverse	AGGCCCCAGTTTGAATTCTT
IL1beta	NM_000576	Forward	GGGCCTCAAGGAAAAGAATC
		Reverse	TTCTGCTTGAGAGGTGCTGA
IL6	NM_000600	Forward	TACCCCCAGGAGAAGATTCC
		Reverse	TTTTCTGCCAGTGCCTCTTT
GAPDH	NM_001289746	Forward	CACATGGCCTCCAAGGAGTAAG
		Reverse	CCAGCAGTGAGGGTCTCTCT
HPRT 1	NM_000194	Forward	ACCCCACGAAGTGTTGGATA
		Reverse	AAGCAGATGGCCACAGAACT
CD80	NM_005191.3	Forward	GGGAAAGTGTACGCCCTGTA
		Reverse	GCTACTTCTGTGCCCACCAT
CD200r	NM_138940.2	Forward	GTTGCCCTCCTATCGCATTA
		Reverse	TGGAAATTCCCATCAGGTGT
CD64	NM_000566.3	Forward	GTCCAAATCTCCAAGTGCGG
		Reverse	CCCAAGTATGAGAGCAGCGT
MRC-1	NM_138806	Forward	GGCGGTGACCTCACAAGTAT
		Reverse	ACGAAGCCATTTGGTAAACG

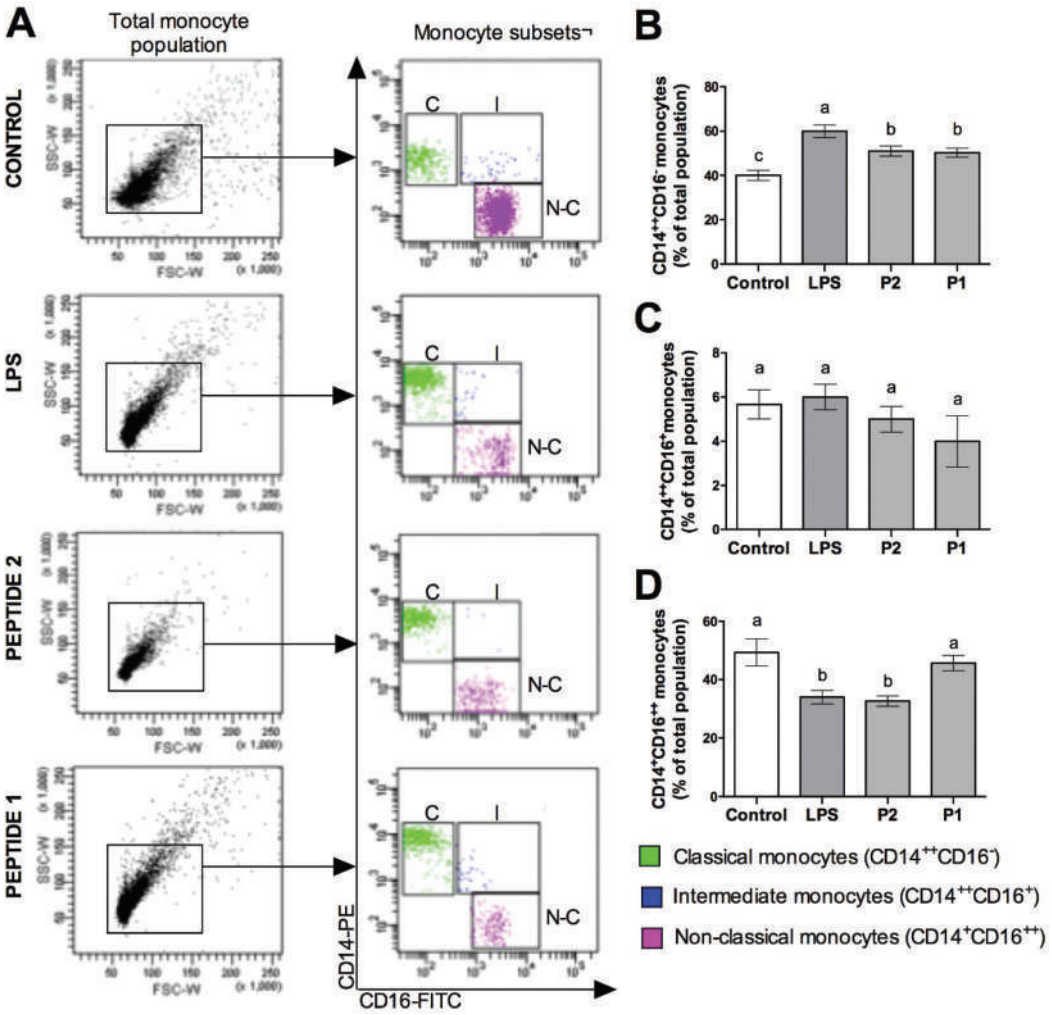

Fig. 1 Effect of GPETAFLR on monocyte subsets. (A) FACS analysis (MFI) of monocyte surface markers CD14 and CD16 after 24 h incubation with or without LPS (100 ng mL^{-1}) and peptide at 50–100 μg mL^{-1} (Peptide 2 and Peptide 1, respectively). (B) Classical $CD14^{++}CD16^{-}$ monocytes, (C) intermediate $CD14^{++}CD16^{+}$ monocytes, and (D) non-classical $CD14^{+}CD16^{++}$ monocytes. Values are presented as means ± SD (n = 3) and those marked with different letters are significantly different ($P < 0.05$).

cytes was analysed using flow cytometry. According to the manufacturer's instructions, 5×10^5 of purified monocytes, after *in vitro* stimulation with or without LPS (100 ng mL^{-1}), were exposed to GPETAFLR at 50–100 μg mL^{-1} for 24 h. Thereafter, cells were incubated with antibodies at room temperature and in the dark for 15 min; erythrocytes were removed with FACS lysing solution (Becton Dickinson). Mean fluorescence intensity (MFI) was measured by using a FACSCanto II flow cytometer (Becton Dickinson) and calibrated using a FACSDiva software (Becton Dickinson). MFI of 10^4 cells was assessed for each sample. Monocytes were gated as forward scatter^{high} (FSC^{high})-side scatter^{high} (SSC^{high}) cells. Expression levels are presented as MFI corrected for the nonspecific binding of isotope control antibodies.

RNA isolation and qRT-PCR analysis

Total RNA was extracted using the Trisure Reagent (Bioline), as instructed by the manufacturer. The A_{260}/A_{280} ratio in a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Madrid, Spain) was used to determinate RNA quality.

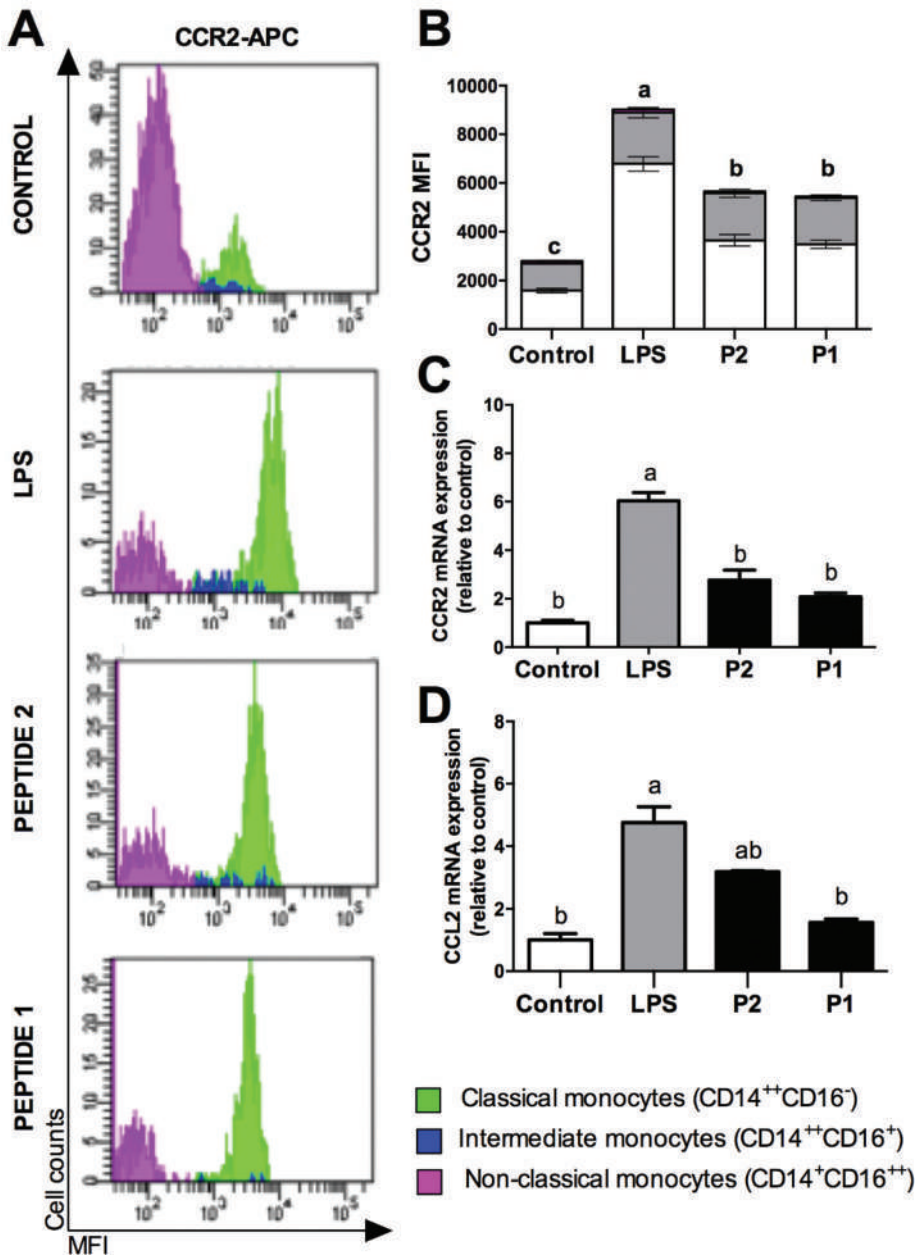

Fig. 2 Effect of GPETAFLR on the CCR2/CCL2 axis. (A) Representative overlay of MFI as a marker for CCR2 in monocyte subsets after 24 h incubation with or without LPS (100 ng mL^{-1}) and peptide at 50–100 μg mL^{-1} (Peptide 2 and Peptide 1, respectively). (B) FACS analysis of monocyte CCR2 expression in subsets by means of CD14 and CD16 surface marker expression. (C, D) Relative expression of CCR2 and CCL2 genes were measured using RT-qPCR. Values are presented as means ± SD (n = 3) and those marked with different letters are significantly different ($P < 0.05$).

Momentarily, RNA (1 μg) was subjected to reverse transcription (iScript, Bio-Rad, Madrid, Spain). An amount of 10 ng of the resulting cDNA was used as a template for real-time PCR amplifications. The mRNA levels for specific genes were determined in a CFX96 system (Bio-Rad). For each PCR reaction, the cDNA template was added to Brilliant SYBR Green QPCR Supermix (Bio-Rad) containing the primer pairs for either gene or for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyltransferase 1 (HPRT) as housekeeping genes (Table 1). All amplification reactions were performed in triplicate and average threshold cycle (Ct) numbers of the triplicates were used to calculate the relative

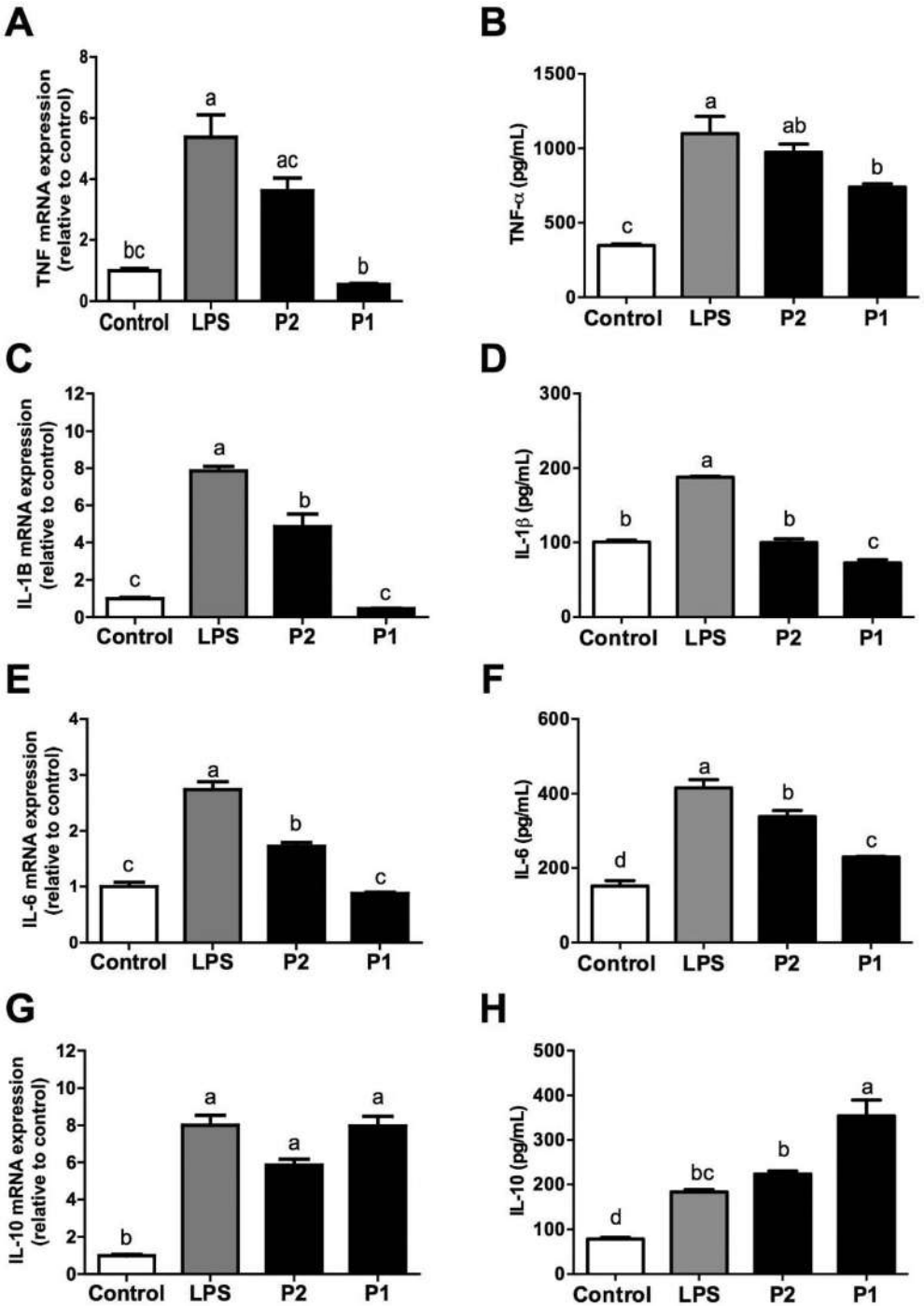

Fig. 3 Effect of GPETAFLR on pro-inflammatory cytokine release in primary human monocytes. (A, C, E, G) Gene expression and (B, D, F, H) and secretion of TNFα, IL-1β, IL-6, and IL-10 in primary human monocytes after 24 h incubation with or without LPS (100 ng mL^{-1}) and peptide at 50–100 μg mL^{-1} (Peptide 2 and Peptide 1, respectively). Values are presented as means ± SD (n = 3) and those marked with different letters are significantly different ($P < 0.05$).

mRNA expression of candidate genes. The magnitude of change of mRNA expression for candidate genes was calculated using the standard $2^{-(\Delta\Delta Ct)}$ method. All data were normalized to endogenous reference (GAPDH and HPRT) gene content and expressed as percentage of controls.

Cytokine quantification

The levels of TNF-α, IL-1β, IL-6, and IL-10 in culture supernatants were determined by the enzyme-linked immunosorbent assay (ELISA), following the indications of the manufacturer (Diaclone, Besancon, France). The cytokine concentrations were expressed in pg per mL, as calculated from the calibration curves from serial dilution of human recombinant standards in each assay.

Statistical analysis

All values are expressed as arithmetic means ± standard deviations (SD). Data were evaluated with GraphPad Prism Version 5.01 software (San Diego, CA, USA). The statistical significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance (ANOVA), following the Tukey multiple comparisons test as *post hoc* test. *P* values less than 0.05 were considered statistically significant.

Results

Effect of GPETAFLR on classical, non-classical, and intermediate monocyte subsets

FACS analysis of the monocyte surface markers CD14 and CD16 showed different results with the different treatments on monocyte subsets (Fig. 1A). Classical monocytes ($CD14^{++}CD16^{-}$) were increased in LPS-treated monocytes; however, the treatment with GPETAFLR decreased this population (Fig. 1B). Intermediate monocytes did not show significant differences when it was exposed with GPETAFLR (Fig. 1C). In contrast, non-classical monocytes ($CD14^{+}CD16^{++}$) were decreased with LPS. However, the treatment with GPETAFLR at 100 μg mL^{-1} increased the non-classical monocyte subset (Fig. 1D).

Effect of GPETAFLR on CCR2/CCL2 axis in monocyte subsets

The CCR2/CCL2 axis was studied (Fig. 2). CCR2 protein expression was analysed in monocyte subsets by FACS analysis (Fig. 2A). GPETAFLR decreased significantly CCR2 expression primarily on classical monocyte subsets compared to LPS (Fig. 2B), although no significantly differences were found on CCR2 MFI in non-classical or intermediate monocytes, there was a reduction tendency. In addition, mRNA expression of

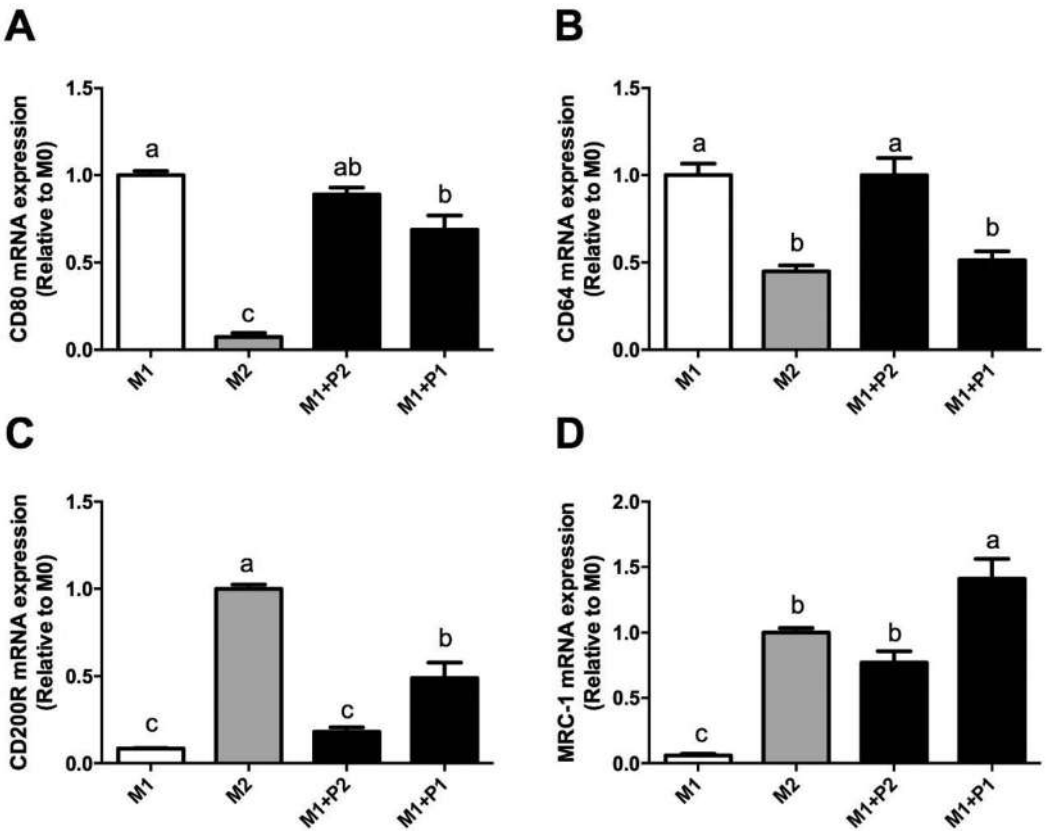

Fig. 4 Effect of GPETAFLR on M1/M2 macrophage polarisation. M0 macrophages were incubated with LPS plus IFNγ (M1 control), IL-4 (M2 control), or with LPS plus IFNγ plus GPETAFLR (50–100 μg mL^{-1}, Peptide 2 and Peptide 1, respectively) for additional 24 h. Relative expression of (A) CD80, (B) CD64, (C) CD200R, and (D) MRC-1 genes were measured by RT-qPCR. Values are presented as means ± SD (n = 3) and those marked with different letters are significantly different ($P < 0.05$).

CCL2 and its receptor, CCR2 was analysed. LPS-treated monocytes showed a CCR2 up-regulation (Fig. 2C). However, those that were treated with GPETAFLR peptide had a lower increase in CCR2 mRNA levels than those that were treated with LPS. Regarding CCL2 mRNA levels, LPS treatment up-regulated its expression, whereas that GPETAFLR treatment down-regulated CCL2 expression compared to these treated with LPS (Fig. 2D).

Effect of GPETAFLR on cytokine expression and release in primary human monocytes

In addition to monocyte subsets, we observed that LPS-induced the release of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and expression of genes encoding these cytokines were diminished by GPETAFLR in LPS-treated human monocytes (Fig. 3A–F). In all these cases, GPETAFLR effects were dose-dependent. Secretion and gene expression of the anti-inflammatory IL-10 was also promoted by GPETAFLR (Fig. 3G and H).

Effect of GPETAFLR on modulation of M1/M2 macrophage polarisation

Macrophage polarization depends on different microenvironment and signals that promote specific phenotypes. These phenotypes correspond to classically (M1) and alternatively (M2) polarized macrophages. Relative expressions of CD80, CD64, CD200R and MRC-1 were measured using RT-qPCR (Fig. 4).

M0 macrophages were incubated with LPS plus IFNγ (M1), IL-4 (M2) or LPS plus IFNγ plus GPETAFLR peptide (M1 + P) to study the phenotype plasticity. M0 macrophages treated with LPS plus IFNγ showed an increased in CD80 and CD64 mRNA levels (Fig. 4A and B, respectively) and a decrease in CD200R and MRC-1 mRNA levels (Fig. 4C and D, respectively). M0 macrophages treated with LPS plus IFNγ plus GPETAFLR peptide down-regulated CD80 and CD64 mRNA levels compared to M1 macrophages, whereas CD200R and MRC-1 mRNA levels were up-regulated compared to M1 macrophages. The results at 100 μg mL^{-1} were more remarkable than with 50 μg mL^{-1}. In addition, cytokine production in the macrophage phenotype was also studied (Fig. 5). TNFα, IL-1β, and IL-6 cytokine levels were increased in M1 macrophages (Fig. 5A, B and C, respectively). M0 macrophages treated with LPS plus IFNγ plus GPETAFLR peptide decreased the pro-inflammatory activity of the M1 phenotype, whereas IL-10 cytokine levels were increased compared to M1 macrophages (Fig. 5D).

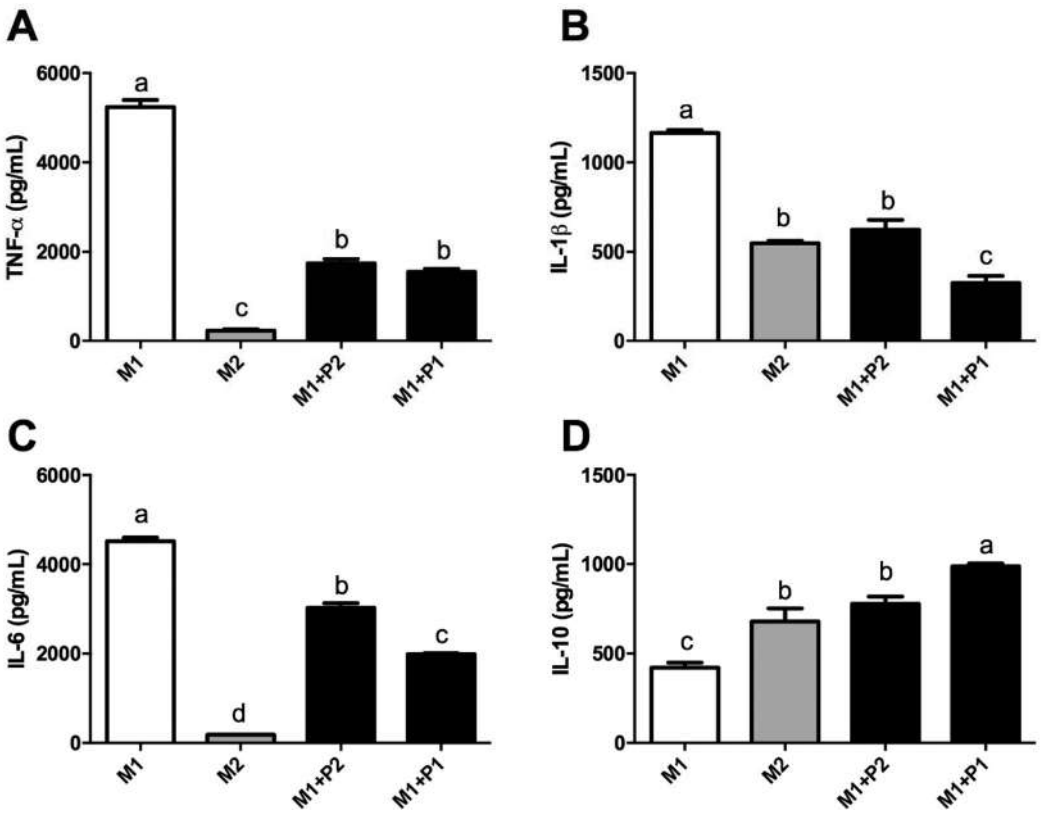

Fig. 5 Effect of GPETAFLR on expression of cytokines in M1 macrophages. M0 macrophages were incubated with LPS plus IFNγ (M1 control), IL-4 (M2 control), or with LPS plus IFNγ plus GPETAFLR (50–100 μg mL^{-1}, Peptide 2 and Peptide 1, respectively) for additional 24 h. Relative expression of (A) TNFα, (B) IL-1β, (C) IL-6, and (D) IL-10 genes were measured using RT-qPCR. Values are presented as means ± SD (n = 3) and those marked with different letters are significantly different ($P < 0.05$).

Discussion

In a world where resources are scarce, food plant processing residues are an alternative source to find proteins or peptides with bioactive properties.[21] Plant-derived bioactive peptides are very interesting compounds that can be used with potential pharmaceutical and nutraceutical benefit.[22]

In the literature, it is possible to find a large number of examples where plant-derived biopeptides are used as anti-inflammatory or antioxidant compounds. One of them is 1,2,3,4,6 penta-*O*-galloyl-β-D-glucose, a naturally occurring polyphenolic compound present in some medicinal herbs as *Rhuschinensis Mill*.[23] *Fagopyrum tataricum*, commonly known as buckwheat, is another example of a bioactive plant. Researchers found that buckwheat extracts may inhibit adipogenesis and inflammatory response during adipocyte differentiation of 3T3–L1 cells.[24] Brazilian red propolis (*Apis mellifera*), *Copaifera* oleoresins, flavonoid fraction of bergamot juice (*Citrus bergamia*), effusanin C (*Isodon japonicus*), oligomeric proanthocyanidins (*Crataegus oxyacantha*) are other isolated compounds with anti-inflammatory actions in activated monocytes and macrophages.[25–29]

Lupinus angustifolius L. is a legume that contents a high level of plant protein.[17] The GPETAFLR peptide was isolated from a lupine protein hydrolysate. This biopeptide showed an anti-inflammatory effect in different line cells such as osteoclasts[30] and THP-1-derived macrophages.[17,31] However, it is the first time that the anti-inflammatory activity of GPETAFLR was shown on primary monocytes.

Macrophage polarisation depends on different microenvironments and signals to promote specific phenotypes. These phenotypes correspond to classically (M1) and alternatively (M2) polarized macrophages that exhibit pro-inflammatory and anti-inflammatory functions, respectively.[32–34] In the present study, a modulation capacity of GPETAFLR on macrophage polarization was observed. This could be explained by the fact that the GPETAFLR peptide increase anti-inflammatory cytokines such as IL-10 while decreasing pro-inflammatory as IL-6 or IL-1β. These cytokines regulate and modulate the macrophage phenotype, stimulating the M1 phenotype in the case of IL-1β or M2 phenotype in the case of IL-10.[35,36]

GPETAFLR could also modulate monocyte subsets. Under LPS stimulation, classical monocytes (monocytes that promote inflammatory reactions) increased their population while non-classical monocytes suffer a decrease with respect to monocytes treated with the peptide. This mean that GPETAFLR can modulate the monocyte subset ratio, stimulating those monocytes that participate in the anti-inflammatory process and decreasing classical monocytes.[37,38]

In conclusion, the GPETAFLR peptide showed an anti-inflammatory effect in monocyte cells. Our findings imply a new understanding of the mechanisms by which GPETAFLR favors a continuous and gradual plasticity process in the human monocyte/macrophage system and offer novel benefits derived from the consumption of *Lupinus angustifolius* L. in the prevention of inflammatory-related diseases.

Abbreviations

CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	Cluster of differentiation
GPETAFLR	Gly-Pro-Glu-Thr-Ala-Phe-Leu-Arg
HLA-DR	Human leukocyte antigen–antigen D related
M-CSF	Macrophage colony-stimulating factor
TLR	Toll-like receptor
TNF	Tumour necrosis factor

Conflicts of interest

All the authors declare that they do not have conflicts of interest.

Acknowledgements

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A lupine (*Lupinus angustifolious* L.) peptide prevents non-alcoholic fatty liver disease in high-fat-diet-induced obese mice†

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Bioactive peptides are related to the prevention and treatment of many diseases. GPETAFLR is an octapeptide that has been isolated from lupine (*Lupinus angustifolius* L.) and shows anti-inflammatory properties. The aim of this study was to evaluate the potential activity of GPETAFLR to prevent non-alcoholic fatty liver disease (NAFLD) in high-fat-diet (HFD)-induced obese mice. C57BL/6J mice were fed a standard diet or HFD. Two of the groups fed the HFD diet were treated with GPETAFLR in drinking water at 0.5 mg kg^{-1} day^{-1} or 1 mg kg^{-1} day^{-1}. To determine the ability of GPETAFLR to improve the onset and progression of non-alcoholic fatty liver disease, histological studies, hepatic enzyme profiles, inflammatory cytokine and lipid metabolism-related genes and proteins were analysed. Our results suggested that HFD-induced inflammatory metabolic disorders were alleviated by treatment with GPETAFLR. In conclusion, dietary lupine consumption can repair HFD-induced hepatic damage possibly *via* modifications of liver's lipid signalling pathways.

Introduction

Nowadays, the population is growing exponentially and meanwhile, natural sources are decreasing due to their overexploitation and climate change. For these reasons, it is necessary to discover new sources of nutritional compounds that have nutritional value and can be easy to obtain and process. This research opens a novel area that takes advantage of agroindustry by-products to further propose adaptive supply regarding the specific demand of nutrients and bioactive compounds.[1,2]

Vegetables are an important source of nutrients. The isolation and purification of peptides are relatively easy; moreover, their growth procedure is cheap and simple. Therefore, peptides are an ideal target for obtaining bioactive compounds. The main sources of peptides are soy, eggs, milk, and fish.[3] The main ways by which the bioactive peptides act on human health are as follows: decreased inflammation, lower blood pressure, anti-obesity, and prevention and improvement in the symptoms related to diabetes.[4] In addition, the diseases that are on the rise in developed countries such as obesity, dyslipidemias, type II diabetes, metabolic syndrome, and non-alcoholic fatty liver disease (NAFLD) have pharmacological treatment that is not fully effective and also has adverse side effects.[5] This is why many scientists are looking for substances of plant origin that can alleviate or prevent these diseases.[6]

Bioactive peptides contain a short sequence of amino acids with different biological activities. Initially, they are found in protein precursors in the organism of origin and they are generally not active.[7,8] Peptides are obtained from plant proteins as a result of enzymatic hydrolysis during digestion *via* fermentation by micro-organisms or by *in vitro* chemicals or enzymatic hydrolysis processes.[7]

Lupinus angustifolius L. is one of the targets for the research of such peptides because it has a high amount of proteins and low fat content.[9,10] GPETAFLR is an octapeptide isolated from *Lupinus angustifolius* L. with the sequence glycine-proline-glutamic acid-threonine-phenylalanine-leucine-arginine. This peptide features a three-dimensional secondary structure of

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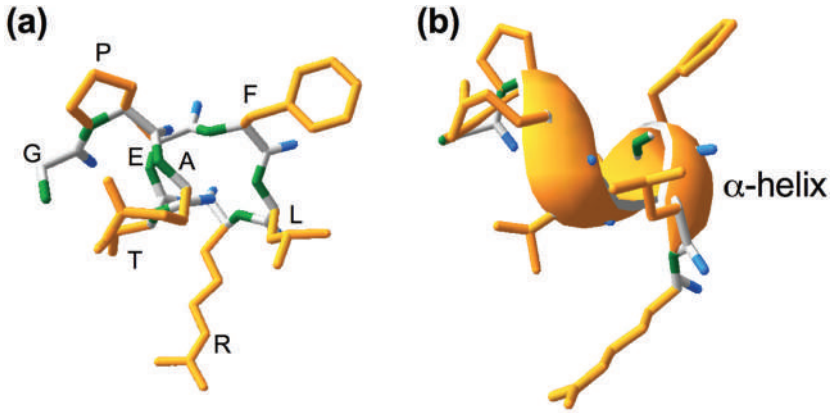

Fig. 1 Chemical (a) and secondary three-dimensional (b) structures of GPETAFLR peptide, an octapeptide isolated from *Lupinus angustifolius* L., for which the amino acid sequence is identified as Glycine (G), Proline (P), Glutamate (E), Threonine (T), Alanine (A), Phenylalanine (F), Leucine (L), and Arginine (R). Yellow color was used for the side chain, green color was used for the amino group, and blue color was used for the carboxyl group.

α-helix (Fig. 1). Its immunomodulatory and anti-inflammatory capacities have been demonstrated.[11] Its effects have been previously investigated in macrophage THP-1-line derivatives,[11] osteoclasts derived from human primary monocytes,[12] and primary human monocytes.[13] Hence, it is an interesting compound to treat or prevent diseases characterized by inflammation.

Non-alcoholic fatty liver disease (NAFLD) is a health problem that affects a large percentage of the world's population with a global prevalence of 25%.[14] The prevalence of this disease is very common in developed countries due to the fact that it typically occurs in people with obesity, type 2 diabetes, and metabolic syndrome among other pathologies related to lifestyle.[15–18] NAFLD consists of the accumulation of fat in the liver tissue, which is derived from a high amount of free fatty acids (FFAs) and triglycerides (TGs) in the circulating blood. These levels may increase after higher caloric and fat intakes. For this reason, fatty liver disease is often associated with obesity and dyslipemias.[15–18] The best models for the study of this disease are animal models of induced obesity; specifically, mice and rat models present pathogenesis more similar to humans.[19] NAFLD has 4 phases: non-alcoholic fatty liver (NAFL), accumulation of fat in the liver; non-alcoholic steatohepatitis (NASH), liver fat accumulation with inflammation; fibrosis, accumulation of fat and inflammation resulting in tissue damage that drifts into fibrosis; and cirrhosis, most of the cells are replaced by fibrosis, thus considerably damaging the cellular hepatic function. This state leads to hepatic dysfunctionality, and the development of NAFLD may culminate in the development of liver cancer.[18] Specifically, cirrhosis (last stage of NAFLD) is the major risk factor for developing hepatocellular carcinoma, the most common liver cancer. Hepatic steatosis is also related to the occurrence of hepatocellular carcinoma.[20] Thus, NAFLD, even in its early stages, promotes the development of the most common type of liver cancer. Herein, the peptide GPETAFLR was used to investigate its role in the development of NAFLD in the hepatic tissues of mice with HFD-induced obesity.

Materials and methods

All animal protocols received appropriate institutional approval (Animal Care and Use Committee of the University of Seville) and were performed according to the official rules formulated in the Spanish law on the care and use of experimental animals (UE Directive of 2010: 2010/63/UE; RD 53/2013).

Isolation, purification, and synthesis of GPETAFLR

Seeds of sweet lupine (*Lupinus angustifolius* L.) were a gift from Koipesol Semillas, S.A. (Seville, Spain). Alcalase 2.4 L was provided by Novozymes (Bagsvaerd, Denmark). Lupine protein isolate was obtained using a previously described method at the pilot plant scale (Plant Protein Unit, Instituto de la Grasa, Seville, Spain).[21] The chemical composition of lupine protein isolate in dry matter was as follows: protein 86.83%, dietary fiber 5.97%, fat 5.08%, ash 0.78%, and carbohydrate 1.34%. Lupine protein isolate was suspended in distilled water (10% w/v), and hydrolysis with Alcalase was performed under the following conditions: pH 8, 50 °C, E/S = 0.3 AU g^{-1} protein, and a hydrolysis time of 15 min. The mixture was then heated at 85 °C for 15 min to inactivate the enzyme and centrifuged at 6500*g* for 15 min, and the supernatant constituted LPH.[11] This fraction was purified by ultrafiltration and chromatographic techniques.[22] The sequence of the purified peptide was identified through sequence analysis using nano-HPLC coupled with a Polaris Q ion-trap mass spectrometer (Mass Spectrometry Unit, Instituto de la Grasa, Seville, Spain). Finally, the peptide was synthesized by an Fmoc solid-phase method (Barcelona Scientific Park Foundation, Barcelona, Spain) at 95% purity measured using HPLC-UV at 220 nm.

Animal diets and experimental design

In order to keep the experimental number of mice as low as possible, forty male mice C57BL/6J were used in the study. They were obtained from the Animal Production and Experimentation Centre at the University of Seville. The mice

Table 1 Composition of standard chow and high-fat diets

Macronutrients		kcal kg^{-1}	%kcal
Standard chow diet (amount per kg)			
Protein	205.9 g	823.8	23.3
Fat	55.1 g	496.4	14
Carbohydrates	554.2 g	2216.8	62.7
Total	815.2 g	3537	100
High-fat diet (amount per kg)			
Protein	200.2 g	800.9	1.6
Fat	359.4 g	3235.2	58.8
Carbohydrates	374.1 g	1496.3	26.7
Total	933.7 g	5505.4	100

were divided into four groups (n = 10 per group), in which 3 of them were fed HFD and the last group was fed a standard chow diet (CD) and was used as a control group (210 SAFE, Augy, France). Mice were fed for 8 weeks. The HFD, which contained 60% energy as fat, was prepared by Panlab Laboratories (HF 260 SAFE) and presented as pellets to the animals (Table 1). Two of the experimental animal groups fed HFD were also treated with the synthesized GPETAFLR peptide, which was added into drinking water and administered at a dose of 0.5 or 1 mg kg^{-1} day^{-1}. Mice were anesthetized by an intraperitoneal injection of ketamine (100 mg kg^{-1}) and diazepam (5 mg kg^{-1}) and then euthanized by cervical dislocation. Hepatic tissues were immediately removed and then frozen at −80 °C until processing. Homogenization was done with TRIsure (Bioline, Memphis, TN, USA).

Dosage information

Doses of 0.5 and 1 mg kg^{-1} day^{-1} of the synthesized GPETAFLR peptide were established according to previous studies[23–28] and the expected water consumption of the C57BL/6J strain.[29] These selected doses for animals were comparable to human equivalent doses (HED) of 40 and 80 μg kg^{-1}.[30]

RNA isolation and real-time quantitative PCR analysis

RNA from hepatic tissues was isolated to quantify gene expression by RT-qPCR. Total RNA was extracted by using TRIsure Reagent (Bioline). RNA quality was assessed by the A_{260}/A_{280} ratio using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Madrid, Spain). Briefly, RNA (250 ng) was subjected to reverse transcription (iScript, Bio-Rad). An amount of 40 ng of the resulting cDNA was used as a template for RT-qPCR amplifications. The mRNA levels for specific genes were determined in a CFX96 system (Bio-Rad). For each PCR reaction, a cDNA template was added to Brilliant SYBR green QPCR Supermix (Bio-Rad) containing the primer pairs for either gene or hypoxanthine phosphoribosyltransferase (HPRT) as a housekeeping gene. All amplification reactions were performed in triplicate and the average threshold cycle (Ct) numbers of the triplicates were used to calculate the relative mRNA expression of the candidate genes. The magnitude of the change in the mRNA expression for candidate genes was calculated by using the standard $2^{-(\Delta\Delta Ct)}$ method. All data were normalized to endogenous reference (HPRT) gene content and expressed as relative fold-change of control. The sequences of the designed oligonucleotides are shown in Table S1.†

Histological analysis

Dissected liver sections were fixed overnight with 4% paraformaldehyde, cryoprotected with 15%–30% sucrose and embedded in Tissue-Tek OCT embedding medium (Sakura Finetek Europe, The Netherlands). Cryostat sections (8–10 microns) were rinsed with PBS and stained with a 0.3% solution of Oil Red O for 10 minutes at room temperature. After washing in PBS, the sections were counterstained with hematoxylin and eosin for 1 minute and washed with water.[31] The percentage of the stained area was determined using Image J software.

Biochemical analysis

The levels of leptin in supernatants were measured by ELISA following the indications of the manufacturer (ThermoFisher). The adipokine concentrations were expressed in ng per mL, as calculated from the calibration curves from a serial dilution of mice recombinant standards. In addition, the TG content in serum and liver tissues was determined by colorimetric enzyme assays (Bio-Science-Medical). The enzyme activities of liver enzymes, alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) were measured using a commercial kit (Bio-Science-Medical) following the instructions. Absorbance was read using a microplate reader.

Statistical analysis

All values in the figures and text are expressed as the arithmetic mean ± SD. Experiments were carried out in triplicate. Data were evaluated with GraphPad Prism Version 7.0 software (San Diego, CA, USA). The statistical significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance (ANOVA) using Tukey's test for multiple comparison analyses. P values < 0.05 were considered statistically significant.

Results

GPETAFLR decreases body and liver weight gain in HFD-induced obese mice

After 8 weeks of diet, the animals showed body weight gain (Fig. 2(a)), and the increase in the body weight of the groups fed HFD was higher than that for the control group (Fig. 2(b)). Weight gain during the 8 weeks with HFD and GPETAFLR treatment at 1 mg kg^{-1} day^{-1} was significantly lower (1.3-fold lower for HFD-GPETAFLR (1 mg kg^{-1} day^{-1}) than HFD) than that for animals fed HFD not containing the peptide. Liver weight was also significantly higher in the obese control (animals fed only HFD) (1.3-fold lower for HFD-GPETAFLR (1 mg kg^{-1} day^{-1}) than HFD) with respect to that for the animals treated with GPETAFLR in drinking water (Fig. 2(c)).

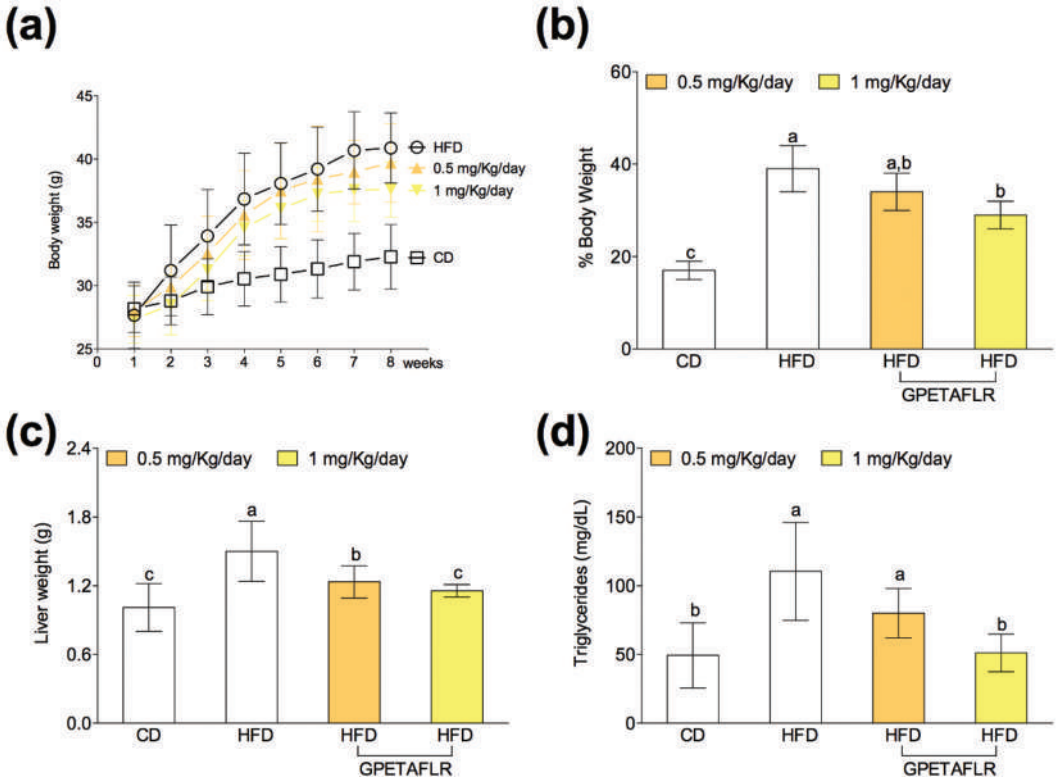

Fig. 2 Effect of GPETAFLR on the evolution of body weight (a), percentage of body weight gain (b), liver weight (c) and blood triglyceride levels (d) in mice fed a standard chow diet (CD), an HFD or an HFD + GPETAFLR at a dose of 0.5 mg kg^{-1} day^{-1} and 1 mg kg^{-1} day^{-1} in drinking water. Values are presented as means ± SD (n = 10) and those marked with different letters are significantly different ($P < 0.05$).

In line with the results described above, the level of TGs in the blood was significantly higher in the obese control group (2.1-fold higher for HFD than HFD-GPETAFLR (1 mg kg^{-1} day^{-1})) with respect to that in the animals that, in addition to HFD, were treated with GPETAFLR in their drinking water (Fig. 2(d)). In this case, similar to that observed for liver weight, the effect of GPETAFLR was dose-dependent.

GPETAFLR decreases hepatic leptin resistance in HFD-induced obese mice

In obesity, the blood levels of leptin increase due to the lack of sensitivity between leptin and its receptor (OB-R), which is similar to that observed in insulin resistance. After 8 weeks of HFD, mice presented higher serum levels of leptin than the animals fed the standard diet (4.2-fold higher for HFD than CD) (Fig. 3(a)). The treatment with GPETAFLR significantly decreased these levels (2.7-fold lower for HFD-GPETAFLR (1 mg kg^{-1} day^{-1}) than HFD). Similarly, the OB-R mRNA levels were significantly higher in the animals fed HFD in contrast to that in the animals that did not receive GPETAFLR through drinking water (3.9-fold lower for HFD-GPETAFLR (1 mg kg^{-1} day^{-1}) than HFD, Fig. 3(b)).

GPETAFLR decreases hepatic steatosis in HFD-induced obese mice

Fig. 4 shows the results of the histological study carried out with ORO and H&E staining for the determination of the lipid content in hepatocytes. Fig. 4(a) shows the representative staining images of the histological slices of livers obtained from each experimental group. After 8 weeks of HFD, mice presented higher levels (3.2-fold higher for HFD than CD) of hepatic lipids than the animals fed a standard diet (Fig. 4(b)). The mice treated with GPETAFLR exhibited significant decrease in the hepatic steatosis induced by HFD (1.9-fold lower for HFD-GPETAFLR (1 mg kg^{-1} day^{-1}) than HFD).

GPETAFLR modulates hepatic lipid metabolism-related genes in HFD-induced obese mice

After 8 weeks of HFD, the levels of TGs in liver tissues were higher compared to that for CD (5.2-fold higher for HFD than CD, Fig. 5(a)). However, the TG levels were lower in the animals that were fed HFD and received GPETAFLR treatment (2.7-fold lower for HFD-GPETAFLR (1 mg kg^{-1} day^{-1}) than HFD). Significantly, GPETAFLR at 1 mg kg^{-1} day^{-1} restored the values of CD. In line with these results, the expression of the fatty acid synthase (FAS) enzyme (Fig. 5(b)) and peroxisome proliferator-activated receptor α (PPARα) were determined (Fig. 5(c)). The FAS mRNA levels in the animals fed HFD were significantly higher than those in the animals fed CD (33.6-fold higher for HFD than CD) or treated with GPETAFLR (18.6-fold higher for HFD than CD). In addition, the transcriptional activity of PPARα increased in the groups that received the octapeptide (6.6-fold higher for HFD-GPETAFLR

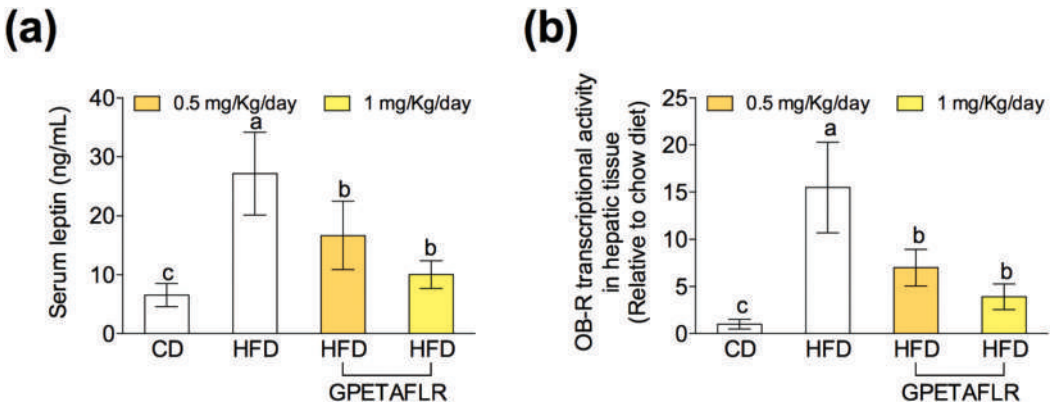

Fig. 3 Effect of GPETAFLR on leptin resistance. Serum leptin levels (a) and gene expression in leptin receptor hepatic tissue (OB-R) (b) in mice fed a standard chow diet (CD), an HFD or an HFD + GPETAFLR at a dose of 0.5 mg kg^{-1} day^{-1} and 1 mg kg^{-1} day^{-1} in drinking water. Values are presented as means ± SD (n = 10) and those marked with different letters are significantly different (P < 0.05).

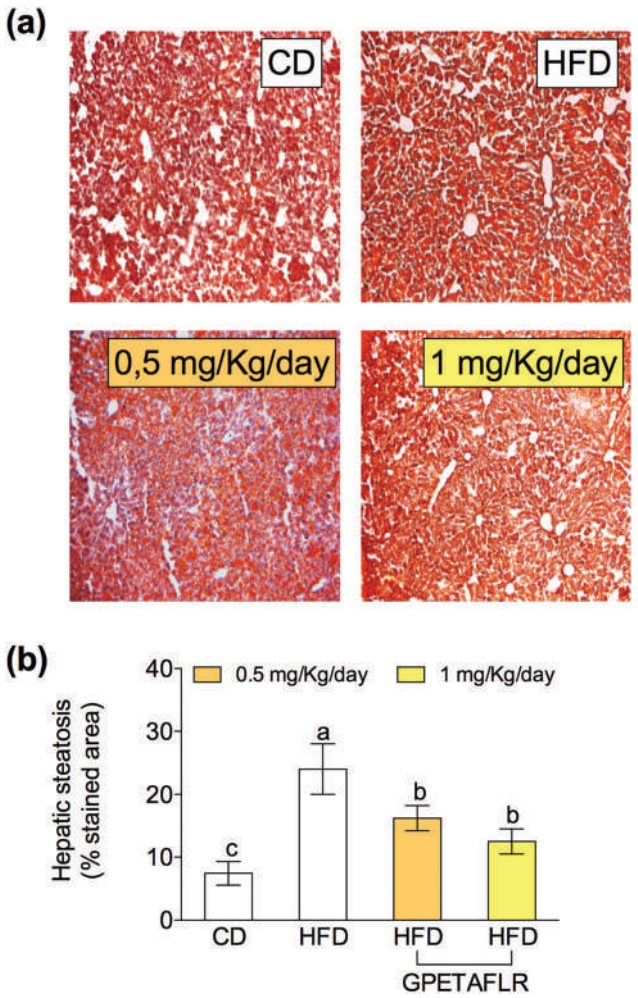

Fig. 4 Effect of GPETAFLR on hepatic steatosis. Representative images (10×) of stained histological slices with Oil Red O (ORO) and hematoxylin–eosin (H&E) (a) and quantification of the stained area with ORO from each experimental group (b) in mice fed a standard chow diet (CD), an HFD or an HFD + GPETAFLR at a dose of 0.5 mg kg^{-1} day^{-1} and 1 mg kg^{-1} day^{-1} in drinking water. Values are presented as means ± SD (n = 10) and those marked with different letters are significantly different (P < 0.05).

(1 mg kg^{-1} day^{-1}) than HFD). Finally, the mRNA levels of mitochondrial decoupling protein 1 (UCP1) were determined (Fig. 5(d)). The treatment with GPETAFLR in drinking water significantly increased the hepatic transcriptional activity of UCP1 in comparison to those groups that did not receive the peptide (4.9-fold higher for HFD-GPETAFLR (1 mg kg^{-1} day^{-1}) than HFD).

GPETAFLR decreases hepatic inflammation in HFD-induced obese mice

After 8 weeks of HFD, the gene expressions of proinflammatory cytokines on hepatic tissues such as tumor necrosis factor α (TNF-α) (Fig. 6(a)), interleukin 1β (IL-1β) (Fig. 6(b)), and interleukin 6 (IL-6) (Fig. 6(c)) were significantly higher in the animals fed exclusively with HFD. In both doses, GPETAFLR significantly decreased the mRNA levels of the pro-inflammatory cytokines. The gene expression of the anti-inflammatory cytokine interleukin 10 (IL-10) significantly increased in the mice that received GPETAFLR in drinking water compared to that in the groups that did not receive the peptide (5.1-fold higher for HFD-GPETAFLR (1 mg kg^{-1} day^{-1}) than HFD, Fig. 6(d)).

GPETAFLR decreases blood markers of liver damage in HFD-induced obese mice

After 8 weeks of HFD, mice showed higher serum activity for AST (5.1-fold higher for HFD than HFD-GPETAFLR (1 mg kg^{-1} day^{-1}), Fig. 7(a)) and ALT (8.2-fold higher for HFD than HFD-GPETAFLR (1 mg kg^{-1} day^{-1}), Fig. 7(b)) than those animals that received the GPETAFLR peptide in drinking water. GPETAFLR restored the values of transaminases to the levels in the animals fed CD. The activity of serum ALP was significantly higher in the obese control (1.4-fold higher for HFD than HFD-GPETAFLR (1 mg kg^{-1} day^{-1}), Fig. 7(c)). GPETAFLR at 1 mg kg^{-1} day^{-1} restored the values of this marker to similar levels to the animals fed CD. On the other hand, the values for the serum activity of the enzyme LDH were higher in the obese control (2.8-fold higher for HFD than

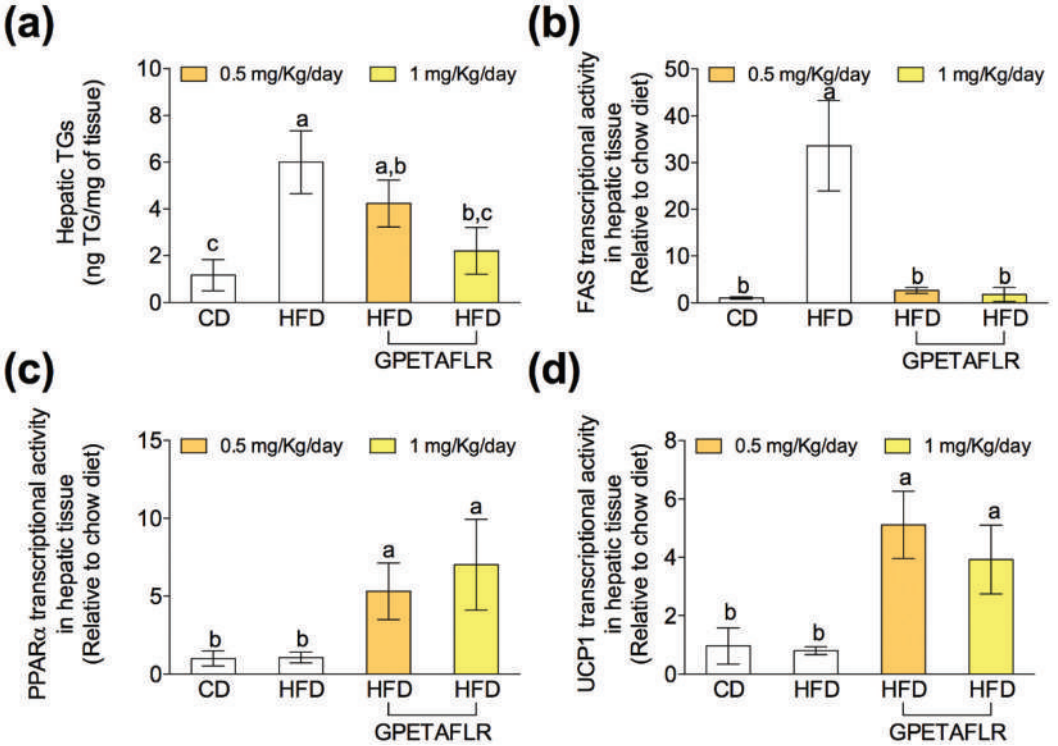

Fig. 5 Effect of GPETAFLR on the TG levels in the liver tissue (a) and the expression of genes related to lipid and energy metabolism, such as the fatty acid synthase (FAS) (b), peroxisome proliferator-activated receptor α (PPARα) (c) and uncoupling protein 1 (UCP1) (d) in mice fed a standard chow diet (CD), an HFD or an HFD + GPETAFLR at a dose of 0.5 mg kg^{-1} day^{-1} and 1 mg kg^{-1} day^{-1} in drinking water. Values are presented as means ± SD (n = 10) and those marked with different letters are significantly different ($P < 0.05$).

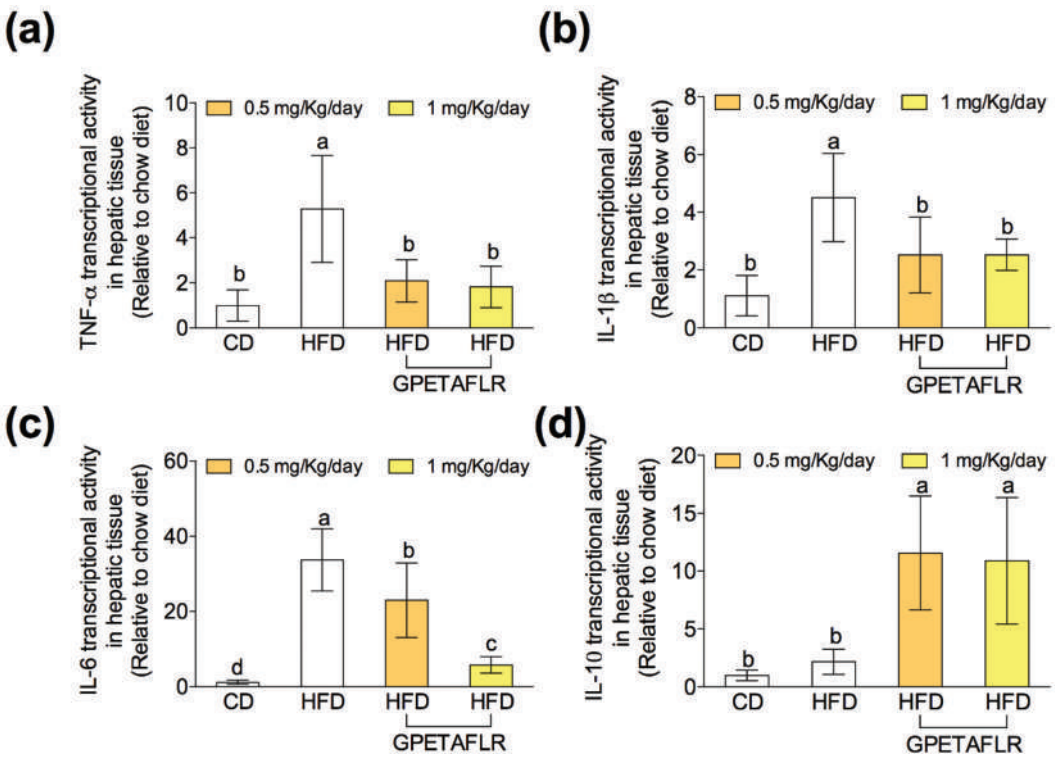

Fig. 6 Effect of GPETAFLR on the expression of pro-inflammatory and anti-inflammatory cytokine genes in mice fed a standard chow diet (CD), an HFD or an HFD + GPETAFLR at a dose of 0.5 mg kg^{-1} day^{-1} and 1 mg kg^{-1} day^{-1} in drinking water. The mRNA levels of TNF-α (a), IL-1β (b), IL-6 (c) and IL-10 (d) were measured in the liver tissue. Values are presented as means ± SD (n = 10) and those marked with different letters are significantly different ($P < 0.05$).

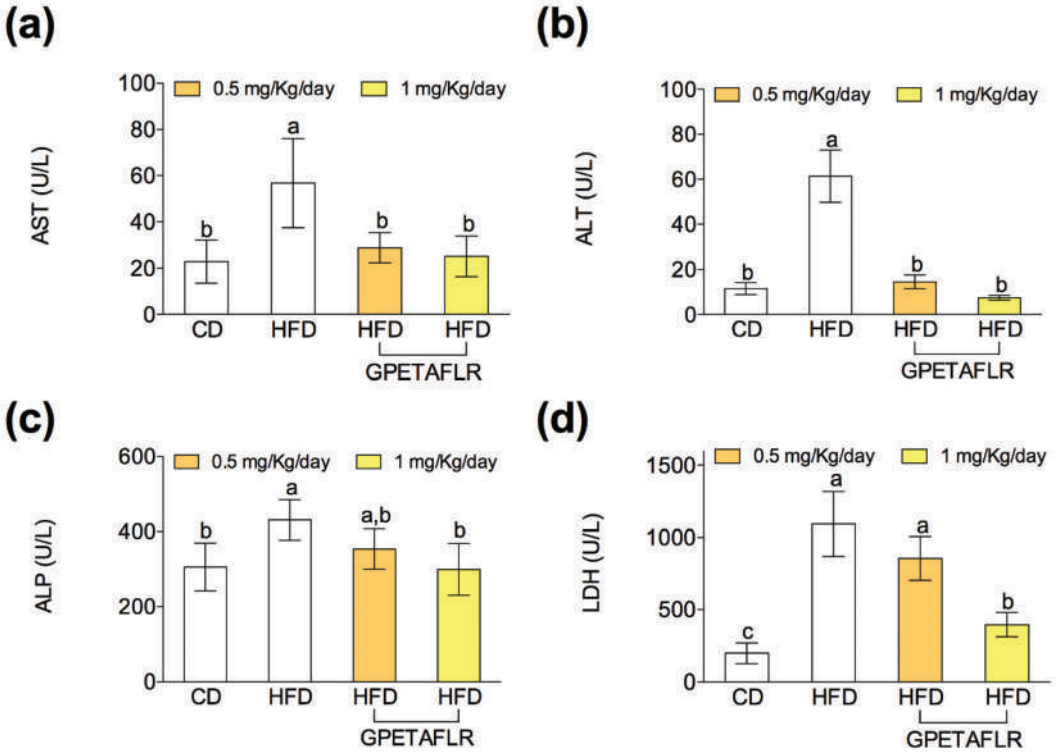

Fig. 7 Effect of GPETAFLR on serum liver damage markers such as aspartate aminotransferase (AST) (a), alanine aminotransferase (ALT) (b), alkaline phosphatase (ALP) (c) and lactate dehydrogenase (LDH) (d) in mice fed a standard chow diet (CD), an HFD or an HFD + GPETAFLR at a dose of 0.5 mg kg^{-1} day^{-1} and 1 mg kg^{-1} day^{-1} in drinking water. CD, chow diet, was used as a control group. Values are presented as means ± SD (n = 10) and those marked with different letters are significantly different ($P < 0.05$).

HFD-GPETAFLR (1 mg kg^{-1} day^{-1}), Fig. 7(d)). GPETAFLR at 1 mg kg^{-1} day^{-1} significantly decreased the values of this enzyme compared to that for the mice fed HFD without the GPETAFLR treatment.

Discussion

The use of bioactive peptides obtained from food is increasingly gaining importance because of their health benefits and use in preventing chronic diseases, whose incidences are increasing worldwide.[4] Many chronic diseases are characterized by a marked inflammatory process. Therefore, GPETAFLR, a novel anti-inflammatory biopeptide obtained from *Lupinus angustifolius* L., can be a potent molecule for the prevention of these types of diseases. Its anti-inflammatory activity has been demonstrated. Specifically, both the peptide and the extracts of hydrolyzed lupin proteins have shown the ability to decrease the expression of pro-inflammatory cytokines and increase the expression of anti-inflammatory cytokines.[11–13]

The liver is the main metabolic organ of the body. NAFLD affects its functionality, endangering the homeostasis metabolic system. This disease is typically associated with obesity,[15–18] where a substance that has the effect of reducing body weight and obesity rates will greatly alleviate the symptoms and development of NAFLD. The octapeptide GPETAFLR prevents the weight gain resulting from a diet rich in fat in contrast to other substances such as isoliquiritigenin with hepatoprotective properties that do not have the capacity to prevent weight gain.[32] It can be said that GPETAFLR interferes by preventing the progression of the main cause of NAFLD: obesity.

On the other hand, the obtained data corroborated that the HFD-induced obese mice presented NAFLD pathophysiology similar to humans. The pathophysiology of this disease is characterized by increased body weight and liver weight, high levels of TGs in the blood, fat accumulation in adipose tissues, hepatic inflammation and elevated markers of liver damage.[33] In our study, the obese control group showed a greater liver weight derived from a greater accumulation of fat in the liver. The accumulation may be due to high levels of TGs in the blood and the increase in the FAS gene expression. In addition, this group presented a pro-inflammatory state provoked by high levels of the expressions of pro-inflammatory cytokines IL-6, IL-1β, and TNF-α since the Kupffer cells increase their expression in response to hepatic fat accumulation.[34] Finally, it presented elevated markers of liver damage since the activities of transaminases, LDH, and ALP in serum were significantly higher. These values were also obtained in other similar studies on HFD-induced obese animals.[19,32,35–37]

All these parameters of the pathology were palliated with the treatment with GPETAFLR owing to the fact that liver weight

and liver fat accumulation were lower in the animals treated with the octapeptide. The factors that can influence the minor accumulation of hepatic fat in these individuals are a decreased gene expression of FAS and an increased expression of the enzymes that increase energy metabolism, such as UCP1 and PPARα. In addition, there is less circulation of TGs in the blood and the availability for its accumulation in different tissues including the liver. GPETAFLR could mediate the accumulation of hepatic fat by influencing the signaling pathways involved in the regulation of the gene expression or the activity of lipid metabolism proteins, such as the kinase pathway activated by AMP (AMPK). AMPK regulates the activity and expression of many important proteins related to metabolism; one of them is mTOR, another metabolic regulator. AMPK decreases the accumulation of fat (down-regulating FAS, between other proteins) and other metabolites and also down-regulates mTOR, the protein that increases the accumulation of fat and also has effects on the gene and protein expressions.[38] In previous studies, GPETAFLR has been demonstrated to present anti-inflammatory activity that is also exhibited by hepatocytes. The octapeptide decreased pro-inflammatory cytokine levels and increased the levels of the anti-inflammatory cytokine IL-10. IL-10 did not show a decrease in expression in the obese control compared to that in the standard control, but its expression greatly increased in the groups treated with GPETAFLR. These data are related to previous studies on other cell lines and primary cultures.[11–13] Pro-inflammatory cytokines present several actions related to metabolism; for example, TNF-α decreases the degradation of fats and increases their accumulation, and IL-6 inhibits lipolysis and increases fat deposition.[35,39] For this reason, in addition to contributing to tissue damage from chronic inflammation, they contribute to the development of NAFLD by boosting fat accumulation in the liver. Chronic inflammation is primarily mediated by the nuclear factor κB (NF-κB) pathway, which stimulates the production of cytokines such as TNF-α.[40] For this reason, GPETAFLR may decrease inflammation by influencing the NF-κB pathway. The NF-κB pathway increases the expression of pro-inflammatory cytokines; this pathway can be activated by the interaction of free fatty acids with TLR4, a membrane receptor expressed by Kupffer cells, which produces a pro-inflammatory state.[34] The down-regulation of this pathway can be determined by the reduction in fat accumulation in the liver associated with the treatment with GPETAFLR.

Another parameter used as a marker of NAFLD pathogenesis in this study was the measurement of blood markers for liver damage, specifically transaminases, ALP, and LDH. The activity of these enzymes in serum was lower in both GPETAFLR treatments compared to that in the obese control group. This effect is probably due to the fact that the peptide decreases liver damage by reducing the accumulation of fat and inflammation (influenced by the lower accumulation of fat) and thus all the tissue damage associated with a chronic inflammatory process.

On the other hand, leptin is an adipokine produced by adipose tissues, and the levels of this hormone in the blood are indicative of the body's fat reserves because it is produced proportionally to the amount of adipose tissues. Therefore, a higher amount of leptin results in a higher amount of fat reserve.[41] Leptin resistance is a characteristic of most obesity-associated diseases. This syndrome is characterized by a rise in serum leptin due to the increase in adipose tissues, but there is a lack of its signaling because the tissues with receptors show resistance. A proof that leptin resistance is occurring is a high value of leptin in serum and the gene expression of its receptor in the tissues that express it, such as that observed in the animals of the obese control group. The liver is one of the organs that can present receptors for leptin and signaling by this molecule increases the catabolic metabolism, increases the degradation of fatty acids, decreases the synthesis of fatty acids and affects glucose metabolism, specifically hypoglycemia.[41,42] Leptin resistance in NAFLD can mediate an increase in the accumulation of hepatic fat in its direct form by a lack of signaling in the liver. In addition, leptin resistance may enhance the progression of NAFLD due to its role in intake and satiety. Leptin can cross the blood–brain barrier once inside the central nervous system (CNS), activate the production of anorexigenic peptides and inhibit the production of orexigenic peptides. This role translates into the inhibition of appetite and therefore into a decrease in caloric intake.[41] Supplementation with GPETAFLR produced a decrease in obesity-associated leptin resistance. These effects may be due to the fact that GPETAFLR decreases body weight or GPETAFLR decreases resistance to the leptin and therefore, there is a decrease in body weight. It may also be considered that both effects are produced. Other plant bioactive substances have been shown to have effects by decreasing the resistance to leptin without significantly affecting the body weight.[32]

Conclusions

To conclude, GPETAFLR decreased body weight gain, liver weight and fat accumulation in the liver as well as TG levels in the blood and leptin resistance associated with obesity. GPETAFLR showed anti-inflammatory properties at the hepatic level, decreasing the mRNA levels of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-10 and increasing the expression of the anti-inflammatory cytokine IL-10 in liver tissues. The octapeptide also reduced blood markers related to liver damage such as AST, ALT, ALP, and LDH. Taken together, our findings suggest that the use of GPETAFLR is a potential treatment as well as an important preventive therapy for NAFLD. Thus, the dietary supplementation with *Lupinus angustifolius* L. would also contribute to a reduction in this pathology.

Conflicts of interest

The authors declare no conflict of interest.

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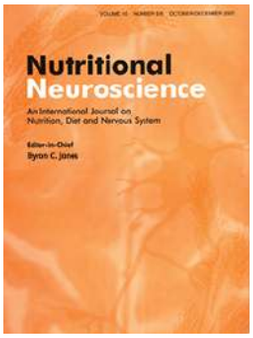

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GPETAFLR, a peptide from Lupinus *angustifolius* L. prevents inflammation in microglial cells and confers neuroprotection in brain

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GPETAFLR, a peptide from Lupinus *angustifolius* L. prevents inflammation in microglial cells and confers neuroprotection in brain

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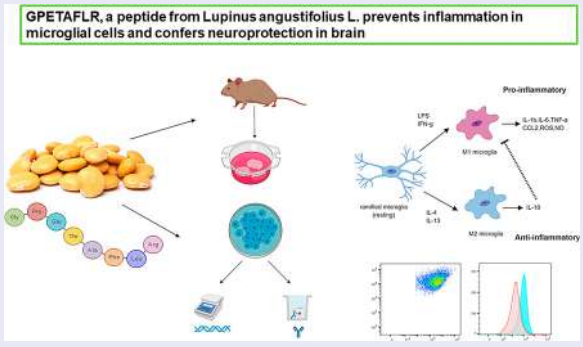
ABSTRACT

Objectives: Neuroinflammation is a complex inflammatory process in the central nervous system (CNS) where microglia may play a critical role. GPETAFLR is a peptide isolated from *Lupinus angustifolius* L. protein hydrolysates with functional activity in mononuclear phagocytes. However, it is unknown whether GPETAFLR has neuroprotective effects.

Methods: We analysed the potential anti-neuroinflammatory activity of GPETAFLR by using two different models of neuroinflammation: BV-2 microglial cells and mice with high-fat diet (HFD)-induced obesity.

Results: GPETAFLR hampered LPS-induced upregulation of pro-inflammatory and M1 marker genes in BV-2 cells. This effect was accompanied by an unchanged expression of anti-inflammatory IL-10 gene and by an increased expression of M2 marker genes. GPETAFLR also increased the transcriptional activity of M2 marker genes, while the microglia population remained unchanged in number and M1/M2 status in brain of mice with high-fat diet (HFD)-induced obesity. Furthermore, GPETAFLR counteracted HFD-induced downregulation of IL-10 and upregulation of pro-inflammatory markers in the mouse brain, both at gene and protein levels.

Discussion: This is the first report describing that a peptide from plant origin robustly restrained the pro-inflammatory activation of microglial cells in cultures and in brain. Our data suggest that GPETAFLR might be instrumental in maintaining CNS homeostasis by inhibiting neuroinflammation.



KEYWORDS

GPETAFLR peptide; BV-2 cell line; neuroinflammation; cytokines; high-fat diet; bioactive; protein hydrolysates; *Lupinus angustifolius*

Introduction

The substitution of animal proteins with plant proteins in the food industry or in our own diet is an important issue for health [1]. Within plant proteins, encrypted peptides of a small amino acid sequence become active when released from the target protein by enzymatic hydrolysis or chemical treatments [2–6]. In previous studies, lupine (*Lupinus angustifolius* L.) protein hydrolysates (LPH) [7] and GPETAFLR (Figure 1) from LPH [8] were shown to exhibit potent anti-inflammatory properties in phorbol ester-stimulated human monocytic THP-1 cells. More recently, GPETAFLR was reported to reduce osteoclastogenic markers during differentiation of human blood monocytes into osteoclasts [9].

Microglial cells, which share a common myeloid origin with osteoclasts, are resident macrophages distributed in all regions of the adulthood brain and spinal cord, and represent between 10 and 15% of the total cells in the central nervous system (CNS) [10]. The principal function of microglia is to destroy and to clear

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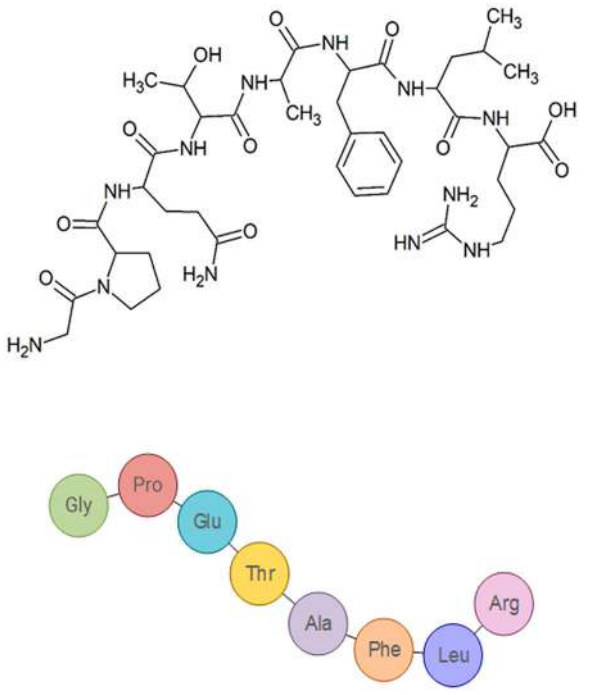

Figure 1. Chemical structure and amino acid sequence scheme of GPETAFLR. The sequence was identified as: Glycine, Proline, Glutamic acid, Threonine, Alanine, Phenylalanine, Leucine, and Arginine.

foreign materials through phagocytic mechanisms [11]. Microglia also contributes to complex dynamics of inflammation and homeostasis by secreting a wide array of cytokines and other signalling molecules, and has a role for antigen-presentation to T cells in the CNS [12]. As for the peripheral macrophage system, two phenotypes of activation in microglial cells in vitro have been proposed: classical M1 and alternative M2 microglial activation states [13,14]. M1 phenotype may produce an excess of inflammatory mediators, including ROS, and pro-inflammatory cytokines and chemokines [15]. On the other hand, M2-activated microglia is believed to generate protective cytokines such as IL-10, IL-4, and IL-13, frequently involved in tissue repair and wound healing [16]. A sustained and not resolved inflammation in the CNS (termed neuroinflammation) is the primary pathological feature of neurodegenerative disorders including Alzheimer's disease, Parkinson's disease, Huntington's disease, tauopathies, age-related macular degeneration, and diseases of autoimmune origin such as multiple sclerosis [17]. Likewise, obesity is well known to be associated with multitude of risk factors that include local and systemic inflammation [18]. Chronic inflammation linked to obesity promotes alterations in circulating levels of cytokines and adipokines as a result of increased production by adipose tissue, liver, and cells of the immune system [19]. Such chronic low-grade inflammation also affects CNS and leads to neuroinflammation [20]. Previous studies have shown that chronic high-fat diet (HFD) may induce hypothalamic inflammation and production of pro-inflammatory cytokines, including tumour necrosis factor (TNF-α), IL-1β, and IL-6 [21], all of which expand inflammation in other brain areas and systemically [22]. However, the role of peptide GPETAFLR in the neuroinflammation field still remains to be elucidated.

Herein, the peptide GPETAFLR was used to investigate its anti-inflammatory activity in LPS-treated microglial BV-2 cells, as alternative model system for primary microglia cultures, and in the brain tissue of mice with HFD-induced obesity.

Materials and methods

All animal protocols received appropriate institutional approval (Animal Care and Use Committee of the University of Seville, AGL2012-40247, July 2017) and were performed according to the official rules formulated in the Spanish law on the care and use of experimental animals (UE Directive of 2010: 2010/63/UE; RD 53/2013).

Isolation, purification, and synthesis of GPETAFLR

Seeds of lupine (*Lupinus angustifolius* L.) were a gift from Koipesol Semillas, S.A. (Seville, Spain). Alcalase 2.4L was provided by Novozymes (Bagsvaerd, Denmark). Lupine protein isolate was obtained using a previously described method [23] at pilot plant scale (Plant Protein Unit, Instituto de la Grasa, Seville, Spain). The chemical composition of lupine protein isolate in dry matter was: protein 86.83%, dietary fibre 5.97%, fat 5.08%, ash 0.78%, and carbohydrate 1.34%. Lupine protein isolate was suspended in distilled water (10% w/v), and hydrolysis with alcalase was performed under the following conditions: pH 8, 50 °C, E/S=0.3 AU/g protein, and a hydrolysis time of 15 min. The mixture was then heated at 85 °C for 15 min to inactivate the enzyme and centrifuged at 6500 × *g* for 15 min, and the supernatant constituted the LPH [11]. This fraction was purified by ultrafiltration and chromatographic techniques [24]. The sequence of the purified peptide was identified through sequence analysis by nanoHPLC coupled to a Polaris Q ion-trap mass spectrometer (Mass Spectrometry Unit, Instituto de la Grasa, Seville, Spain). Finally, the peptide was synthesized by Fmoc solid-phase method (Barcelona Scientific Park Foundation, Barcelona, Spain) at 95% purity, measured by HPLC-UV at 220 nm.

Animal diets and experimental design

Male mice C57BL/6J were obtained from the Animal Production and Experimentation Centre at the University of Seville. At eight weeks old, mice ($n = 40$) were divided into four groups (10 animals per group) to receive for 8 weeks: standard chow diet, HFD, HFD plus GPETAFLR at a dose of 0.5 mg/kg/d, or HFD plus GPETAFLR at a dose of 1 mg/kg/d. GPETAFLR was added to the drinking water. HFD, which contained 60% energy as fat, was prepared by Panlab Laboratories (HF 260 SAFE) and presented as pellets to animals (Table supplementary 1).

Mice were anesthetized by an intraperitoneal injection of ketamine (100 mg/kg) and diazepam (5 mg/kg), and were underwent transcardial perfusion with 0.9% saline prior to harvesting the brain. Brain tissues were removed from the skull and frozen at −80 °C until processing. Homogenization was done with TRIsure (Bioline, Memphis, TN, USA) [25].

Dosage information

Doses of 0.5 and 1 mg/kg/d for GPETAFLR were established according to previous studies [26–31] and to expected water consumption of the C57BL/6J strain [32]. These selected doses for animals were comparable to human equivalent doses (HED) of 40 and 80 µg/kg, respectively [33].

BV-2 cell culture and treatments

BV-2 is a murine cell line used extensively in research related to neurodegenerative disorders [34,35]. BV-2 microglial cells were obtained from the Cell Biology Unit at the Instituto de la Grasa (Seville, Spain). The cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum and 1% penicillin/streptomycin. They were maintained in 5% CO_2 at 37 °C in a CO_2 incubator (Thermo Con Electron Corporation, Waltham, MA, USA). Experiments were done in twelve-well plates. BV-2 microglial cells were seeded at a density of 5×10^5 cells/well. Four different conditions were used: Untreated cells (negative control); cells exposed to LPS at 100 ng/mL (positive control); and cells exposed to LPS at 100 ng/mL + GPETAFLR at 50 or 100 µg/mL. LPS (*E. coli* 055: B5) (Sigma-Aldrich) was added for 1 h and then GPETAFLR. After incubation for an additional period of 24 h, cells were collected for RNA extraction.

Cell viability assay

BV-2 microglial cells were incubated in complete medium with different concentrations (10, 50, 100, 250, and 500 µg/mL) of GPETAFLR in 96-well plates (1×10^5 cells/well) during 24 h. Afterwards, the MTT reagent (Sigma-Aldrich) was added to each well until a purple precipitate was visible. MTT-formazan crystals were solubilized with DMSO (Sigma-Aldrich), and the absorbance was measured at 570 nm corrected to 650 nm with a microplate reader [36]. Cell survival was expressed as the percentage of absorbance compared with that obtained in the negative control, untreated cells.

RNA isolation and quantitative real-time PCR analysis

RNA from BV-2 microglial cells and from frozen brain samples was isolated to quantify gene expression by qRT-PCR. Total RNA was extracted by using TRIsure Reagent (Bioline). RNA quality was assessed by A_{260}/A_{280} ratio in a NanoDrop ND-1000 Spectrophotometer (ThermoFisher Scientific, Madrid, Spain). Briefly, RNA (250 ng) was subjected to reverse transcription (iScript, Bio-Rad). An amount of 40 ng of the resulting cDNA was used as template for qRT-PCR amplifications. The mRNA levels for specific genes were determined in a CFX96 system (Bio-Rad). For each PCR reaction, cDNA template was added to Brilliant SYBR green QPCR Supermix (Bio-Rad) containing the primer pairs for either gene or for hypoxanthine phosphoribosyltransferase (HPRT) as a housekeeping gene. All amplification reactions were performed in triplicate and average threshold cycle (Ct) numbers of the triplicates were used to calculate the relative mRNA expression of candidate genes. The magnitude of change of mRNA expression for candidate genes was calculated by using the standard $2^{-(\Delta\Delta Ct)}$ method. All data were normalized to endogenous reference (HPRT) gene content and expressed as relative fold-change of control. The sequences of the designed oligonucleotides are shown in Table supplementary 2.

Immunostaining analysis by FACS

Brain cells were stained, in the dark and for 15 min, with fluorescent antibodies directed against CD45 (PE-Cy7 anti-mouse CD45, Beckton Dickinson, Madrid, Spain), CD11b (PerCP-Cy5.5 anti-mouse CD11b, BD), F4/80 (APC anti-mouse APC, Miltenyi Biotec, Madrid, Spain), CD80 (FITC anti-mouse FITC, Miltenyi Biotec), and CD200R (PE anti-mouse CD200R, Miltenyi Biotec). Mean fluorescence intensity (MFI) of 10^4 counted cells

was measured in each sample with a flow cytometer FACSCanto II (BD, Palo Alto, CA, USA). Myeloid cells were gated on high side scatter (SSC^{high})-$CD45^+$ cells. The population of microglial cells was then gated as $CD11b^+F4/80^+$ [37]. Pro-inflammatory M1-like and anti-inflammatory M2-like subsets were further gated as $CD80^+$ and $CD200R^+$ microglial cells, respectively. Expression levels were presented as MFI corrected for non-specific binding of isotype control antibodies.

Measurement of cytokine release

The levels of IL-10 and IL-6 in brain tissue homogenate supernatants were measured by ELISA, following the indications of the manufacturer (Diaclone, Besancon, France). Cytokine concentrations were expressed as pg/mL, according to calibration curves from serial dilution of mice recombinant standards in each assay.

Statistical analysis

All values in the figures and text are expressed as the arithmetic mean ± SD. All experiments were carried out in triplicate and repeated at least three times independently, except for the whole animal experiments. Data were evaluated with GraphPad Prism Version 7.0 software (San Diego, CA, USA). The statistical significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance (ANOVA), using Tukey's test for multiple comparison analysis. The statistics details (P values, F values, and degrees of freedom) were listed in Table supplementary 3. P values of < 0.05 were considered statistically significant.

Results

GPETAFLR on BV-2 microglial cell viability

BV-2 microglial cells were incubated with GPETAFLR at concentrations up to 500 µg/mL for 24 h. The cell viability evaluated by the MTT method was not affected by any GPETAFLR concentration (Figure 2).

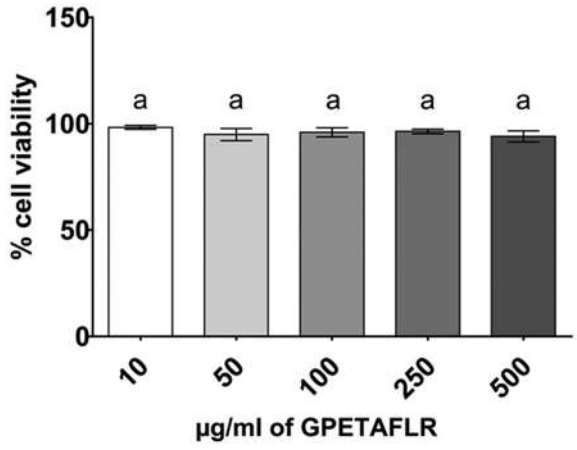

Figure 2. Effect of GPETAFLR on viability of BV-2 microglial cells. Cells were treated with GPETAFLR (10–500 µg/mL) in complete medium for 24 h. According to MTT assay, cell viability was expressed as percentage of absorbance relative to control (untreated) cells. Experiments were carried out in triplicate, at least three independent times. Values are presented as means ± SD and those marked with different letters are significantly different ($P < 0.05$), as determined through one-way ANOVA, followed by a post hoc Tukey test.

GPETAFLR on cytokine gene expression in BV-2 microglial cells

Gene expression of cytokines TNF-α, IL-1β, IL-6, and IL-10 was measured by qRT-PCR in BV-2 microglial cells. As expected, LPS increased mRNA levels of TNF-α, IL-1β, and IL-6 genes (Figure 3A, 3B, and 3C). However, these LPS-induced changes were blocked in the presence of GPETAFLR at 50 or 100 µg/mL. Both doses of GPETAFLR were effective to keep the expression levels of IL-6 gene close to those found in untreated cells. GPETAFLR also showed a statistically significant dose-dependent effect on IL-1β gene expression. Furthermore, LPS induced a decrease in mRNA levels of IL-10 gene that was blocked by GPETAFLR (Figure 3D).

GPETAFLR on plasticity in BV-2 microglial cells

Most tissue macrophages, including microglial cells, are highly plastic cells as they can polarize by exhibiting some of the characteristics of M1- and M2-like phenotypes upon appropriate stimulus in vitro [38–40]. To observe polarization of BV-2 microglial cells after LPS in the absence or presence of GPETAFLR at 50 µg/mL or 100 µg/mL, we extracted mRNA and performed qRT-PCR. LPS increased mRNA levels of M1-like gene markers (Ccr7, Figure 4A and iNOS, Figure 4B), but decreased mRNA levels of M2-like gene markers (Arg-1, Figure 4C and Ym-1, Figure 4D) in BV-2 microglial cells. Interestingly, the lowest dose of GPETAFLR completely suppressed the transcriptional activity of Ccr7 and iNOS genes. GPETAFLR also blocked LPS-induced decreased expression of Arg-1 and Ym-1 genes.

GPETAFLR on plasticity in microglia of brain from mice with HFD-induced obesity

Mean daily food intake (standard chow diet, 2.92 ± 0.62; HFD, 2.35 ± 0.24; HFD plus GPETAFLR 0.5 mg/kg/d,

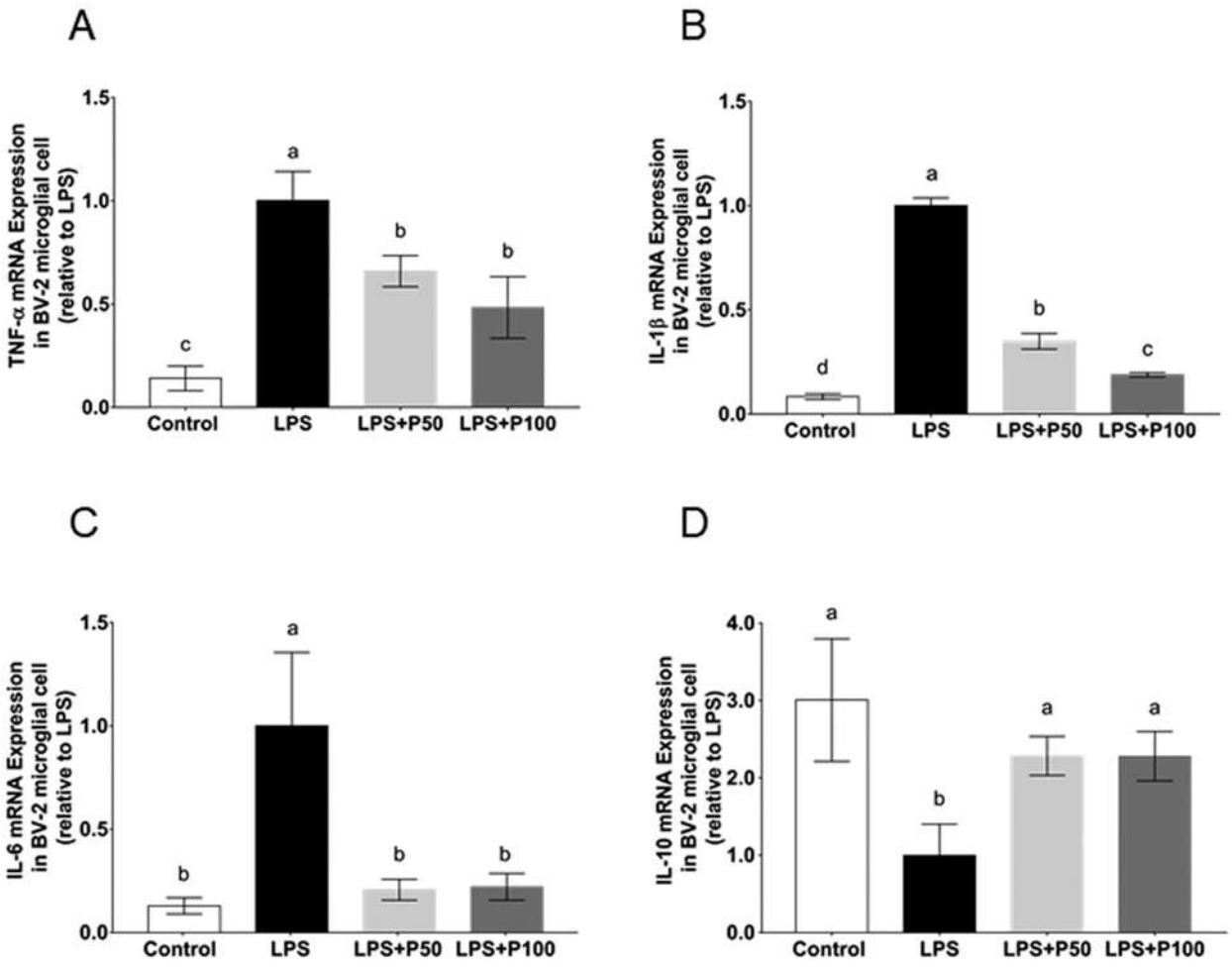

Figure 3. Effect of GPETAFLR on expression of pro-inflammatory and anti-inflammatory cytokine genes in LPS-treated BV-2 microglial cells. The mRNA levels of TNF-α (A), IL-1β (B), IL-6 (C), and IL-10 (D) genes were measured after the treatment of cells with LPS (100 ng/mL) in the absence or presence of GPETAFLR at 50 (P50) and 100 (P100) μg/mL. Control means untreated cells. Experiments were carried out in triplicate, at least three independent times. Values are presented as means ± SD and those marked with different letters are significantly different ($P < 0.05$), as determined through one-way ANOVA, followed by a post hoc Tukey test.

2.47 ± 0.25; HFD plus GPETAFLR 1 mg/kg/d, 2.37 ± 0.27 g/mouse) and water intake (standard chow diet, 3.00 ± 0.89; HFD, 2.95 ± 0.92; HFD plus GPETAFLR 0.5 mg/kg/d, 3.23 ± 0.78; HFD plus GPETAFLR 1 mg/kg/d, 4.02 ± 1.37 mL/mouse) did not statistically differ among the four diet groups. However, mean energy daily intake per mouse was lower ($P < 0.05$) with the standard chow diet (10.33 ± 2.19 kcal) than with HFD in the absence (12.94 ± 1.32 kcal) or presence of GPETAFLR at 0.5 mg/kg/d (13.60 ± 1.38 kcal) and at 1 mg/kg/d (13.05 ± 1.49 kcal).

To study plasticity of microglia as a consequence of an HFD-induced obesity in brain of mice during administration of GPETAFLR, we extracted mRNA and performed qRT-PCR as well as obtained primary microglia cells and used FACS to check their surface markers. As shown in Figure 5A, GPETAFLR at a dose of 1 mg/kg/d was able to decrease mRNA levels of Ccr7 gene as a marker for M1-like phenotype in the brain of mice on HFD, even to levels lower than those seen in mice fed with the standard chow diet. This effect was not evident for the iNOS gene (Figure 5B). It was noteworthy that GPETAFLR at a dose of 1 mg/kg/d also increased mRNA levels of Arg-1 (Figure 5C) and Ym-1 (Figure 5D) genes as markers for M2-like phenotype in the same brains. This effect of GPETAFLR on Ym-1 gene expression was dose-dependent.

Using FACS, the microglia population from mouse brain was gated as $CD45^{high}CD11b^{+}F4/80^{+}$ cells (Figure 6A). The expression of microglial activation markers CD80 for M1-like phenotype and CD200R for M2-like phenotype was then determined. The HFD-induced increase of total microglial population in the brain was

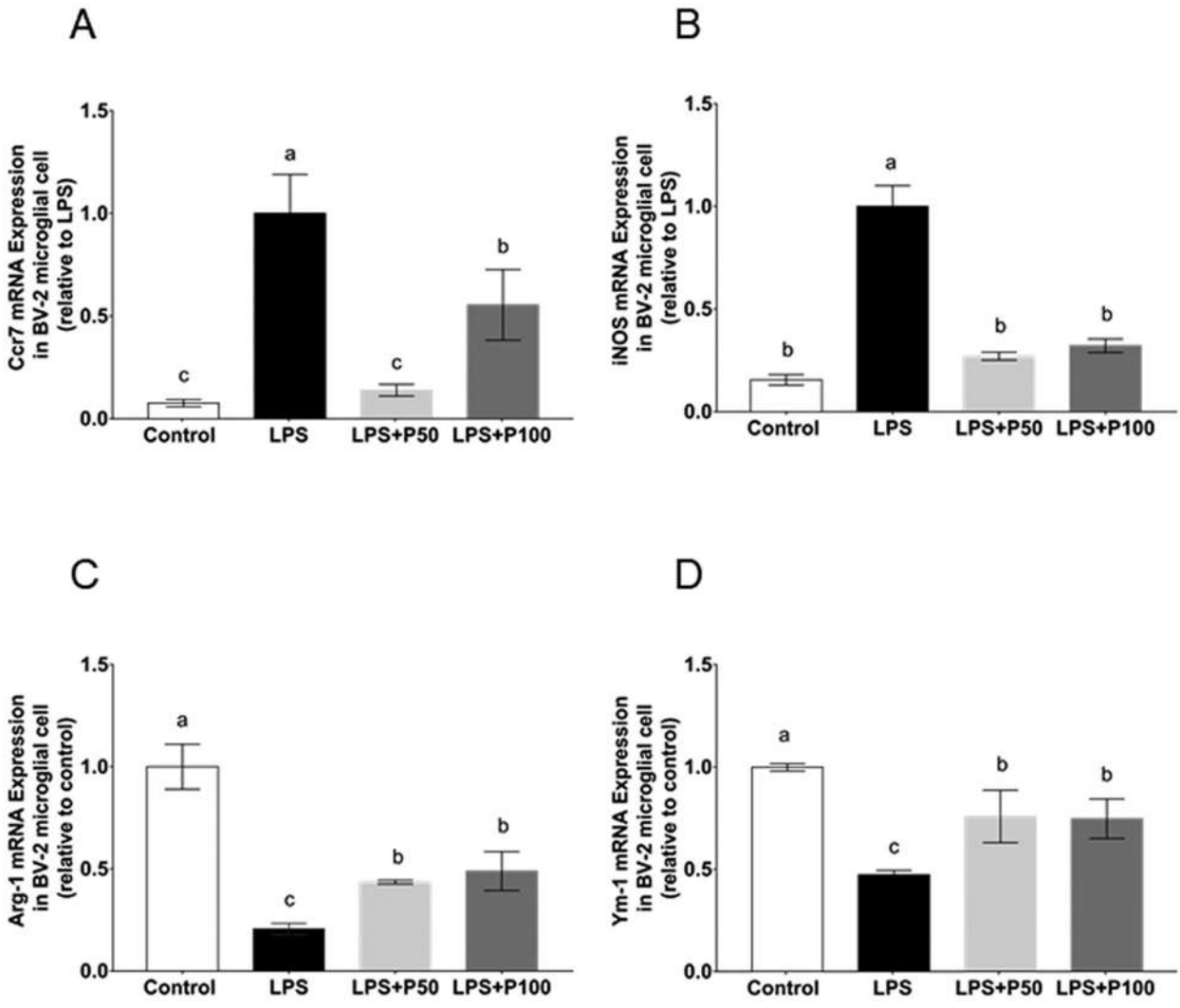

Figure 4. Effect of GPETAFLR on expression of M1 and M2 marker genes in LPS-treated BV-2 microglial cells. The mRNA levels of CCR7 (A), iNOS (B), Arg-1 (C), and Ym-1 (D) genes were measured after the treatment of cells with LPS (100 ng/mL) in the absence or presence of GPETAFLR at 50 (P50) and 100 (P100) µg/mL. Control means untreated cells. Experiments were carried out in triplicate, at least three independent times. Values are presented as means ± SD ($n = 3$) and those marked with different letters are significantly different ($P < 0.05$), as determined through one-way ANOVA, followed by a post hoc Tukey test.

completely abrogated by GPETAFLR (Figure 6B). No differences were observed on microglial expansion in animals fed with the HFD that received GPETAFLR at a dose of 0.5 mg/kg/d in drinking water compared to animals fed with the standard chow diet. Similar expression patterns were seen for CD80, the brain of mice on HFD doubling the number of $CD80^+$ microglial cells found in the brain of mice on HFD plus GPETAFLR or on standard chow diet (Figure 6C). The HFD-induced decrease of $CD200R^+$ in the brain was also completely abrogated by GPETAFLR (Figure 6D), with only the half of $CD200R^+$ microglial cells in the brain of mice fed on HFD regarding those found in the brain of mice on HFD plus GPETAFLR or on standard chow diet.

GPETAFLR on cytokine gene and protein expression in brain from mice with HFD-Induced obesity

Gene expression of cytokines TNF-α, IL-1β, IL-6, and IL-10 was measured by qRT-PCR in brain of mice with HFD-induced obesity during administration of GPETAFLR. The protein level of IL-6 and IL-10 was also measured by ELISA in the supernatant of brain tissue homogenates. GPETAFLR protected in a dose-dependent fashion from the HFD-induced mRNA levels of TNF-α gene in brain (Figure 7A). GPETAFLR at a dose of 1 mg/kg/d also decreased the expression of IL-1β gene (Figure 7B). Furthermore, the expression of IL-6 gene was dose-dependently repressed by GPETAFLR, reaching levels below those detected in brain of mice on standard chow diet

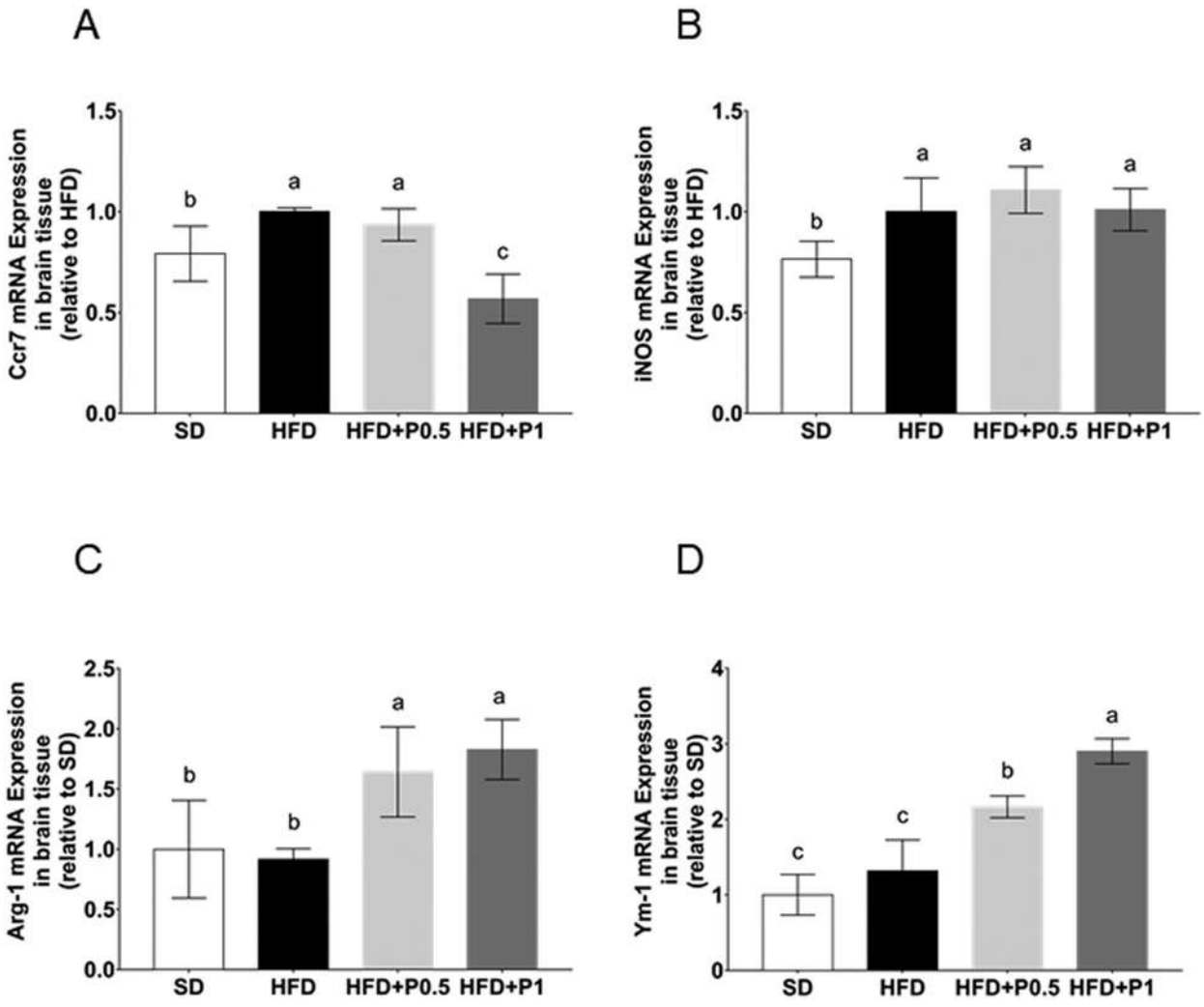

Figure 5. Effect of GPETAFLR on expression of M1 and M2 marker genes in brain of mice with HFD-induced obesity. The mRNA levels of CCR7 (A), iNOS (B), Arg-1 (C), and Ym-1 (D) genes were measured in brain of mice fed with a standard chow diet (SD), an HFD or an HFD + GPETAFLR at a dose of 0.5 mg/kg/d (HFD + P0.5) and 1 mg/kg/d (HFD + P1) in drinking water. Ten animals were used for analysis, and each sample was subjected to triplicate analyses. Values are presented as means ± SD and those marked with different letters are significantly different ($P < 0.05$), as determined through one-way ANOVA, followed by a post hoc Tukey test.

(Figure 7C). Despite HFD reduced the expression of IL-10 gene in brain, co-administration with GPETAFLR counteracted this effect (Figure 7D). In addition, GPETAFLR protected from the HFD-induced increase of IL-6 (Figure 7E) and reduction of IL-10 (Figure 7F) in brain tissue homogenates.

Discussion

There is growing interest in obtaining biologically and functionally active peptide sequences that are encrypted inside native food proteins [41,42]. In previous studies, we reported that LPH [7] and GPETAFLR derived therefrom [8] can inhibit LPS-induced inflammatory response in human monocytic THP-1 cells. GPETAFLR was also shown to exert anti-osteoclastogenic activity in human blood monocyte-derived osteoclasts [9]. Herein, we now report that GPETAFLR has the ability to prevent the pro-inflammatory activation of microglial cells in cultures and in brain of mice with HFD-induced obesity. Although it remains to be determined whether GPETAFLR is capable of reversing the inflammatory profile once obesity has been achieved.

Microglia, the set of professional phagocytes in the brain, usually exists in a quiescent or resting state, continuously monitoring their microenvironment, and can be activated in response to surrounding stimuli that could perturb the CNS homeostasis [43]. Upon a complex multistage activation, microglial cells become strong producers of pro-inflammatory mediators that include a broad spectrum of cytokines involved in degeneration or trauma of neuronal structures, dysfunction, and death

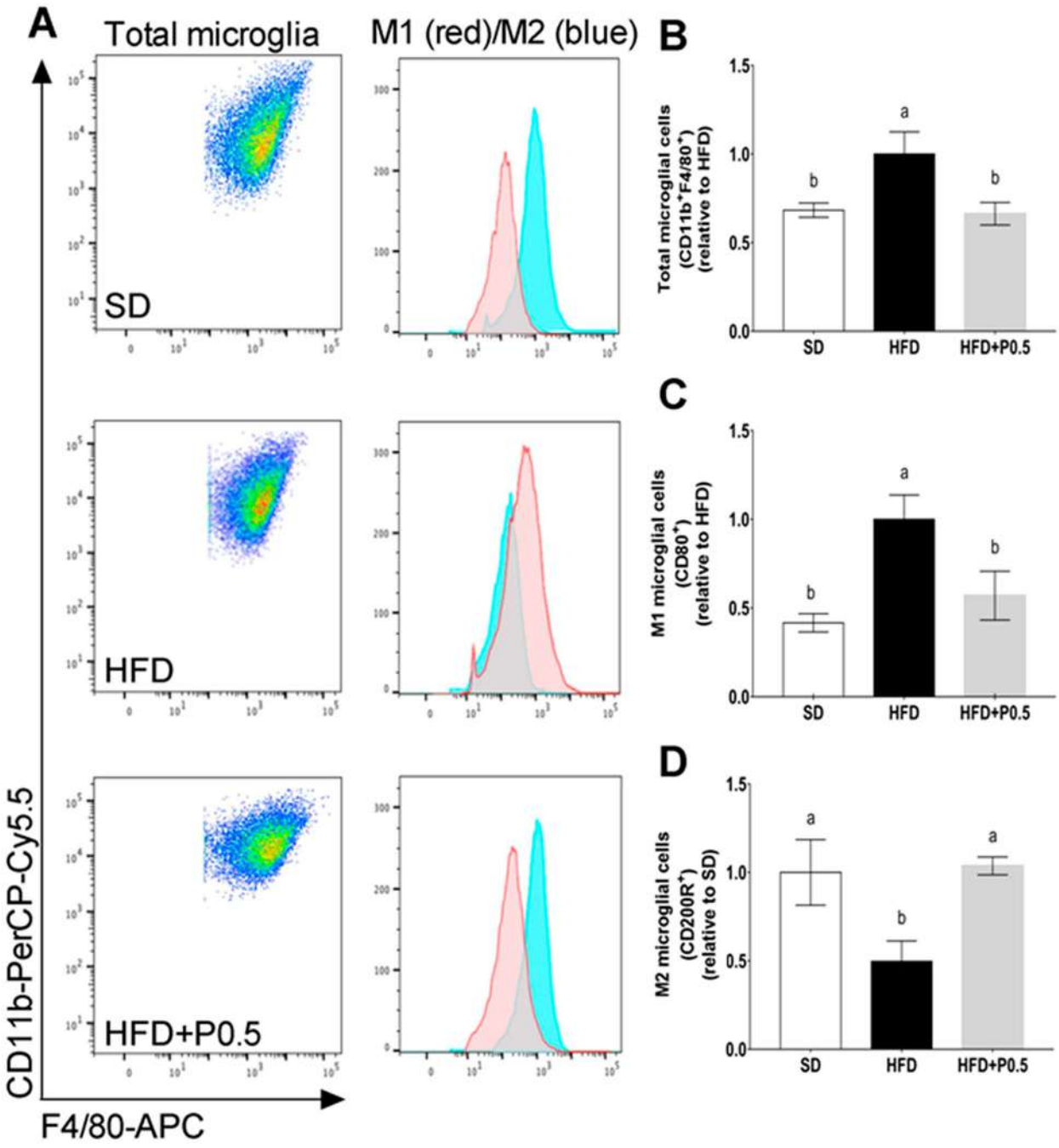

Figure 6. Representative dot plots showing the gating strategy for identification of microglia populations in brain tissue of mice with HFD-induced obesity. Brain cells of mice fed with a standard chow diet (SD), an HFD or an HFD + GPETAFLR at a dose of 0.5 mg/kg/d (HFD + P0.5) in drinking water were stained with multiple fluorescently conjugated antibodies directed to CD45, CD11b, F4/80, CD80, and CD200R. A region was created on a bivariate plot of CD45 vs SSC-A to select $CD45^{high}CD11b^{+}F4/80^{+}$ microglial cells (A). M1 and M2 subsets were quantified from histograms in total microglial cells (B) and in $CD80^{+}$ (C) and $CD200R^{+}$ (D) cells, respectively. Ten animals were used for analysis, and each sample was subjected to triplicate analyses. Values are presented as means ± SD and those marked with different letters are significantly different ($P < 0.05$), as determined through one-way ANOVA, followed by a post hoc Tukey test.

[44]. Under prolonged microglial pro-inflammatory activation, the neuroinflammation turns into a driving force in the development of various neurodegenerative disorders [17,37,45]. In the process of developing new strategies to modulate the inflammatory response in the brain, a number of phytochemicals, mainly phenolic compounds, have been shown to target neuroinflammation in Alzheimer's disease due to their anti-oxidant and anti-inflammatory activities [46]; however, some of the benefits of these natural products were further dependent on their poor bioavailability or activity when metabolically transformed into conjugates, apart from disease

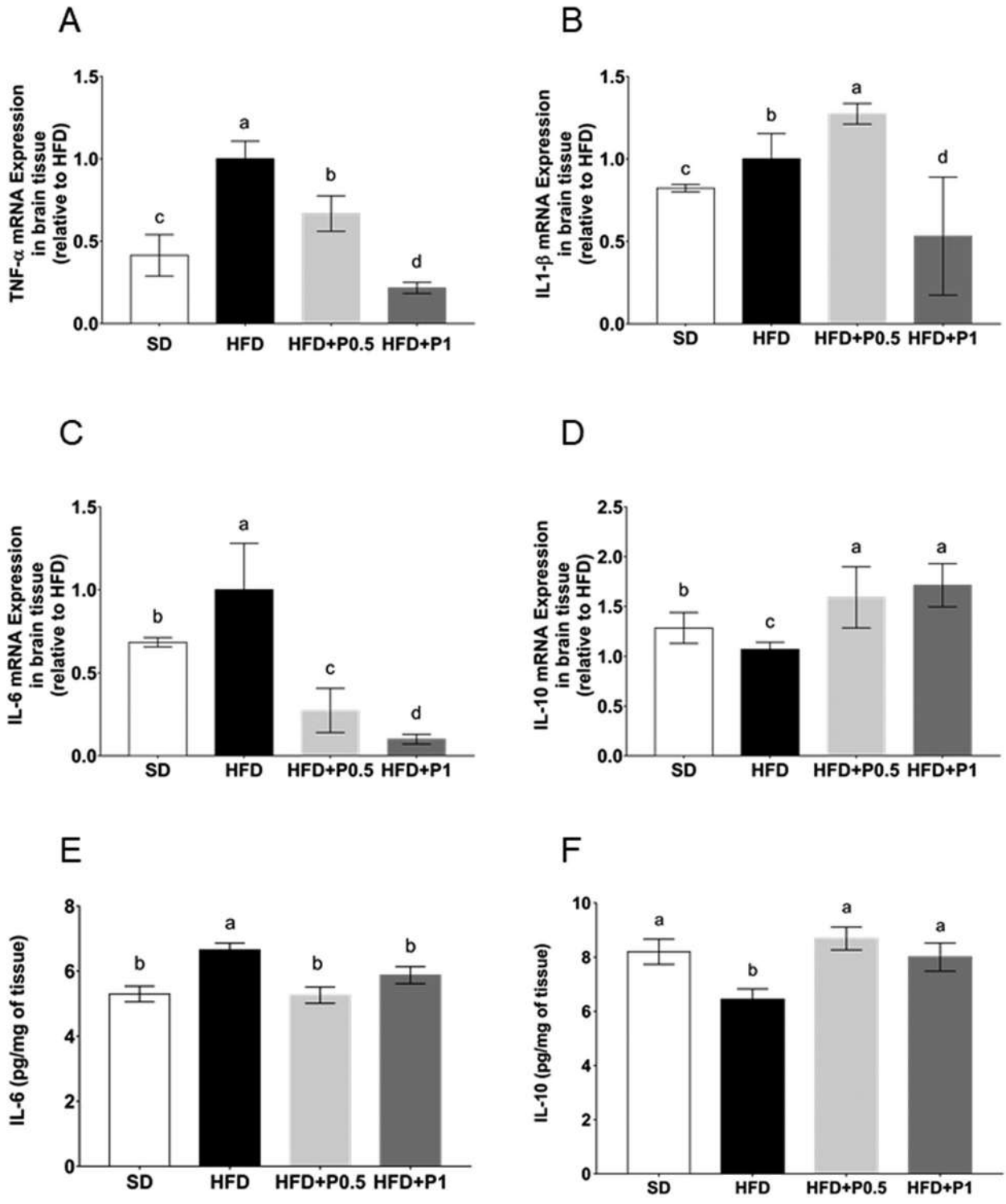

Figure 7. Effect of GPETAFLR on expression of pro-inflammatory and anti-inflammatory cytokine genes and proteins in brain of mice with HFD-induced obesity. The mRNA levels of TNF-α (A), IL-1β (B), IL-6 (C), and IL-10 (D) genes, and the amount of IL-6 (E) and IL-10 (F) were measured in brain of mice fed with a standard chow diet (SD), an HFD or an HFD + GPETAFLR at a dose of 0.5 mg/kg/d (HFD + P0.5) and 1 mg/kg/d (HFD + P1) in drinking water. Ten animals were used for analysis, and each sample was subjected to triplicate analyses. Values are presented as means ± SD and those marked with different letters are significantly different ($P < 0.05$), as determined through one-way ANOVA, followed by a post hoc Tukey test.

stage and treatment of duration. One promising attempt for an improved and safety therapy of neurodegenerative diseases by naturally occurring compounds has been recently reported with the soy-derived peptide lunasin [47]. Lunasin was able to rescue Aβ42 mediated neurodegeneration by blocking cell death in retinal neurons,

and resulted in restoration of axonal targeting from retina to brain. Inspired in part by these studies and in our own findings on anti-inflammatory activity of GPETAFLR in mononuclear phagocytes, we envisioned that GPETAFLR might be an ideal candidate to test its efficacy in mitigating a neuroinflammatory environment by using both in vitro and in vivo models. To date there is little if any studies on the impact of a peptide from plant origin on microglial activation.

In the current study, we found that GPETAFLR has significant anti-inflammatory effects by markedly blocking the transcriptional activity of TNF-α, IL-1β, and IL-6 genes in LPS-treated BV-2 microglial cells. Importantly, IL-1β and IL-6 gene expression levels remained unchanged during LPS stimulation if GPETAFLR was added to the cultures. Given the robust relevance of these pro-inflammatory cytokines for microglia status [48] and of LPS as a potent trigger of neuroinflammation [49], our findings suggest that GPETAFLR may protect against pro-inflammatory activation of microglia, remaining intact its physiological capacity or resilience to an ex vivo LPS challenge. The improvement of the molecular repertoire to stop the inflammatory phase of activated microglial cells, which was supported by the abrogation of LPS-induced decrease in the expression of anti-inflammatory IL-10 gene upon addition of GPETAFLR to the cultures, may also establish that GPETAFLR could have benefits on microglial function. Even though more research is needed to gain further insights into this possibility, IL-10 has been shown to be produced by brain cell types other than microglia, having the property to inhibit the release or the effects of pro-inflammatory cytokines and to promote survival of neurons and neuronal homeostasis [50]; other molecules enable the healing and functional recovery of neuron-glial interactions, such as resolvins, protectins, and maresins [51].

Microglial plasticity in vitro depends on different microenvironments and signals that promote specific phenotypes, which may correspond to a classically (M1) or an alternatively (M2) activation state [52]. However, this paradigm of microglial polarization may represent an oversimplified model of microglial states in vivo [53,54] and should be carefully managed. M1-like activated microglia has been proposed to secrete pro-inflammatory cytokines, whereas M2-like activated microglia to be involved in reconstructing damaged neuron networks by the removal of toxic aggregates, the upregulation of neurotrophic factors and anti-inflammatory cytokines, and the downregulation of pro-inflammatory cytokines [55]. Therefore, in a context of sustained microglial M1 status, the M1-to-M2 microglial switch could protect the brain from inflammation [56]. We used two independent strategies to test if GPETAFLR was able to influence on microglial plasticity. Firstly, our study found that GPETAFLR suppresses the transcriptional activity of M1 genes and promotes that of M2 genes in LPS-treated BV-2 microglial cells. Secondly, we observed that GPETAFLR not only to some extent reduces the transcriptional activity of genes but also markedly attenuates the expression of surface marker proteins corresponding to canonical M1-like activation in brain microglia of mice fed on HFD. Furthermore, the expression levels of M2 marker genes in brain and of M2 marker proteins in brain microglial cells are increased by GPETAFLR. Collectively, together with the analysis of the balance between brain gene and protein expression patterns of pro-inflammatory and anti-inflammatory cytokines, this study demonstrates both in vitro and in vivo that GPETAFLR may improve the subsets ratio of M1/M2 microglial cells under conditions of inflammatory insult. Obesity can induce neuroinflammation [57] and can produce a chronic damage in the CNS [58]. HFD feeding is also associated with the pro-inflammatory activation of microglial cells and the increase of inflammatory tone in the CNS [19,59]. Hence, our findings are consistent with the notion that GPETAFLR could be a bioactive peptide against neuroinflammation and thereby in maintaining CNS homeostasis. Interestingly, our in vivo data showed that this activity remains following gastrointestinal transition of GPETAFLR. In a recent study, the dipeptide Gly-Arg (GR) from soybean protein hydrolysates was shown to induce neurotrophins in astrocyte cultures and neurogenesis in mouse brain after oral or intraperitoneal administration for a period of 2 weeks [60]. Strikingly, the amino acid sequence of GR is coincident with amino acids on ends of our peptide. Whether these positions occupied by Gly and Arg can represent structural requirements for anti-inflammatory activity of GPETAFLR in microglial cells should be further explored in the future.

To the best of our knowledge, this is the first report describing that a peptide from plant origin robustly restrained the pro-inflammatory activation of microglial cells in cultures and in brain. Our data suggest that GPETAFLR might be instrumental in maintaining CNS homeostasis by inhibiting neuroinflammation.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

Ethics approval

All animal protocols received appropriate institutional approval (Animal Care and Use Committee of the University of Seville, AGL2012-40247-C02) and were performed according to the official rules formulated in the Spanish law on the care and use of experimental animals (UE Directive of 2010: 2010/63/UE; RD 53/2013).

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