TITLE: LUNASIN PEPTIDE IS A MODULATOR OF THE IMMUNE RESPONSE IN THE HUMAN GASTROINTESTINAL TRACT

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Abbreviations

conventional dendritic cells (cDC); cyclooxygenase (COX); healthy controls (HC); inflammatory bowel diseases (IBD); interleukins (IL); lamina propria mononuclear cells (LPMC); macrophages (Mϕ); nitric oxide (NO); nuclear factor-kappa B (NF-κB); tumor necrosis factor-α (TNF-α)

Keywords

antigen presenting cells; bioactive peptides; immunomodulation; intestinal inflammation; lunasin

Abstract

Scope: Lunasin is a soybean bioactive peptide with a variety of beneficial properties against chronic disorders. However, its effect in human primary intestinal cells remains unknown. Hence, we aimed to characterize its *ex vivo* biological activity in the human intestinal mucosa.

Methods and results: Human intestinal biopsies, obtained from healthy controls, were *ex vivo* conditioned with lunasin both in the presence/absence of lipopolysaccharide (LPS). Peptide maintained its stability during biopsy culture by HPLC-MS/MS analysis. Lunasin was bioactive in the human mucosa as it induced *IL-1β, TNF-α, IL-17A, CCL2,* and *PGE2/COX-2* gene expression together with an increased expression of tolerogenic *IL-10* and *TGFβ* while it also downregulated the expression of *iNOS* and subunit *p65* from *NF-κB*. Indeed, lunasin also abrogated the LPS-induced pro-inflammatory response, downregulating *IL-17A*, *IFNγ,* and *IL-8* expression, and inducing *IL-10* and *TGFβ* expression. These results were also mirrored in the cell-free culture supernatants at the protein level by Multiplex. Moreover, lunasin further induced a regulatory phenotype and function on human intestinal conventional dendritic cell and macrophage subsets as assessed by flow cytometry.

Conclusions: We hereby have characterized lunasin as an immunomodulatory peptide with potential capacity to prevent immune and inflammatory-mediated disorders in the human gastrointestinal tract.

1. INTRODUCTION

The gastrointestinal tract represents the main interface between dietary compounds and the organism. Beyond its essential role at processing foods and absorbing both water and nutrients, it also provides a multifaceted barrier against luminal substances. Hence, it harbours numerous receptors and secretes several regulatory signals which, together, act as modulators of innate and adaptive immune responses.[1] Therefore, maintenance of the gut health is a key element not only for the prevention of gastrointestinal disorders but also to maintain the general health status.[2,3] Dietary compounds are a source of luminal antigens that can modulate both the gut microbiota and the mucosal immune system, and have therefore a direct impact on gut health.[4,5] Among food compounds, bioactive peptides have demonstrated a variety of biological functions in the organism with an increasing interest in the last years towards their effect at gastrointestinal level.[6,7]

Lunasin peptide corresponds to a small sub-unit of 43-amino acids encoded within soybean 2S albumin. Since its discovery in 1997, the scientific interest on this peptide has continuously grown, and it has become one of the natural peptides with higher potential against chronic disorders.[8] The main biological effects proved for lunasin are antiproliferative, antioxidant, anti-inflammatory, hypocholesterolemic, and modulatory properties over the nervous, cardiovascular and immune systems.[8–10] Some of its preventive properties have been described in the gastrointestinal system. Hence, lunasin was able to inhibit the proliferation of different colon cancer cell lines including HT-29, HCT-116, KM12L4, and RKO cells,[11–14] and counteract the induction of oxidative stress in chemically-challenged hepatocytes HepG2[15] and intestinal Caco-2 cells[16]. Its

chemopreventive and antioxidant potential has been also linked to inhibitory properties over inflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukins (IL), and nitric oxide (NO) in LPS-activated RAW 264.7 murine macrophages.[17–20] Indeed, it has been recently suggested that a lunasin-enriched soybean extract may reduce the histopathological inflammatory index and the expression of cyclooxygenase (COX)-2 in murine models of dextran sodium sulfate induced-colitis.[21]

Although most of the current research on lunasin has been carried out in cell lines or animal models,[8] the biological relevance of the peptide in the human setting remains elusive. In this context, lunasin inhibited cell proliferation, induced cell cycle arrest and apoptosis, and suppressed nuclear factor-kappa B (NF-κB) activation and the subsequent downstream production of IL-6, IL-8, and matrix metalloproteinase-3 in IL-1β-activated synovial fibroblasts isolated from patients with rheumatoid arthritis.[22] However, the effect of lunasin in human primary intestinal cells is currently unknown. Therefore, we here aimed to investigate, for the first time to our knowledge, the *ex vivo* biological activity of lunasin in the human intestinal mucosa. To that end, we used intestinal biopsies from healthy controls (HC) which were overnight cultured with synthetic lunasin in resting conditions and in the presence of pro-inflammatory LPS in order to assess the effects of the peptide over the mucosal relative gene expression and the cytokine milieu. Moreover, we also studied the immunomodulatory capacity of lunasin over the phenotype and function of gut antigen presenting cells (macrophages –Mϕ– and conventional dendritic cells –cDC–) to provide a mechanistic basis for the bioactivity of lunasin in the human intestinal mucosa.

2. EXPERIMENTAL SECTION

2.1. Patients and biological samples

Intestinal biopsies were obtained during colonoscopy from a total of 18 HC without autoimmune disease or malignancy. HC were referred due to changes in bowel transit, colorectal cancer screening or rectal bleeding. In all cases, they showed macroscopically and histologically normal mucosa. Demographics of all HC [38.9% males; 55.7 ± 15.4 years (mean ± standard deviation); 30-73 age interval] used in each experiment are shown in Supporting Information Table S1. Samples were obtained following written informed consent after ethics approval from the Ethics Committee at La Princesa Hospital (Madrid, Spain) (SFT_Lunasin-2017).

Lunasin peptide (SKWQHQQDSCRKQLQGVNLTPCEKHIMEKIQGRGDDDDDDDDD) was synthesized by the conventional Fmoc solid-phase synthesis method by Chengdu KaiJie Biopharm Co., Ltd. (Chengdu, Sichuan, China). The purity was verified by analytical HPLC-MS through peptide peak area integration.

2.2. Biopsy processing and culture

Intestinal biopsies from HC (*n* = 10) were obtained during colonoscopy, immediately preserved in ice-chilled complete medium [Dutch modified RPMI 1640 (Sigma-Aldrich, Dorset, UK) containing 100 µg/mL penicillin/streptomycin, 2mM L-glutamine, 50 µg/mL gentamicine (Sigma-Aldrich) and 10% foetal calf serum (TCS cellworks, Buckingham, UK)] and processed within 30 min.[23] Biopsies were cultured [1 biopsy per 1 mL of complete medium per well in 24-well culture plates (Corning Inc., Corning, NY, USA)] with/out peptide lunasin (5, 50, and 200 µM) both in the presence or absence of LPS (100 ng/mL,

Sigma-Aldrich), as well stablished pro-inflammatory stimuli,[24] for 18 h at 37 °C. Peptide concentration was established based on previous studies showing no cell toxicity on this range.[20,22,25,26] After culture, medium was harvested, centrifuged, and the cell-free culture supernatants (biopsy secretomes) were immediately cryopreserved at -80 °C until analysis. Biopsies were withdrawn and immediately stored in RNAlater® (Applied Biosystems, Carlsbad, CA, USA), according to manufacturer's instructions.

The presence and integrity of peptide during the biopsy culture was evaluated by HPLC-MS/MS as previously described.[25] Briefly, biopsies were conditioned with lunasin (10 µM) in the absence/presence of LPS (100 ng/mL). Supernatant aliquots were taken immediately after adding the peptide to the culture as well as after 18 h. Experiments were performed in duplicate and two independent injections were analyzed for each sample. The area under the curve of the extracted molecular ions of lunasin and their sodium and potassium adducts, when formed, were measured. HPLC-MS/MS analysis showed that lunasin maintained its stability during biopsy culture up to 90-95% hence discarding its degradation in our culture system in the presence of human intestinal mucosa.

2.3. Biopsy gene expression

RNAlater®-preserved biopsies were lysed by Ultraturrax (IKA-Werke, Staufen, Germany). Total RNA was extracted by using NucleoSpin RNA Kit (Macherey-Nagel Gmbh & Co., Düren, Germany). RNA concentration and purity were determined by NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesized using PrimeScript RT reagent kit (TaKaRa Bio Inc., Shiga, Japan). Quantitative

PCR amplification was performed in a real-time thermocycler (ViiA™ 7 Real-Time PCR System, Applied Biosystems, Foster, USA) in 384 wells microplates with SYBR Premix ExTaq II (TaKaRa Bio Inc.). cDNA samples were evaluated in triplicate and controls were included to confirm the absence of primer dimer formation (no-template control). All real-time PCR assays amplified a single product as determined by melting curve analysis. All primers used in this study were selected from bibliography or home designed. Primer pairs, optimized conditions of thermal cycling and efficiency obtained for each gene are summarized in Supporting Information Tables S2 and S3. Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method[27] normalizing data to the expression of the *GADPH* gene.

2.4. Intestinal cytokine milieu

Cytokine analysis of biopsy secretomes was performed using the Human Inflammation Panel (LEGENDplex™, BioLegend, San Diego, CA, USA) following the manufacturer's recommendations. This panel allows the simultaneous quantification of 13 human cytokines/chemokines, including IL-1β, IFN-α2, IFN-γ, TNF-α, chemokine (C-C) motif ligand 2 (CCL-2), IL-6, IL-8 (chemokine (C-X-C) motif ligand 8), IL-10, IL-12p70, IL-17A, IL-18, IL-23, and IL-33, based on fluorescence-encoded beads suitable for flow cytometry. Multiplex immunoassay was performed as previously described.[23] Samples were acquired on a BD FACSCanto™ II flow cytometer (BD Biosciences) and analyzed using the Biolegend´s LEGENDplex™ Data Analysis Software (version 8.0). IL-8 and IL-17A were over the detection limit while IL-12p70 was below the lower threshold in all the analyzed samples, thus they were excluded from the analysis.

2.5. Lamina propria mononuclear cells culture

Intestinal biopsies from HC (*n* = 6) were processed to obtain intestinal lamina propria mononuclear cells (LPMC) as previously described[28]. Briefly, LPMC were obtained following two incubations (30 min each) of biopsies with Hanks balanced salt solution (Gibco BRL, Paisley, Scotland, UK) containing 1 mM DTT and 1 mM EDTA solutions to remove the associated mucus/bacteria and epithelial layer, respectively. Biopsies were further digested in the presence of 1 mg/mL of collagenase D, 20 µg/mL of liberase (Roche Diagnostics Ltd, Lewes, UK), and 25 U/mL of DNAse (Pierce Universal Nuclease for Cell lysis, Thermo Fisher Scientific). LPMC were subsequently passed through a 100 µm cell strainer and collected by centrifugation. LPMC were further cultured [2×10^6 cells in 1 mL of complete medium per tube in polystyrene test tubes (Corning Inc.)] in the absence/presence of LPS (100 ng/mL) with/out lunasin (200 µM). Following culture, LPMC were washed with PBS containing 1 mM EDTA and 0.02% sodium azide (FACS buffer) and stained with fluorochrome-conjugated antibodies, as detailed below.

2.6. Antibody labelling and flow cytometry

LPMC were stained with monoclonal antibodies and characterized by flow cytometry. The specificity, clone, conjugate, and manufacturer of all the anti-human monoclonal antibodies used in this study are shown in Supporting Information Table S4. In all cases, a Live/Dead™ fixable near-IR dead cell stain kit (Molecular Probes, Thermo Fisher Scientific) was added to the cells prior to performing antibody staining, hence allowing the exclusion of dead cells from the analysis. LPMC were labelled in FACS buffer in ice and in the dark for 20 min following Fc block incubation (Becton Dickinson). For the assessment of intracellular cytokines, LPMC were permeabilized (Leucoperm, Abd

Secrotec, Oxford, UK) following surface staining and stained with intracellular antibodies. LPMC were further washed in FACS buffer, fixed with 2% paraformaldehyde in FACS buffer on ice in the dark for 10 min, and washed again in FACS buffer before they were stored at 4 °C prior to acquisition on the flow cytometer.

LPMC were acquired on a BD LSR-Fortessa™ II flow cytometer (BD Biosciences). All cells were analyzed within the singlet viable fraction. Positive and negative gatings were set by the fluorescence minus one method. The results were analyzed using FlowJo (version 10.1) (Flowjo LLC, Ashland, OR, USA).

2.7. Statistical analysis

All statistical analyses were performed by using GraphPad Prism 6.01 software (San Diego, CA, USA). The significance of differences was analyzed by one-way ANOVA with subsequent Tukey correction or Mann-Whitney test, as detailed in the figure legends. Differences with a p-value < 0.05 were considered statistically significant.

3. RESULTS

3.1. Effects of lunasin over human intestinal gene expression

Our main goal was to study whether peptide lunasin has the capacity to regulate the mucosal immune response in the human gut. Hence, we first determined if lunasin displayed modulatory effects on the mucosal mRNA expression levels of several immune mediators in human intestinal biopsies. Our results showed that synthetic lunasin was bioactive in the human intestinal mucosa where it significantly induced the gene expression of *IL-1β, TNFα, IL-17A, CCL-2,* and *PGE2-COX2*, usually in a dose-dependent manner, relative to control biopsies cultured in the absence of peptide (**Figure 1**). Noteworthy, lunasin was able to enhance the expression of tolerogenic cytokines *IL-10* and *TGFβ* up to 2.1- ($p < 0.001$) and 3.0-fold ($p < 0.0001$), respectively, while it also downregulated the expression of *iNOS* (5 µM: 0.7-fold, $p < 0.0001$; 50 µM: 0.8-fold, $p < 0.01$; 200 µM: 0.8-fold, $p < 0.05$) and subunit *p65* from *NF-κB* (5 µM: 0.9-fold, $p < 0.01$; 50 µM: 0.7-fold, $p < 0.001$). No significant change in the mRNA transcription of *IL-6, IFNγ, IL-8,* or *IL-33* genes was detected at any of the concentrations tested (**Figure 1**).

Having confirmed the immunomodulatory effects of lunasin in the human intestinal mucosa, we next studied whether that was maintained in the presence of a pro-inflammatory insult like the Gram-negative bacteria endotoxin LPS. Referred to the resting cultures, LPS significantly induced mucosal gene expression of pro-inflammatory *IL-1β* (2.3-fold, $p < 0.01$), *IL-17A* (2.8-fold, $p < 0.0001$), *IFNγ* (2.7-fold, $p < 0.01$), *IL-8* (2.2-fold, $p < 0.05$), *CCL-2* (1.8-fold, $p < 0.05$), *IL-33* (1.9-fold, $p < 0.05$), and *PGE2-COX2* (3.1-fold, $p < 0.001$) (**Figure 2**). Noteworthy, lunasin had the capacity to revert the LPS-induced pro-inflammatory effects at its highest dose (200 µM). Hence, lunasin inhibited

the expression of LPS-induced *IL-17A*, *IFNγ,* and *IL-8* ($p < 0.05$) and tended to restore *IL-33* and *PGE2-COX2* expression ($p > 0.05$). Moreover, lunasin also increased the mucosal expression of tolerogenic *IL-10* and *TGFβ* in the presence of LPS ($p < 0.05$) in a dose dependent manner (**Figure 2**). Together, these results not just reveal the immunomodulatory capacity of lunasin in the human intestinal mucosal but also confirm its anti-inflammatory effects.

3.2. Regulation of the profile of mucosal cytokine production

In order to confirm the immunomodulatory role of lunasin, we next evaluated the soluble levels of 13 intestinal cytokines/chemokines in the paired secretomes of the biopsies. IL-8, IL-17A, and IL-12p70 were outside the range of standards and therefore excluded from the analysis (data not shown). Overall, the effects over the expression of mucosal mRNA were mirrored in the production of cytokines into the secretomes since lunasin significantly increased the secretion of intestinal IL-1β and TNFα cytokines ($p < 0.05$) in a dose dependent manner as well as that of IL-10 ($p < 0.05$) (**Figure 3**). Main difference however was found in the case of CCL2, which may suggest a local effect at the autocrine or paracrine level of this chemokine, reason why it was increased at the mRNA level (**Figure 1**) but decreased in the culture supernatant (**Figure 3**). Moreover, lunasin at 200 μM significantly abrogated the secretion of IL-6 and IFNγ ($p < 0.01$).

We next evaluated whether these anti-inflammatory effects of lunasin were validated in the presence of LPS. Lunasin had decreased LPS-induced mRNA expression of *IL-17A*, *IFNγ,* and *IL-8* (**Figure 2**). Although cytokine production of IL-17A and IL-8 was out of the range of standards (and therefore could not be analyzed), lunasin reverted LPS-induced

IFNγ secretion ($p < 0.05$) (Supporting Information Figure S1). Nevertheless, increased expression of *IL-10* (**Figure 2**) was not mirrored in the LPS-challenged secretomes (Supporting Information Figure S1) again suggesting an auto- or paracrine effect for this cytokine.

3.3. Lunasin´s immunomodulatory activity in intestinal lamina propria mononuclear cells

In order to further confirm the previous findings, including the local effects on the cytokines, and provide a mechanism for lunasin immunomodulatory action, we next studied its effect of human intestinal Mϕ and cDC subsets. To that end, LPMC were *ex vivo* cultured for 18 h with/out lunasin (200 µM as determined to be optimal in the previous experiments) in the presence/absence of LPS. Following culture, intestinal Mϕ were identified within singlet viable cells as $CD45^+CD14^+CD64^+$ while cDC were identified within singlet viable cells as $CD45^+CD14^-CD64^-HLA\text{-}DR^+CD11c^+$. cDC were further divided into regulatory $CD103^+$ cDC and $CD103^-$ cDC,[29] while tissue resident Mϕ were differentiated from newly arrived pro-inflammatory monocytes based on the expression of CD11c[24,30] (Supporting Information Figure S2).

The proportion of total leukocytes, Mϕ (including pro-inflammatory $CD11c^+$ monocytes and resident $CD11c^-$ Mϕ), and cDC (including $CD103^+$ and $CD103^-$ cDC) were not altered after culture confirming no major effects on cell viability of the different culture conditions (Supporting Information Table S5). Next, we assessed the effect of lunasin, both in resting conditions and in the presence of LPS, on both cDC and Mϕ phenotype (CD40, CD86, ICOS-L, and PD-L1) and function (intracellular production of IL-10, IL-1β,

and TNFα). Our results showed that lunasin acted over the intracellular cytokine profile as significantly induced the expression of IL-10 in total cDC and total Mϕ both in the absence and presence of LPS, as well as the levels of IL-1β in response to LPS (**Table 1**). In addition, LPS-challenged total Mϕ displayed enhanced expression of CD86 and PD-L1 that was significantly restored by lunasin ($p < 0.05$).

We next aimed to identify the specific cDC and Mϕ subsets modulated by lunasin. This approach revealed that regulatory PD-L1 displayed a tendency to be more expressed on $CD103^+$ cDC referred to $CD103^-$ cDC ($p = 0.073$) and, indeed, lunasin increased its expression on $CD103^-$ cDC while, in the presence of LPS, it downregulated ICOS-L expression on the former subset (**Figure 4A**). In a similar manner, regulatory $CD11c^-$ Mϕ tended to have higher expression of PD-L1 referred to their $CD11c^+$ counterparts ($p = 0.088$) while lunasin downregulated its expression in the presence of LPS (**Figure 4B**). Noteworthy, lunasin also modulated cDC and Mϕ function, as assessed by cytokine production. Hence, and although $CD103^-$ cDC produced lower amounts of IL-10 ($p < 0.05$), lunasin increased its expression both in resting conditions and in the presence of LPS, at the time that it downregulated TNFα on resting $CD103^-$ cDC as well as in $CD103^+$ cDC in the presence of LPS (**Figure 4C**). Last, but not least, the ability of lunasin to expand intestinal IL-10 production was more notable on resident $CD11c^-$ Mϕ which displayed increased IL-10 levels in both resting ($p = 0.108$) and LPS conditions ($p < 0.05$) (**Figure 4D**). Together, these results not just identify the immunomodulatory action of lunasin in the human intestinal mucosa, but they also suggest for the first time its capacity to modulate cDC and Mϕ phenotype and function providing therefore a potential basis for its mechanism of action.

4. DISCUSSION

To the best of our knowledge, this is the first study investigating the direct effect of peptide lunasin in the human intestinal mucosa under *ex vivo* resting conditions and in the presence of the gut endotoxin LPS associated to the pathophysiology of intestinal inflammation.[31] First of all, we confirmed that synthetic lunasin maintained its stability in our intestinal biopsy culture model and showed similar peptide patterns in the HPLC-MS/MS analysis of both resting and LPS conditions (data not shown). Approximately 5% of peptide could have been hydrolyzed or metabolized by intestinal tissue, although not derived peptides were identified. This in agreement with previous results using the model of colon cancer Caco-2 cells.[25] Moreover, we found that lunasin was bioactive in the human intestinal mucosa as it modulated mucosal mRNA expression and cytokine production inducing a regulatory phenotype. Indeed, these effects were also elicited in the presence of pro-inflammatory LPS confirming its anti-inflammatory properties. Last, but not least, we hereby also provide mechanistic basis for lunasin through intestinal antigen presenting cells, including cDC and Mϕ subsets. Nevertheless, given that these effects have been evaluated for synthetic lunasin, which secondary and tertiary structure may differ from that of natural sources, different behavior might be observed with food-derived lunasin.

Eliminado: Since

The regulatory role of the immune response is essential to maintain tissue homeostasis in all organs and systems of the body. Unfortunately, when this mechanism is dysregulated, an exacerbated immune response may lead to chronic inflammation and represent the underlying pathological cause of many disorders.[32] In the gastrointestinal tract, this situation is particularly relevant in immune-mediated disorders such as IBD.

346 Both food and microbiota are one of the main environmental factors that can impact
347 the mucosal immunity and thus the status of the gastrointestinal health. Bioactive
348 peptide lunasin is not only partially resistant to the action of digestive enzymes, but it is
349 also absorbed so it can be detected in the human plasma after soy protein intake.[8]
350 Despite the gastrointestinal tract is an obvious target for bioactive ingested
351 compounds,[33] little is known about the physiological relevance of lunasin in human
352 intestinal cells.
353
354 The effect of soybean lunasin in the differential gene expression of RAW 264.7
355 macrophages cultured with/out LPS-challenge was previously compared.[34] These
356 authors evidenced the modulatory action of this peptide naturally extracted from
357 soybean over genes associated to cellular growth, proliferation and function, apoptosis
358 and cell cycle, and cell to cell interaction and signaling. The mechanisms underlying the
359 effect in this cell line could involve modulation of COX-2/PGE2, iNOS/NO, and NF-κB
360 pathways,[20,35] in agreement with our findings in the human intestinal mucosa using
361 synthetic lunasin. Moreover, our transcriptomic results were further validated at the
362 protein level as the secretion of IL-1β, TNF-α, and IL-10 cytokines was expanded while
363 that of IL-6, CCL2, and IFN-γ was almost abrogated by lunasin. Indeed, production of the
364 latter cytokine was also neutralized in the presence of LPS, although the high inter-
365 individual variability did not allow us to confirm the effects of lunasin in the global
366 cytokine milieu of LPS-challenged intestinal secretomes.
367
368 The inhibitory effects of lunasin from both natural or synthetic origins over the
369 production of pro-inflammatory mediators have been mostly investigated by using

cellular models of RAW 264.7 macrophages.[17–20] Similar anti-inflammatory findings along with inhibition of activation of the inflammasome complex have been also proved in THP-1 human macrophages.[36] When this cell line was challenged with LPS, soybean lunasin ameliorated the expression of NF-κB-dependent markers of inflammation by inhibiting translocation of p50 and p65 subunits and Akt phosphorylation.[26] This effect was coupled to peptide cellular internalization, suggested to be mediated by interaction of the lunasin cell adhesion RGD-motif with αVβ3 integrins which is amplified during inflammatory conditions.[37] The anti-inflammatory action of synthetic lunasin in macrophages has provided additional effects as shown in recent studies using co-culture with 3T3-L1 adipocytes[38] and 4T1 breast cancer cells[39] which raise attention to the role of this peptide against adipose tissue inflammation and tumor inflammatory microenvironment. In our model, we found that synthetic lunasin displayed a stimulatory effect in human intestinal biopsies through an innate immune response mainly characterized by IL-1β and TNF-α gene and protein expression. In basal conditions, it also down-regulated gene expression of both *iNOS*, enzyme that synthesizes NO and is associated with early-onset IBD,[40] and *p65*, RelA subunit from NF-κB transcriptional factor, central mediator of redox homeostasis and precursor of inflammatory response.[41] NF-κB activation is inhibited by TGFB, which has additional suppressive effect in pro-inflammatory cytokine production.[42] Hence, our findings showed that inhibition of *NF-κB* by lunasin was linked to an up-regulation of *TGFβ* expression, which was demonstrated both in homeostatic conditions and even after challenge with LPS.

A previous study, focused on type 2 cDC purified from peripheral blood mononuclear cells of healthy donors, found that synthetic lunasin-treated cDC not only expressed higher levels of co-stimulatory markers, cytokines and chemokines, but also they induced higher proliferation of allogeneic $CD4^+$ T cells hereby the peptide acting as a vaccine adjuvant in mice.[43] Likewise, this immunomodulatory action was also able to ameliorate the allergic inflammation in the airway mucosa and promote the accumulation of antigen-specific regulatory T cells in two murine models of asthma.[44]. Based on these observations, and in order to shed some light on the potential mechanism of action elicited by lunasin in the gut, we hypothesized that it had the capacity to modulate human cDC and Mϕ subsets, as the main intestinal antigen presenting cells.[45] cDC not only determine the outcome (pro-inflammatory/tolerogenic) of the mucosal immune response against food antigens, commensal microbiota or pathogens, but also regulate its location imprinting tissue-specific homing markers.[46] In a similar manner, Mϕ are also key contributors to gut homeostasis by maintenance of local inflammation, epithelial renewal and clearance of microbes[47] while both populations also collaborate to induce and maintain regulatory T cells. Hence, inappropriate activation of their function or changes in this delicate balance are related to gut autoimmune and chronic inflammatory diseases such as coeliac disease, food allergies and IBD.[48] In this context, we found that lunasin modulated the intracellular production of IL-10 and IL-1β cytokines of both mucosal cDC and Mϕ, in line with our results on the mRNA/protein expression of biopsies. Specifically, we identified $CD103^-$ cDC and $CD11c^-$ Mϕ as the main subsets targeted by the peptide. Hence, lunasin significantly induced IL-10 and PD-L1, and down-regulated TNFα expression on the $CD103^-$ cDC subset, while it also ameliorated PD-L1 as well as

expanded the levels of IL-10 in resident CD11c$^-$ Mϕ.[24] This is of particular interest given that CD103$^+$ cDC are reduced in the inflamed colon of IBD patients[29] where they do not generate regulatory T cells, and display higher expression of *IL-6, IL-23A, IL-12p35* and *TNFα* in conjunction with increased counts of IFNγ-, IL-13- and IL-17-producing CD4$^+$ T cells.[49]

In summary, we hereby have demonstrated that, to the best of our knowledge, this is the first time in which lunasin is identified as immunoactive in the human intestinal mucosa where it has the capacity to modulate the intestinal cytokine milieu both at the messenger and protein levels. Hence, synthetic lunasin displayed its anti-inflammatory effect by abrogating the production of pro-inflammatory cytokines and expanding the production of tolerogenic IL-10 and *TGFβ* even in the presence of LPS. Our data suggest that the immunomodulatory capacity of lunasin may be related to regulatory effects over intestinal cDC and Mϕ, being these effects predominantly displayed in the CD103$^-$ and CD11c$^-$ subsets, respectively. However, more studies including microscopy and metabolic signaling approaches are still needed to further understand the exact mechanism of action of lunasin in the human intestine. Altogether, in the present *ex vivo* settings, the potential of lunasin as a novel functional compound for the prevention of immune and inflammatory-mediated intestinal disorders is proposed. It remains to be further characterized whether this capacity may also modulate the mucosal immune response in the presence of *in vivo* inflammation like in the context of IBD patients. Moreover, confirmation of the effects of synthetic lunasin with the peptide extracted from natural sources should be evaluated to evidence the potential of this food-derived peptide against intestinal inflammatory disorders.

Eliminado: intestinal cells

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Author contributions

Study concept and design was performed by SFT, BHL and DB. Patients were recruited and biological samples obtained by JAMM, CS, MC and JPG. Experiments were performed by SFT and IMG (sample processing and culture); PIR and LPR (qRT-PCR); SFT, LOM and MBM (Multiplex); PIR and BHL (HPLC-MS/MS); SFT, IMG and ACM (flow cytometry). Data analysis and interpretation was performed by SFT and DB. JPG, MC, BHL and DB obtained the funds required to perform all the experiments. The manuscript was drafted by SFT under DB guidelines. All authors reviewed and approved the final version of the manuscript.

Conflict of interest

The authors declare that they have no competing interests.

REFERENCES

[1] L. W. Peterson, D. Artis, *Nat. Rev. Immunol.* **2014**, *14*, 141–153.

[2] S. C. Bischoff, G. Barbara, W. Buurman, T. Ockhuizen, J. Schulzke, M. Serino, H. Tilg, A. Watson, J. M. Wells, *BMC Gastroenterol.* **2014**, *14*, 189.

[3] M. L. Y. Wan, K. H. Ling, H. El-Nezami, M. F. Wang, *Crit. Rev. Food Sci. Nutr.* **2019**, *59*, 1927–1936.

[4] J. R. Marchesi, D. H. Adams, F. Fava, G. D. A. Hermes, G. M. Hirschfield, G. Hold, M. N. Quraishi, J. Kinross, H. Smidt, K. M. Tuohy, L. V. Thomas, E. G. Zoetendal, A. Hart, *Gut* **2016**, *65*, 330–339.

[5] T. J. Ashaolu, *Biomed. Pharmacother.* **2020**, *130*, 110625.

[6] O. Martínez-Augustin, B. Rivero-Gutiérrez, C. Mascaraque, F. Sánchez de Medina, *Int. J. Mol. Sci.* **2014**, *15*, 22857–22873.

[7] T. J. Ashaolu, *Appl. Microbiol. Biotechnol.* **2020**, *104*, 9009–9017.

[8] S. Fernández-Tomé, B. Hernández-Ledesma, *Food Res. Int.* **2019**, *116*, 71–78.

[9] C. C. Hsieh, C. Martínez-Villaluenga, B. O. de Lumen, B. Hernández-Ledesma, *J. Sci. Food Agric.* **2018**, *98*, 2070–2079.

[10] C. Chatterjee, S. Gleddie, C. W. Xiao, *Nutrients* **2018**, *10*, 8–11.

[11] V. P. Dia, E. G. Mejia, *Cancer Lett.* **2010**, *295*, 44–53.

[12] V. P. Dia, E. Gonzalez de Mejia, *Cancer Lett.* **2011**, *313*, 167–180.

[13] V. P. Dia, E. Gonzalez de Mejia, *Mol. Nutr. Food Res.* **2011**, *55*, 623–634.

[14] S. Fernández-Tomé, F. Xu, Y. Han, B. Hernández-Ledesma, X. Hang, *Int. J. Mol. Sci.* **2020**, *21*, 537.

[15] S. Fernández-Tomé, S. Ramos, I. Cordero-Herrera, I. Recio, L. Goya, B. Hernández-Ledesma, *Food Res. Int.* **2014**, *62*, 793–800.

[16] M. J. García-Nebot, I. Recio, B. Hernández-Ledesma, *Food Chem. Toxicol.* **2014**, *65*, 155–161.

[17] B. Hernández-Ledesma, C. C. Hsieh, B. O. de Lumen, *Biochem. Biophys. Res. Commun.* **2009**, *390*, 803–808.

[18] G. Ren, Y. Zhu, Z. Shi, J. Li, *J. Sci. Food Agric.* **2017**, *97*, 4110–4116.

[19] Y. Zhu, E. Nadia, Y. Yao, Z. Shi, G. Ren, *J. Biosci. Bioeng.* **2018**, *126*, 1–8.

[20] V. P. Dia, W. Wang, V. L. Oh, B. O. De Lumen, E. G. De Mejia, *Food Chem.* **2009**, *114*, 108–115.

[21] K. Kusmardi, N. Nessa, A. Estuningtyas, A. Tedjo, *Int. J. Physiol. Pathophysiol. Pharmacol.* **2018**, *10*, 154–162.

[22] S. Jia, S. Zhang, H. Yuan, N. Chen, *Biomed Res. Int.* **2015**, *2015*, 346839.

[23] S. Fernández-Tomé, A. C. Marin, L. Ortega Moreno, M. Baldan-Martin, I. Mora-Gutiérrez, A. Lanas-Gimeno, J. A. Moreno-Monteagudo, C. Santander, B. Sánchez, M. Chaparro, J. P. Gisbert, D. Bernardo, *Nutrients* **2019**, *11*, 2605.

[24] D. Bernardo, A. C. Marin, S. Fernández-Tomé, A. Montalban-Arques, A. Carrasco, E. Tristán, L. Ortega-Moreno, I. Mora-Gutiérrez, A. Díaz-Guerra, R. Caminero-Fernández, P. Miranda, F. Casals, M. Caldas, M. Jiménez, S. Casabona, F. De La Morena, M. Esteve, C. Santander, M. Chaparro, J. P. Gisbert, *Mucosal Immunol.* **2018**, *11*, 1114–1126.

[25] S. Fernández-Tomé, J. Sanchón, I. Recio, B. Hernández-Ledesma, *J. Food Compos. Anal.* **2018**, *68*, 101–110.

[26] A. Cam, E. G. de Mejia, *Mol. Nutr. Food Res.* **2012**, *56*, 1569–1581.

[27] K. J. Livak, T. D. Schmittgen, *Methods* **2001**, *25*, 402–408.

[28] S. Fernández-Tomé, A. Montalban-Arques, A. Díaz-Guerra, J. M. Galvan-Roman,

A. C. Marin, I. Mora-Gutiérrez, L. Ortega Moreno, C. Santander, B. Sánchez, M. Chaparro, J. P. Gisbert, D. Bernardo, *J. Funct. Foods* **2019**, *52*, 459–468.

[29] H. Matsuno, H. Kayama, J. Nishimura, Y. Sekido, H. Osawa, S. Barman, T. Ogino, H. Takahashi, N. Haraguchi, T. Hata, C. Matsuda, H. Yamamoto, M. Uchino, H. Ikeuchi, Y. Doki, M. Mori, K. Takeda, T. Mizushima, *Inflamm. Bowel Dis.* **2017**, *23*, 1524–1534.

[30] A. Bujko, N. Atlasy, O. J. B. Landsverk, L. Richter, S. Yaqub, R. Horneland, O. Øyen, E. M. Aandahl, L. Aabakken, H. G. Stunnenberg, E. S. Bækkevold, F. L. Jahnsen, *J. Exp. Med.* **2018**, *215*, 441–458.

[31] E. Im, F. M. Riegler, C. Pothoulakis, S. H. Rhee, *Am. J. Physiol. - Gastrointest. Liver Physiol.* **2012**, *303*, 490–497.

[32] M. Reale, L. Conti, D. Velluto, *J. Immunol. Res.* **2018**, *2018*, 7197931.

[33] S. Fernández-Tomé, B. Hernández-Ledesma, *Mol. Nutr. Food Res.* **2020**, *2000401*, 1–12.

[34] V. P. Dia, E. G. de Mejia, *Peptides* **2011**, *32*, 1979–1988.

[35] E. G. de Mejia, V. P. Dia, *Peptides* **2009**, *30*, 2388–2398.

[36] S. J. Price, P. Pangloli, V. P. Dia, *Food Funct.* **2017**, *8*, 4449–4458.

[37] A. Cam, M. Sivaguru, E. Gonzalez de Mejia, *PLoS One* **2013**, *8*, e72115.

[38] C. C. Hsieh, M. J. Chou, C. H. Wang, *PLoS One* **2017**, *12*, e0171969.

[39] C. C. Hsieh, C. H. Wang, Y. S. Huang, *Int. J. Mol. Sci.* **2016**, *17*, DOI 10.3390/ijms17122109.

[40] S. S. Dhillon, L. A. Mastropaolo, R. Murchie, C. Griffiths, C. Thoni, A. Elkardri, W. Xu, A. Mack, T. Walters, C. Guo, D. Mack, H. Huynh, S. Baksh, M. S. Silverberg, J. H. Brumell, S. B. Snapper, A. M. Muise, *Clin. Transl. Gastroenterol.* **2014**, *5*, e46.

[41] R. Liu, Y. Hao, N. Zhu, X. Liu, J. Kang, R. Mao, C. Hou, Y. Li, *Nutrients* **2020**, *12*, 1138.

[42] L. Kubiczkova, L. Sedlarikova, R. Hajek, S. Sevcikova, *J. Transl. Med.* **2012**, *10*, 183.

[43] C. Y. Tung, D. E. Lewis, L. Han, M. Jaja, S. Yao, F. Li, M. J. Robertson, B. Zhou, J. Sun, H. C. Chang, *Vaccine* **2014**, *32*, 5411–5419.

[44] X. Yang, J. Zhu, C. Y. Tung, G. Gardiner, Q. Wang, H. C. Chang, B. Zhou, *PLoS One* **2015**, *10*, e0115330.

[45] V. Cerovic, C. C. Bain, A. M. Mowat, S. W. Milling, *Trends Immunol.* **2014**, *35*, 270–277.

[46] D. Bernardo, M. Chaparro, J. P. Gisbert, *Mol. Nutr. Food Res.* **2018**, *62*, e1700931.

[47] C. C. Bain, A. M. Mowat, *Immunol. Rev.* **2014**, *260*, 102–117.

[48] C. Caër, M. J. Wick, *Front. Immunol.* **2020**, *11*, 410.

[49] M. K. Magnusson, S. F. Brynjólfsson, A. Dige, H. Uronen-Hansson, L. G. Börjesson, J. L. Bengtsson, S. Gudjonsson, L. Öhman, J. Agnholt, H. Sjövall, W. W. Agace, M. J. Wick, *Mucosal Immunol.* **2016**, *9*, 171–182.

559 **Table 1. Immunomodulatory effect of lunasin over human intestinal dendritic cells and macrophages.**

Cells	Condition	% CD40		% CD86		% ICOS-L		% PD-L1		% IL-10		% IL-1β		% TNFα	
		Mean (SEM)	*p*	Mean (SEM)	*p*	Mean (SEM)	*p*	Mean (SEM)	*p*	Mean (SEM)	*p*	Mean (SEM)	*p*	Mean (SEM)	*p*
Total cDC	Basal	3.4 (1.3)		46.1 (10.5)		77.0 (3.7)		8.5 (0.8)		64.9 (4.9)		18.1 (4.2)		25.2 (3.1)	
	Lunasin 200 µM	3.0 (0.9)	ns	45.4 (9.9)	ns	72.6 (3.0)	ns	14.8 (3.6)	ns	73.2 (5.5)	*	24.5 (7.1)	ns	21.1 (2.2)	n
	LPS	3.0 (1.2)		47.9 (11.3)		75.0 (3.9)		8.3 (1.4)		55.5 (7.5)		21.2 (4.3)		22.0 (1.6)	
	LPS + Lunasin 200 µM	3.2 (1.0)	ns	48.2 (11.2)	ns	69.8 (3.8)	ns	7.6 (0.9)	ns	68.1 (6.4)	#	28.3 (5.5)	#	23.5 (2.2)	n
Total Mϕ	Basal	1.9 (0.7)		54.8 (9.1)		5.1 (0.9)		50.3 (3.9)		78.6 (4.0)		17.0 (3.3)		20.9 (7.9)	
	Lunasin 200 µM	1.6 (0.6)	ns	51.9 (8.5)	ns	3.5 (0.7)	ns	53.6 (4.4)	ns	84.0 (2.5)	*	13.7 (6.3)	ns	20.7 (8.2)	n
	LPS	4.4 (2.5)		60.0 (8.7)		5.2 (1.2)		56.9 (5.8)		73.9 (4.1)		17.7 (2.3)		25.3 (9.5)	
	LPS + Lunasin 200 µM	2.9 (1.6)	ns	52.1 (7.7)	#	3.6 (0.5)	ns	52.7 (4.4)	#	79.7 (3.4)	#	21.2 (3.4)	#	21.6 (8.3)	n

cDC: conventional dendritic cells; Mϕ: macrophages; ns: not significant

Human intestinal lamina propria mononuclear cells were cultured with medium (basal) and LPS (100 ng/mL) in the absence and presence of lunasin (200 µM) during 18 h. Following culture, cDC were identified within singlet viable cells as $CD45^{+}CD14^{-}CD64^{-}HLA\text{-}DR^{+}CD11c^{+}$, while Mϕ were identified within singlet viable cells as $CD45^{+}CD14^{+}CD64^{+}$. Results are shown as percentage of positive cells for each marker (mean (SEM), *n*=6)). Statistical differences elicited by lunasin were determined by one-way ANOVA with subsequent Tukey correction and indicated by *($p<0.05$), significantly different from basal, and #($p<0.05$), significantly different from LPS.

FIGURE CAPTIONS

Figure 1. Effect of lunasin on the relative gene expression of human intestinal biopsies. Biopsies were cultured during 18 h in the presence of lunasin (5, 50, and 200 µM) as well as in resting conditions (control). mRNA expression levels were measured by real-time RT-PCR. Data are means ± SEM (*n*=10). Results are shown as relative gene expression level of control (fold change value 1). Statistical differences between control and each condition were determined by Mann-Whitney test and indicated by *(p<0.05), **(p<0.01), ***(p<0.001), ****(p<0.0001), significantly different from control.

Figure 2. Effect of lunasin on the relative gene expression of LPS-challenged human intestinal biopsies.

Biopsies were cultured during 18 h with medium (control) and LPS (100 ng/mL) in the presence/absence of lunasin (5, 50, and 200 µM). mRNA expression levels were measured by real-time RT-PCR. Data are means ± SEM (*n*=10). Results are shown as relative gene expression level of control (fold change value 1, represented as dotted line). Statistical differences between control and LPS or LPS and each condition were determined by Mann-Whitney test and indicated by *(p<0.05), **(p<0.01), ***(p<0.001), ****(p<0.0001), significantly different from control, and #(p<0.05), significantly different from LPS.

Figure 3. Intestinal cytokine milieu of culture supernatants from human intestinal biopsies treated with lunasin.

Biopsies were cultured with medium in the absence (0) and presence of lunasin (5, 50, and 200 µM) during 18 h in resting conditions, and the mucosal cytokine production was

measured by Multiplex immunoassay. Data are means ± SEM (*n*=10). Results are shown as cytokine levels (pg/mL). Statistical differences between control and each condition were determined by one-way ANOVA with subsequent Tukey correction and indicated by *($p<0.05$), **($p<0.01$), significantly different from control.

Figure 4. Immunomodulatory effect of lunasin over human intestinal dendritic cell and macrophage subsets.

Human intestinal lamina propria mononuclear cells were cultured with medium (basal) and LPS (100 ng/mL) in the absence and presence of lunasin (200 μM) during 18 h. **(A, C)** Conventional dendritic cells (cDC) and **(B, D)** macrophages (Mϕ) were identified by flow cytometry and further divided into subsets based on the expression of CD103 and CD11c integrins, respectively. Results are shown as percentage of positive cells for each **(A, B)** surface, and **(C, D)** intracellular marker (*n*=6). Statistical differences elicited by lunasin were determined by one-way ANOVA with subsequent Tukey correction and indicated by *($p<0.05$), significantly different from basal, and #($p<0.05$), significantly different from LPS.