1 *Ex vivo and in vitro* assessment of anti-inflammatory activity of seed β -conglutin proteins
2 from *Lupinus angustifolius*

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Abstract

The development of functional food ingredients using legume seed proteins has potential for nutraceutical use. We purified recombinant β -conglutin proteins (r β 1, r β 2, r β 3, r β 4, and r β 6) from narrow-leaved lupin using affinity-chromatography, and evaluated their anti-inflammatory activity using *ex vivo* and *in vitro* systems. The r β 1, r β 3, and r β 6 proteins produced lower levels of pro-inflammatory mediators such as nitric oxide (about –34.0-fold in all cases), iNOS mRNA (–7.15, –7.97, –7.41-fold, respectively), interleukin 1 β (–12.05, –11.64, –12.16-fold, respectively), chemokine CCL5 (–16.0, –18.0, –19.0-fold, respectively), and cytokines including TNF- α , INF- γ , IL-1 β , IL-2, IL-6, IL-8, and IL-12 (β 1: –28, –100, –8, –2, –49, –45, –127-fold; β 3: –22, –400, –9, –3, –33, –10, –2.54-fold; β 6: –72, –122, –11, –3, –2000, –13, –338-fold, respectively). These results suggest that the β 1, β 3, and β 6 conglutins have potential as functional food components in nutraceuticals and that can provide alternative therapies for the prevention and treatment of inflammatory-related diseases.

Key words: Anti-inflammatory proteins; β -conglutins; chemokine CCL5; IL-1 β ; iNOS; *Lupinus angustifolius*; vicilin.

1. Introduction

There is an increasing demand for plant-derived proteins as components of functional foods in the nutraceutical industry and as an alternative to the environmentally expensive production of animal protein. In this regard, legumes are an economical dietary source of good quality protein and higher in protein than most other plant foods. In addition, legumes, particularly lupin proteins and peptides, have positive health effects (Arnoldi et al., 2015).

Epidemiological studies on lupins, especially species from the ‘sweet lupin’ group, have shown that the consumption of seed proteins may provide preventive and protective effects for several chronic diseases such as cardiovascular disease, cancer, familial hypercholesterolemia, obesity and diabetes, hypertension (blood pressure-lowering), and control glycaemia (Villarino et al., 2016). Recently, the molecular basis for type 2 diabetes (T2D) prevention and treatment (Lima-Cabello et al., 2017), or cholesterol reduction (Lammi et al., 2016) has been elucidated, where bioactive peptides from the main seed storage protein family in narrow-leafed lupin (NLL; *Lupinus angustifolius*), the β -conglutins are important players in the promotion of these health benefits.

Chronic inflammation is involved in the onset and progress of T2D, and pancreatic tissue is an important target for this progression. Different factors or stressors promote the mechanisms underlying defective insulin secretion and responses, as well as insulin resistance in T2D. Among these, the most frequent are glucotoxicity, lipotoxicity, oxidative stress, misfolded proteins triggering endoplasmic reticulum (ER) stress, and alterations in gut microbiota that increase lipopolysaccharides (LPS) in blood or form amyloid deposits in pancreatic islets that stimulate immune-response-mediated inflammation (Montane et al., 2014). Continued and excessive inflammation promoted by the uncontrolled production of pro-inflammatory cytokines such as tumor necrosis factor (TNF), interferon gamma (INF γ), interleukin 6 (IL-6), interleukin 1 (IL-1), chemokines (i.e. CCL2, CCL5), reactive oxygen

1 species (ROS), adhesion molecules (i.e. ICAM-1, VCAM-1), and the overproduction of nitric
2 oxide (NO) and nitrogen intermediates might elicit a characteristic situation of T2D
3 progression (Turner et al., 2014). Thus, reducing the production and/or activity of pro-
4 inflammatory mediators has potential as an anti-inflammatory treatment that can also result in
5 reducing T2D progression.

6 The search for natural compounds with anti-inflammatory properties has increased as the
7 incidence of inflammation-related diseases has soared. The health-promoting properties of
8 phenolic compounds in legumes have been well-studied. Phenolic acids and flavonoids are
9 abundant in legume seeds and have potential anti-inflammatory properties among other health
10 benefits (Boudjou et al., 2013).

11 Few studies have reported on the anti-inflammatory properties of bioactive polypeptides and
12 protein hydrolysates. Interestingly, the enzymatic plant protein hydrolysates in field pea seeds
13 have shown anti-inflammatory properties throughout inhibition of mediators production such
14 as NO, TNF- α , IL-1 β , and IL-6, without affecting cell viability (Ndiaye et al., 2012), and a
15 few small and specific peptides have been isolated from them. Lunasin, a plant 2S-albumin-
16 derived peptide found in soybean, barley, wheat, rye, and triticale has been studied for its
17 benefits for cancer, cholesterol lowering, and cardiovascular disease (Malaguti et al., 2014).
18 In soybean, the anti-inflammatory properties of this peptide were related to its capacity to
19 suppress the NF- κ B pathway, an effect that is mediated by a lunasin- α V β 3 integrin
20 interaction through the Arg-Gly-Asp (RGD) motif (Cam and de Mejia, 2012).

21 Blue lupine seed protein hydrolysates have potential anti-inflammatory properties due to the
22 inhibition of enzymes involved in the inflammation pathway (phospholipase A2 and
23 cyclooxygenase-2) (Millán-Linares et al., 2014). Bioactive peptides generated after two food-
24 grade proteases also modulated the expression of pro- and anti-inflammatory mediators and
25 attenuated the cell migratory response. The sequence of one of these peptides was

1 characterized as GPETAFLR (Millán-Linares et al., 2015), which identifies 100% with
2 *Glycine max* lectin-like protein, and 88% with *Arabidopsis thaliana* 2S albumin (BLAST,
3 <https://blast.ncbi.nlm.nih.gov/>).

4 The seeds of sweet lupin species, particularly NLL, have high nutritional value and beneficial
5 nutraceutical effects. Despite these features, NLL is largely underexploited, and its
6 consumption is at a long base, but is increasing. Alternative applications such as the
7 incorporation of NLL seeds in new functional foods may help further increase the use of NLL
8 grain. Recently good progress has been made in the identification and characterization of the
9 seed storage proteins in NLL, which are called conglutins and fall into four families of which
10 β -conglutin being the largest and most highly expressed (Foley et al., 2015).

11 There have been little published studies on the anti-inflammatory properties of legume seed
12 compounds, and particularly about NLL seed proteins. An initial study primarily focused on
13 the ability of the NLL β -conglutins to modulate gene expression and protein markers level of
14 the insulin signaling pathways in patients suffering from T2D for disease amelioration (Lima-
15 Cabello et al., 2017). T2D is associated with an inflammatory chronic state, and in this study
16 was also showed preliminary evidence that some β -conglutins may be also involved in
17 reduction of pro-inflammatory mediators. In the present study, we evaluated the anti-
18 inflammatory effects of five NLL individual recombinant β -conglutin isoforms on
19 lipopolysaccharide (LPS)-stimulated *ex vivo* and *in vitro* cells model.

20 21 **2. Material and Methods**

22 *2.1. Expression plasmids*

23 We used a system for bacterial expression modified from the pET28a(+) vector containing b-
24 conglutin-6xHis-Tag for β 1, β 2, β 3, β 4, and β 6 (GenBank accession number HQ670409,
25 HQ670410, HQ670411, HQ670412, and HQ670414, respectively).

2.2. Beta-conglutin protein isoforms expression and purification

β -conglutin protein isoforms were expressed in Rosetta™ 2(DE3) pLysS Singles™ Competent Cells (Novagen) following an auto-induction method (Jimenez-Lopez et al., 2016). Purification of recombinant conglutin isoforms β 1, β 2, β 3, β 4, and β 6 was performed using bacterial pellets. The purity of the protein samples was >95% and typical yields were ~10–25 mg/ml.

2.3. β -conglutin antibody production

The synthesis of a synthetic peptide commonly shared in the five NLL β -conglutin protein isoforms was used for the immunization of rabbits and to produce polyclonal antiserum (Agrisera, Sweden), which was affinity-purified and IgG quantified by ELISA.

2.4. Cell culture and treatment

PANC-1 cells were grown in poly-L-lysine-coated 75 cm² flasks (~2.0–2.5 × 10⁶ cells/ml) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine and 10% heat-inactivated fetal bovine serum in the presence of 5% CO₂/95% air at 37°C.

Cells were routinely subcultured and used in the exponential growth phase for all experiments. Cells were grown as monolayers and detached from cultures by trypsinization. Cells were washed twice with phosphate-buffered solution (PBS, Sigma) and treated with 0.25% tryp-EDTA (Lonza) for 10 min in 5% CO₂/95% air at 37°C humidified atmosphere. The trypsinization effect was neutralized with culture medium. The cells were collected after centrifugation at 1000 rpm for 5 min and washed with PBS for viability assays. Cell counting and viability were checked using a Countess II FL Automated Cell Counter (Thermo Fisher)

1 at the beginning and end of each experiment using representative wells. Viability was always
2 >95%.

3 Cells were cultured to 80% confluence and then treated with LPS (1 µg/ml) for 24 h. The cells
4 were treated with β1, β2, β3 β4, or β6 purified proteins for 24 h alone or in combination with
5 LPS. Aliquots of β1, β2, β3 β4, and β6 were stored at –20°C in PBS. The purified congenitins
6 were thawed just before use and diluted in DMEM to desired concentrations. 20 µg of each
7 purified congenitin was added to cultures. After the treatments, cells were harvested for further
8 analyses.

10 2.5. MTT assay for cell viability

11 Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
12 bromide (MTT). Briefly, PANC-1 cells were seeded in 96-well plates in the normal culture
13 medium: 1×10^3 cells in 100 µL of medium per well in 200 µL of DMEM with FBS and
14 antibiotics. After overnight incubation, cells were treated with LPS alone, or each of the β1,
15 β2, β3 β4, and β6 purified protein as a combination of all five proteins at indicated doses in
16 two replicates. After the treatments, the cells were thoroughly washed with PBS (at least three
17 times) to avoid any interference of the phenolic compounds with the MTT. Next, 200 µL of
18 free red-phenol DMEM containing 1 mg/mL of MTT was added, and the cells incubated for 3
19 h. Viable cells with active metabolism convert MTT into formazone crystals (purple color),
20 which was solubilized with 200µL of DMSO, and the absorbance measured at test (570 nm)
21 and reference (690 nm) wavelengths using a microplate reader (iMark microplate reader,
22 BioRad).

24 2.6. Participant information

1 Fourteen healthy control subjects and nine type 2 diabetic patients were recruited for this
2 study. The subjects were unrelated and diagnosed from the coverage area of the ‘Pedro
3 Martínez’ hospital (A.G.S. North East Granada, Spain). Samples consisted of venous blood
4 collected from the cubital vein in 4 ml lithium–heparin tubes. All study procedures were
5 performed using a protocol previously approved by the ethics committee of ‘Pedro Martínez’
6 hospital (A.G.S. North East Granada, Spain). All participants provided full written and
7 informed consent for the procedures.

9 *2.7. Whole blood culture*

10 Venous blood was drawn into lithium–heparin tubes (BD Vacutainer System, Heidelberg,
11 Germany) in the morning. Participants fasted for 12 h before blood collection (water only, no
12 food intake). Within 3 h, whole unseparated blood was diluted 1:3 with DMEM and HEPES
13 2.4% (Invitrogen, Karlsruhe, Germany), and agitated gently in 50 ml tubes (Greiner Bio-one,
14 Solingen, Germany); 1 ml aliquots were seeded in 24-well plates (Nunc, VWR International
15 GmbH, Langenfeld, Germany) and cultured for 24 h at 37°C and 5% CO₂. From each blood
16 draw, we performed triplicate incubations in parallel with positive and negative controls —
17 separate cultures that included each purified β -conglutin (20 μ g). The same lots of purified β -
18 conglutin (β 1, β 2, β 3, β 4, β 6), LPS and PBS were used for all experiments. Blood cultures
19 were removed from each well and centrifuged at 700 g for 5 min at 20°C. The supernatants
20 were aliquoted and stored at –20°C until further analysis.

21 Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by
22 density-gradient centrifugation on Histopaque 1077 (Sigma, St. Louis, MO), washed three
23 times in Hanks’ balanced salt solution (Life Technologies, Grand Island, NY), and
24 resuspended in complete medium consisting of RPMI 1640 (Sigma) supplemented with

1 penicillin, streptomycin, and L-glutamine (100 U/ml, 100 µg/ml, and 2 mM, respectively)
2 (Sigma).

3 4 2.8. Polymerase chain reaction (PCR) array for diabetes-inflammatory response

5 Total RNA was extracted from PBMC using an RNeasy Mini Kit (Qiagen). 2 µg total RNA
6 was converted to complementary DNA (cDNA) using an RT² First-Strand kit (Qiagen). The
7 resulting cDNA was mixed with RT² SYBR Green qPCR (Qiagen) and aliquoted into a
8 commercially available diabetes-obesity and inflammation pathway-specific real-time RT²
9 Profiler PCR Array (PAHS-023Z, Qiagen). Quantitative real-time PCR (qPCR) was
10 performed using a Light Cycler[®] 480 (Roche), where samples were run as duplicates for 40
11 cycles: 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 15 s, and 40 cycles of 60°C for 1
12 min. Cycle thresholds were measured, and the relative expression of genes was calculated by
13 comparing Ct values. The difference in gene expression was evaluated by calculating the
14 fold-change in expression levels based on at least a 2-fold up or down change in comparison
15 to the gene expression levels of the control group. All gene expression levels were normalized
16 to the housekeeping genes — Actin beta, hypoxanthine phosphoribosyl transferase 1, beta-2-
17 microglobulin, and glyceraldehyde-3-phosphate dehydrogenase — which did not vary
18 significantly between the study groups.

19 20 2.9. Quantitative real-time PCR

21 Real-time quantitative PCR technology was used to assay IL-1β, iNOS, and TNF-α mRNA
22 expression from each experimental group. Total RNA was isolated from PANC-1 cells using
23 the RNeasy Tissue RNA isolation kit (Qiagen). First-strand cDNA was synthesized using a
24 High-Capacity cDNA Archive Kit (Applied Biosystems). For gene expression assays, cDNA
25 was prepared, diluted and subjected to real-time PCR, and amplified using TaqMan

1 technology (LightCycler 480 quantitative PCR System, Roche). Primers and probes were
2 used from the commercially available TaqMan Gene Expression Assays (Hs01555410_m1,
3 Hs01075529_m1, Hs00174128_m1, respectively, Applied Biosystems). Relative changes in
4 gene expression levels were determined using the $2^{-\Delta\Delta C_t}$ method. The cycle number that
5 detected the transcripts (CT) was normalized to the cycle number for β -Actin
6 (Hs99999903_m1, Applied Biosystems, used as the housekeeping gene) detection, referred to
7 as ΔC_t , where the relative mRNA levels were presented as unit values of $2^{[CT(\beta\text{-Actin}) - CT(\text{gene of interest})]}$, having CT as the threshold cycle value defined as the fractional cycle
8 number at which the fluorescent target signal passes a fixed threshold above the baseline.
9
10 PCR efficiency was determined by TaqMan analysis on a standard curve for targets and
11 endogenous control amplifications, which were highly similar.

12 13 *2.10. ELISA assays for cytokines, INF- γ and TNF-quantification*

14 After cell culture, the cells were counted and plated in six well plates (106 cells per well),
15 with two wells per group. All experiments were made in duplicate to confirm data and reduce
16 variation among samples.
17 After 24 h incubation, the treated culture media was removed and cells washed with 4°C PBS.
18 During protein extraction, the plates were kept on ice to avoid denaturation of cytokines. Each
19 well was filled with 100 μ L of buffer (150 mM sodium chloride, 1.0% NP-40, 50 mM Tris pH
20 8), supplemented with 1 μ L of protease inhibitor (Sigma), and remained in contact with the
21 cells for 15 s. The cells were then vigorously scraped from the bottom of the wells and
22 transferred to a micro centrifuge tube. The tubes were centrifuged in a refrigerated centrifuge
23 (4°C) at 12,000 rpm for 15 min. After centrifugation, the supernatant was collected and
24 diluted to a 1:4 ratio for the ELISA sets for IL-1 β , IL-2, IL-6, IL-8, IL12, IL-17, INF- γ , and

1 TNF- α (Diaclone) following the manufacturer's instructions. Data were statistically analyzed
2 using the t-test.

3 4 *2.11. Nitric oxide detection*

5 The total amount of NO production including nitrite/nitrate contents from cultured blood
6 supernatants of control subjects and T2D patients was measured following the recommended
7 protocol using a commercial assay kit ab65328 (Abcam, Cambridge, UK). Briefly,
8 supernatants were collected and centrifuged to remove cell debris. Plasma from all
9 experimental groups was deproteinized according to the manufacturer's instructions. An equal
10 amount (30 μ l) of samples and standards were loaded into 96-well microtiter plates. A volume
11 of nitrate reductase, enzyme cofactor, and assay buffer was added, followed by 1 h incubation
12 at room temperature by Enhancer, Griess Reagent R1 and Griess Reagent R2. Immediately
13 after incubation, absorbance at 540 nm was detected with a microplate reader (iMark Biorad),
14 and the value of the blank control (medium without cells) was subtracted. Total nitrite/nitrate
15 concentrations were calculated with the calibrated standard curve.

16 17 *2.12. Statistical analysis*

18 Values shown are expressed as means $\pm$ S.E.M. of the three individual experiments. Statistical
19 significance of the data was assessed using SPSS software by analysis of variance followed
20 by Dunnett analysis. P-values $\leq$ 0.05 were considered statistically significant.

21 22 **3. Results and Discussion**

23 *3.1. Purification of anti-inflammatory proteins*

24 Purification of β -conglutins was performed according to the set-up methodology of Jimenez-
25 Lopez et al. (2016). Elution was performed with the linear gradient of imidazole (0–250 mM).

1 The SDS–PAGE analyses of the eluted fractions showed a single protein band of
2 approximately 68 kDa (Fig. 1), where recombinant β -conglutins exhibited a level of purity
3 >95%, and a concentration of 10-25mg/mL. Analysis by immunoblotting using the anti- β -
4 conglutin protein antibody confirmed the identity of the recombinant protein (Fig. 1).

5 6 *3.2. Beta-conglutins do not affect the cellular viability of PANC-1 pancreatic cells*

7 Before assessing anti-inflammatory activity, we evaluated cell viability and the potential
8 cytotoxicity of the β -conglutins. To investigate whether LPS and β -conglutin isoforms have a
9 cytotoxic effect, an MTT assay was performed on PANC-1-pancreatic cells after separately
10 adding LPS plus each β -conglutin, as well as a mix of the five β -conglutins at increasing
11 concentrations to condition DMEM with FBS and antibiotic culture medium for 24 h. The
12 LPS plus individual β -conglutins had no significant effects on cell viability, nor did the mix of
13 β -conglutins (Table 1), when compared to the untreated control group. For all samples, even
14 though a degree of proliferative activity was observed, which was not significant, the cells
15 remained in the quiescent phase for longer. The addition of 10% FBS was used as a positive
16 control.

17 To complete the feasibility studies of the β -conglutins, cell viability was assayed using trypan
18 blue exclusion in PANC-1-pancreatic cells treated with increasing concentrations of LPS and
19 each β -conglutin, and the mix of conglutins, for 24 h. As expected, there were no differences
20 in cell viability after 24h of incubation in the presence of higher concentrations of each β -
21 conglutin, or in the mix of β -conglutins, which ranged from 10–20 μ g when compared to the
22 control (Table 1).

23 These results suggest that β -conglutins (individually or together) do not compromise the
24 integrity of PANC-1 pancreatic cells. Moreover, cell viability did not differ significantly from

the control assays upon exposure to higher concentrations of β -conglutins. Hence, β -conglutins did not affect membrane integrity in this cell model.

3.3. Participant information

The clinical parameters of the population studied are summarized in Table 2. As expected, the T2D patients showed classical parameters for BMI, and in the levels of fasting glycaemia ($p < 0.001$), blood pressure ($p < 0.03$), heart rate ($p < 0.001$), and HbA1c ($p < 0.001$), as expected compared to the control study group of healthy volunteers with no signs of T2D that were chosen as the control study group.

3.4. Effect of β -conglutin peptides on the inflammatory process

Different diseases, such as T2D, obesity, and metabolic syndrome, are associated with and chronically sustained by inflammation. Multiple mechanisms could be underlying inflammatory response diseases, where multiple stressor factors affect pancreatic islets function, and contributing to the pathology depending on aspects such as genetic background and environment (Halban et al., 2014). Diabetes is a chronic disease that occurs when pancreatic islets fail to produce sufficient insulin and/or the sensitivity of glucose-metabolizing tissues to insulin decreases.

During the establishment of T2D, chronic hyperglycemia may lead to organ damage.

Pancreatic beta cells mediate tissue damage and promote inflammatory responses together with immune system cells by releasing pro-inflammatory mediators and other cell attractants as chemokines, establishing a feed-forward process that further increases immune system cell content and promotes a chronic inflammatory state (Donath and Shoelson, 2011).

Increased levels of cytokine IL-1 β or iNOS as a consequence of stressor factors are among the main contributors to inflammatory development since IL-1 β -mediated pancreatic β -cell

dysfunction is involved in the pathogenesis of pancreatic β -cell dysfunction and establishment of T2D. For instance, sustained exposure of the pancreatic β -cell to IL-1 β activates the expression of iNOS resulting in excessive production of NO, which induces the expression of pro-inflammatory genes (Wang et al., 2010).

Therefore, we evaluated the ability of purified β -conglutins (β 1, β 2, β 3, β 4, and β 6) to modulate the expression levels of pro-inflammatory mediators (possible potential anti-inflammatory effects) as IL-1 β and iNOS mRNA in PANC-1-pancreatic cells (Fig. 2).

β 1+LPS, β 3+LPS, and β 6+LPS significantly suppressed iNOS mRNA expression in PANC-1 [−7.15, −7.97, and −7.41–fold, respectively, *versus* LPS treated culture cells] (Fig. 2A). In contrast, iNOS mRNA levels remained significantly high [+8.42, and + 6.93 - fold, respectively, *versus* LPS-treated culture cells] (Fig. 2A) when β 2+LPS and β 4+LPS were used in challenge assays. β 1, β 3, and β 6 did not alter mRNA levels of iNOS in PANC-1 cell culture (Fig. 2A). However, mRNA levels of iNOS significantly increased using β 2 [+8.08 - fold *versus* culture cells] and β 4 [+7.21–fold *versus* culture cells] (Fig. 2A).

In this regard, gene expression levels of IL-1 β followed a similar pattern to iNOS (Fig. 2). Figure 2B shows a significant reduction in mRNA expression levels of IL-1 β in PANC-1 cell when challenged with β 1+LPS, β 3+LPS, and β 6+LPS [−12.05, −11.64, and −12.16–fold (Fig. 2B), respectively, *versus* culture cells]. No differences in IL-1 β cytokine levels were observed when challenges were performed with β 1, β 3 and β 6 when compared to the control group (Fig. 2B). Nevertheless, a large induction of IL-1 β cytokine levels was observed after the LPS+ β 2 and LPS+ β 4 challenges [+6.42, and +6.93, respectively, *versus* culture cells] in comparison to LPS+ β 1, LPS+ β 3, and LPS+ β 6 challenge assays (Fig. 2B). In addition, the mRNA expression levels of IL-1 β remained significantly high when β 2 and β 4 were used in challenge assays [+7.64, and +7.65–fold (Fig. 2B), respectively, *versus* culture cells].

1 These results indicate that β -conglutins reduce the pro-inflammatory capacity of pancreatic
2 cells PANC-1 by diminishing iNOS and cytokine expression, thus assisting in the
3 amelioration of the inflammatory process. Furthermore, these results on PANC-1 cell are in
4 accordance with previous preliminary analysis showing comparative decreased expression
5 levels of iNOS and IL-1 β mRNA in T2D blood culture using β -conglutins challenges (Lima-
6 Cabello et al., 2017).

7 Challenge assays were also performed using a mix of β -conglutin isoforms
8 (β 1+ β 3+ β 6+ β 2+ β 4) in PANC-1 cell culture to measure variations of iNOS and IL-1 β levels
9 using qRT-PCR. The β -conglutin pool suppressed the induction levels of iNOS and the pro-
10 inflammatory mediator IL-1 β in the β -conglutin mix and LPS+ β -conglutin mix treated cells
11 [mix: -6.0, and -7.03-fold *versus* LPS-treated culture cells (Fig. 2A); LPS+mix: -5.02 and -
12 5.27-fold *versus* LPS treated culture cells (Fig. 2B)].

13 It has been well established that local production and/or systemic IL-1 β play an important role
14 in the progression of pancreatic tissue dysfunction, particularly β -cell apoptosis in T2D. This
15 process is produced by a sustained inflammatory stage initiated and amplified by IL-1 β -
16 mediated iNOS synthesis and NO production (Russell and Morgan, 2014). NLL β 1-, β 3-, and
17 β 6-conglutin proteins can reverse this state by diminishing the pro-inflammatory IL-1 β and
18 iNOS mRNA levels.

19 20 *3.5. β -conglutin peptides inhibit the production of multiple cytokines and inflammation* 21 *mediators*

22 Cytokines are implicated in the regulation of pancreatic β -cell function. Besides plasma
23 circulating cytokines, pancreatic β -cells produce various cytokines in response to different
24 physiological and pathological stimuli, playing key roles in regulating β -cells function (Wang
25 et al., 2002). Insulin resistance, increase the production of deleterious (pro-inflammatory)

cytokines in β -cells and plasma. In a stage of T2D progression, this abnormal situation is characterized by an imbalance in the profile of pro-inflammatory cytokines and mediators that play an important role in triggering β -cell dysfunction and T2D since crosstalk among cytokines in pancreatic β -cells or other tissues may be strongly involved in regulation of β -cell function (Tanabe et al., 2017). Thus, restoring the balance in favor of protective cytokines in β -cells and plasma could prevent and treat β -cell dysfunction and T2D progression.

Biological activities of peptides from legume seeds have been observed. However, only a few studies have characterized the anti-inflammatory effects of plant-derived short peptides, which are usually mediated through the regulation of pro-inflammatory nitric oxide, cytokines (i.e., interleukins), TNF- α , and INF- γ . Bioactive peptides in soybean flour significantly inhibited inflammatory mediators including NO, iNOS, COX-2, and TNF- α in LPS-induced macrophages (Ndiaye et al., 2012). Moreover, the peptide lunasin, a 43 amino acid peptide derived from the soybean 2S albumin seed protein, reduced the production of ROS in LPS-induced macrophages and inhibited the release of TNF- α and IL-6 (Millán-Linares et al., 2014; 2015).

We evaluated the potential anti-inflammatory effect of different β -conglutins (β 1, β 2, β 3, β 4, and β 6) and their capacity to modulate the concentration of pro-inflammatory mediators including TNF α , INF γ and cytokines IL-1 β , IL-2, IL-6, IL-8, IL-12, and IL-17 in PANC-1-pancreatic cells (Fig. 3, Supplementary Table 1 and 2). The cytokines and other mediators were estimated under basal conditions, after cell treatment with LPS, by challenging the cell culture with β -conglutins after LPS and by adding LPS+ β -conglutinin together.

The production (protein levels) of cytokines TNF α , INF γ and interleukins IL-1 β , IL-2, IL-6, IL-8, IL-12, and IL-17 proteins significantly increased (several fold) in LPS + β 2 and LPS + β 4 challenges [LPS + β 2: +29, +6, +23, +6.2, +59, +12, +94, +33–fold *versus* C, respectively] and [LPS + β 4 :+29, +6, +28, +3.8, +59, +12, +62, +32–fold *versus* C, respectively] (Fig. 3A

to H; supplementary Table 1); while the LPS+ β 1, LPS+ β 3, and LPS+ β 6 challenges resulted in a substantial reduction (several fold) in protein levels [LPS+ β 1: -28, -100, -8, -2, -49, -45, -127, -13-fold *versus* LPS treated cells, respectively; LPS+ β 3: -22, -400, -9, -3, -33, -10, -2.54, -2-fold *versus* LPS-treated cells, respectively; LPS+ β 6: -72, -122, -11, -3, -2000, -13, -338, -9-fold *versus* LPS treated cells, respectively] (Fig. 3A to H; supplementary Table 2).

β 1, β 3, and β 6 did not alter protein levels of TNF α , INF γ , or all analyzed interleukins in PANC-1 cell culture (Fig. 3). However, protein levels of TNF α , INF γ , and interleukins IL-1 β , IL-2, IL-6, IL-8, IL-12, and IL-17 significantly increased in challenges performed using β 2 [+39, +70, +8.4, +24, +38, +6, +78, +12 - fold *versus* C], and β 4 [+19, +6, +21, +6, +39, +12, +70, +40 - fold *versus* C] (Fig. 3A to H; supplementary Table 1). This data is in accordance with the results obtained using RT-qPCR, where PANC-1-cells had reduced mRNA expression levels of the pro-inflammatory interleukin IL-1 β (Fig. 2).

Furthermore, to study the combinatorial effect of the purified β -conglutin proteins on the protein levels of TNF α , INF γ , and interleukins IL-1 β , IL-2, IL-6, IL-8, IL-12, and IL-17, we performed assays in PANC-1 cell culture using a mix of the β -conglutin isoforms (β 1+ β 3+ β 6+ β 2+ β 4). Challenges using the β -conglutin mix + LPS strongly reduced the protein levels of all pro-inflammatory cytokines [-19, -4.8, -18, -5, -45, -108, -21, -10-fold *versus* LPS treated cultured cells, respectively] (Fig. 3A to H; supplementary Table 2) but no differences were observed using the β -conglutin mix in PANC-1 cells.

We demonstrated that NLL β 1-, β 3-, and β 6-conglutin proteins alone or by joint action decreased the production levels of pro-inflammatory mediators. This wide anti-inflammatory capacity may be able to cope with multiple situations that promote a feed-forward process in the establishment of a chronic inflammatory state that is advocated by T2D. Thus, β -conglutins may be able to reverse the harmful effects of an inflammatory course of various cellular states as different levels as described below.

1) A sustained elevated level of fatty acids that induce the release of IL-1 β , IL-6 and IL-8. This continued exposure of pancreatic β -cells to these kinds of cytokines promotes excessive production and release of ROS and inhibits of insulin secretion, which resulted in the apoptosis of the pancreatic β -cells (Imai et al., 2013). In this regard, challenging T2D blood with β 1, β 3, and β 6 isoforms decreased the mRNA expression level of IL-6 in PBMC samples [–27.5, –28.0, –27.0–fold *versus* T2D, respectively] (Table 3).

2) Pancreatic β -cell apoptosis induction by IL-1 β and IFN- γ is activated by ER stress (Gurzov et al., 2009).

3) Pancreatic islet apoptosis was induced by the synergistic action of TNF- α and IFN- γ through activation of Ca²⁺ channels, which promote NO synthesis and subsequently activate the ER stress pathway (Ramadan et al., 2011), leading to mitochondrial dysfunction and caspases activation (Chang, 2004).

4) The synergistic combination of two (IL-1 β + IFN- γ) or three (IL-1 β + IFN- γ + TNF- α) cytokines in pancreatic tissues increased NO production by directly increasing iNOS protein synthesis, or indirectly affecting mRNA expression of iNOS islets, resulting in islet cell destruction (Sekine et al., 2000).

In the current study, the mRNA expression levels of two of the three main apoptosis-inducer factors (TNF- α and IFN- γ) were declined in PBMC isolated from T2D blood samples after treatment with β 1, β 3, and β 6-conglutin isoforms [TNF α : –28, –32, –31-fold *versus* T2D, respectively; and INF γ : –6.09, –1.07, –5.8–fold *versus* T2D, respectively] (Table 3), which may have a positive effect on pancreatic β -cell survival (Salim et al., 2016).

5) IFN- γ positively affects IL-12 mRNA levels, while IL-12 acts as a signaling molecule for increasing IFN- γ synthesis (Liu et al., 2010). The reduction in IL-12 mRNA levels in T2D blood cells after treatment with β 1, β 3, and β 6 [–25.7, –27.4, –27.0–fold *versus* T2D,

1 respectively] (Table 3), may reduce IFN- γ synthesis (and increase of IL-12 production) and its
2 detrimental inflammatory effects.

3 6) Interleukin-17 (IL-17A) acts as a highly potent inflammatory cytokine and a potent T2D
4 inducer via ROS production, playing an important role in insulin resistance resulting in tissue
5 damage. In addition, IL-17 and IFN- γ have a synergistic collaboration to stabilize diabetes
6 (Marwaha et al., 2014). IL-17 has pleiotropic activities including the induction of various
7 inflammatory cytokines (e.g., IL-6 and TNF- α) and chemokines [e.g. monocyte
8 chemoattractant protein 1 (MCP1)] from a large variety of cells (Kawaguchi et al., 2004).
9 Thus, the reduction in IL-17 protein levels (Fig. 3) may contribute to the reduced pro-
10 inflammatory effects of IL-6 (Table 3) and TNF- α (Fig. 3, Table 3), pancreatic islet cell
11 apoptosis, and immune cell local recruitment to enhance the inflammation process in T2D
12 progression.

13 7) The pro-inflammatory effects induced by IL-1 β through synthesis induction of IL-6, IL-8,
14 TNF- α , and chemokines CCL2 production attracted macrophages and other immune cells to
15 pancreatic tissues, which enhance the feed-forward inflammation progression in islets
16 (Vanbervliet et al., 2002). This effect could be reduced by diminishing levels of IL-1 β
17 production (Fig. 3) and IL-6 (Table 3), IL-8, and TNF- α (Fig. 3, Table 3).

18 8) IL-2 and chemokine CCL5 induce the activation and proliferation of immune cells natural
19 killer (NK), which enhances pro-inflammatory molecular processes (Wu et al., 2017). In the
20 current study, the reduction in IL-2 and CCL5 mRNA levels (Figure 3, Table 3) by β 1, β 3,
21 and β 6 congenitins may help to reduce this pro-inflammatory effect.

22 9) The nuclear factor NF κ B is a key player in inflammatory diseases as it controls the global
23 inflammatory response. NF κ B regulates the expression of a plethora of pro-inflammatory
24 genes in most metabolic tissues, including enzymes [e.g. cyclooxygenase (COX)-2, 5-
25 lipooxygenase (LOX), iNOS], cytokines [e.g. interleukin (IL)-1, IL-6, IL-8, chemokines CCL2,

CCL5, and TNF], and adhesion molecules (e.g. ICAM-1, VCAM-1, cell cycle regulatory molecules, and angiogenic factors) (Lawrence, 2009). The activation of NFκB, particularly in patients with a chronic inflammatory state, is critically associated with a wide variety of human diseases, including diabetes, asthma, atherosclerosis, and cancer. In the current study, the mRNA expression level of NFκB1 (p50), a dimeric component of the NFκB complex, was strongly inhibited by β1, β3, and β6 [−20.6, −25.0, −26.3-fold *versus* T2D, respectively] (Table 3), which may contribute to a reduction in the formation and activation of the NFκB complex. This reduction in NFκB mRNA expression level may also be indirectly facilitated by the reduction in TNFα levels promoted by the same β-conglutin isoforms (Fig. 3, Table 3) since this cytokine is a potent inducer of NFκB expression (Lawrence, 2009). The activation of NFκB expression also induces the production of adhesion (i.e., ICAM-1), and chemotaxis (i.e., CCL2, CCL5) molecules (Naamane et al., 2007). Thus, a reduction in NFκB levels prompted by β-conglutins (Table 3) may facilitate the same effect on ICAM-1, chemokine CCL5 and the CCR2 receptor expression (Table 3) and contribute to diminishing inflammation molecular processes, thus reducing immune cell recruitment.

3.6. Effect of β-conglutin isoforms on NO in T2D blood culture

Nitric oxide is a signaling molecule that plays a key role in the pathogenesis of inflammation. NO is considered a pro-inflammatory mediator that induces inflammation when over-produced in abnormal situations. The link between pancreatic β-cell stress and inflammation in T2D activates the expression of a variety of pro-inflammatory genes of cytokines and chemokines (e.g., TNF-α, IL-6, IL-12, CCL2, CCL5, and other pro-inflammatory mediators such as NO), in addition to the excessive production of ROS. This pro-inflammatory cytokines expression would activate inflammatory signaling pathways and the attraction of

immune cells that further aggravate local inflammation, causing β -cell apoptosis and T2D (McDaniel et al., 1996).

NO production by inducible NO synthase (iNOS) is a sign of inflammation progression. The over-production of NO is detrimental for the host, leading to the development of inflammatory-related diseases (Fujimoto et al., 2005).

In the current work, The effect on the NO production was determined in T2D blood cell culture challenged with individual β 1, β 2, β 3, β 4, β 6 isoforms, or β -conglutins mix after LPS treatment for 24h, and also adding at the same time LPS+ β -conglutin isoforms or LPS+mix. LPS added to blood cell culture significantly increased NO production, compared to the control group (Fig. 4). A range of β -conglutin treatments was tested to see if they could reverse the effects of LPS on NO production. The blood cultures of patients with T2D had significantly elevated levels of NO compared to blood cultures from normal donors (Fig. 4), adding β 1, β 3, or β 6 conglutins to the T2D blood cultures able to reduce NO production [–34, –34.2, –34-fold, respectively, *versus* T2D blood culture alone], but adding β 2 or β 4 conglutins had no effect. Interestingly, adding the β -conglutin mix (β 1+ β 2+ β 3+ β 4+ β 6) to T2D blood cultures also strongly suppressed NO production [–63–fold *versus* T2D blood culture alone] (Fig. 4).

Soybean lunasin, a short 2S-albumin-derived peptide, has anti-inflammatory effects by inhibiting NO production in macrophages (de Mejia and Dia, 2009). However, this is the first time that a legume protein belonging to *L. angustifolius* has suppressed NO production in blood culture cells from T2D patients, with potential anti-inflammatory effects.

3.7. β -conglutin proteins inhibit chemotaxis and cell adhesion capacity

The pathogenesis of pancreatic islet inflammation in T2D is a complex process that involves immune cell infiltration, cytokine production, β -cell apoptosis, islet amyloid peptide

deposition and fibrosis (Montane et al., 2014). Inflammatory pathways contribute to impaired insulin-stimulated glucose uptake in adipose tissue due to TNF α and chemokines inhibition in the liver, and skeletal muscle cells, as well as diminished pancreatic production of insulin and dysregulation of hepatic gluconeogenesis and glycogenolysis (McArdle et al., 2013).

Expression levels of cytokines such as TNF α , IL-1 β , IL-6, and IL-18, and chemokines such as CCL2 (MCP-1) and CCL5 or RANTES, strongly contribute to localized inflammation and islet dysfunction in T2D by recruiting immune cells into adipose tissue and pancreatic islet, and driving insulin resistance and hyperglycemia. This recruitment of immune cells is enhanced by the vicious cycle of IL-1 β auto-stimulation, and amyloid peptide deposition that may interact with immune cells to promote IL-1 β synthesis (Turner et al., 2014).

We hypothesized that NLL β -conglutins might have a beneficial effect on the chemotaxis mechanism by modulating the CCR/CCL axis. To examine this, we evaluated the expression of CCR2 and chemokine CCL5 in PANC-1 pancreatic cells after 24 h of incubation with individual β -conglutinin isoform proteins. It is clear that β -conglutins markedly modulated the CCR/CCL axis. CCR2 expression decreased dramatically in the β 1, β 3, and β 6 challenges [–33.6, –34.6, –27–fold *versus* T2D PBMC, respectively], but there was no effect in the β 2 and β 4 challenges compared to T2D PBMC (Table 3). Similarly, the expression of the chemokine CCL5 declined in the β 1, β 3, and β 6 challenges [–16.0, –18.0, –19.0–fold *versus* T2D PBMC, respectively] (Table 3).

Thus, results indicate that treatment of T2D blood cultures with β 2 or β 4 conglutins increased the expression of both receptor CCR2 and chemokine CCL5 and, consequently, the capacity of chemotaxis to increase immune cell migration to inflammatory tissues. In contrast, treatment with β 1, β 3, or β 6 conglutins reduced CCR2 and CCL5 expression levels, which directly ameliorated the inflammatory state by inhibiting immune cell recruitment and migration, and reduced the levels of inflammatory mediators (iNOS, TNF α , INF γ),

interleukins mRNA and protein production (Fig. 3, Table 3), and NO production (Fig. 4). The inhibition of CCR2 and chemokines CCL2 and CCL5 significantly improves pancreatic pathology by increasing the number of pancreatic islets and the near complete reversal of islet hypertrophy (Cardona et al., 2014). In addition, pancreatic functional recovery may be a reflection of the reduced severity of diabetes, as insulin decreased CCR2 levels, improved the systemic response, and increased hepatic glycogen content to ensure normalization.

It is well established that chronic inflammation plays a critical role in the pathogenesis of various diseases such as T2D via multiple mechanisms. One such mechanism is the activation and increased expression of vascular cell adhesion molecules (i.e. ICAM-1, VCAM-1) that facilitate the adhesion, transmigration and recruitment of immune cells, particularly monocytes and T-cells, with further induction and release of pro-inflammatory cytokines, macrophages migration, and lipid oxidation that promote and sustain feed-forward inflammatory responses (Muller, 2013). Our results showed that, in addition to reducing the capacity to recruit immune cells by decreasing the expression of CCR/CCL axis mediators, conglutin isoforms $\beta 1$, $\beta 3$, and $\beta 6$ have the capacity to reduce chemotaxis due to the strong reduction in the expression of the cell adhesion molecule ICAM-1 in T2D blood samples [–28.7, –22.1, –26.8–fold *versus* T2D PBMC, respectively] (Table 3). Thus, joint modulation of chemotaxis and cell adhesion by lowering the expression of CCR/CCL axis and ICAM-1 molecules by β -conglutin proteins may reduce immune cell recruitment and hence ameliorate inflammatory progress. This effect would also facilitate the reduction in TNF α levels (Fig 3, Table 3) since this cytokine can induce ICAM-1 expression (Thichanpiang et al., 2014).

4. Conclusions

Islet inflammation contributes to the loss of β -cell function with T2D development and progression. In this study, treatments with particular NLL β -conglutin protein isoforms ($\beta 1$,

1 β 3, and β 6) to LPS-stimulated *ex vivo* (blood samples from T2D diagnosed patients), and in
2 vitro (PANC-1 pancreatic) culture cells assays promoted the attenuation of mRNA expression
3 in key pro-inflammatory mediators of β -cell damage in T2D including interleukin IL-1 β ,
4 chemokine CCL5 and chemokine receptor MCP-1, the adhesion factor ICAM-1, cytokines
5 (TNF α , INF γ), and mediator NF κ B1. In addition, protein production declined significantly
6 for pro-inflammatory cytokine TNF α , INF γ , IL-1 β , IL-2, IL-6, IL-8, IL-12, and IL-17.
7 Moreover, conglutins β 1, β 3, and β 6 inhibited NO production and mRNA and protein levels
8 of iNOS.

9 This study is the first to describe the anti-inflammatory effect of a legume protein family,
10 namely NLL β 1-, β 3-, and β 6-conglutin proteins, with strong evidence to suggest that NLL β -
11 conglutins play a role as a functional food component with novel therapeutic options for the
12 prevention and treatment of disease-related inflammation. Further studies will be conducted to
13 reveal the mechanisms underlying the NLL β -conglutins promoting these anti-inflammatory
14 effects.

16 **5. Acknowledgement**

17 This work was supported by the European Research Program MARIE CURIE (FP7-PEOPLE-
18 24 2011-IOF) for the grant ref. number PIOF-GA-2011-301550 to JCJ-L, KBS and JDA. JCJ-
19 L thanks the Spanish Ministry of Economy, Industry and Competitiveness for the grant ref.
20 number RYC-2014-26 16536 (Ramon y Cajal Research Program).

22 **6. Conflict of Interest Statement**

23 The authors have declared that no competing interests exist.

25 **7. Authors' Contribution**

Conceived and designed the experiments: JCJ-L, EL-C. Performed the experiments: JCJ-L,
EL-C. Analyzed the data: JCJ-L, EL-C, SM-S, SM, KHMS, KBS, JDA. Contributed
reagents/materials/analysis tools: JCJ-L, VA, JDA. Wrote the paper: all authors.

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FIGURE LEGENDS

Figure 1. Purification of conglutin proteins $\beta 1$, $\beta 2$, $\beta 3$, $\beta 4$, and $\beta 6$.

Five purified β -conglutin proteins were stained with Coomassie, with a high level of purity (>95%). Immunoblotting shows the same five purified β -conglutin proteins identified by the anti- β -conglutin antibody (asterisks). MW, molecular weight standard (kDa).

Figure 2. Effect of NLL $\beta 1$ -, $\beta 3$ -, $\beta 6$ -, $\beta 2$ -, and $\beta 4$ -conglutin proteins on mRNA expression of iNOS and IL1 β genes.

PANC-1-pancreatic cells were incubated for 24h with LPS alone, each purified β -conglutin proteins ($\beta 1$, $\beta 3$, $\beta 6$, $\beta 2$, $\beta 4$) alone or in combination with LPS. mRNA levels determined by real-time qRT-PCR of iNOS and IL1 β . The bar graph shows iNOS PANC-1-pancreatic cells mRNA relative levels (A) and IL1 β PANC-1-pancreatic cells mRNA relative levels (B). Data represent three independent experiments.

C: Untreated control culture cells; **LPS:** LPS-treated culture cells; **$\beta 1$, $\beta 3$, $\beta 6$, $\beta 2$, and $\beta 4$:** β -conglutin challenge culture cells; **$\beta 1$ +LPS, $\beta 3$ +LPS, $\beta 6$ +LPS, $\beta 2$ +LPS, $\beta 4$ +LPS:** β -conglutin+LPS challenge; **LPS+mix:** LPS-treated culture cells + $\beta 1$ + $\beta 3$ + $\beta 6$ + $\beta 2$ + $\beta 4$ challenge culture cells; **mix:** $\beta 1$ + $\beta 3$ + $\beta 6$ + $\beta 2$ + $\beta 4$ challenge culture cells. $p < 0.05$ represent statistically significant differences associated with each figure.

C: Untreated healthy control subject blood culture cells; **T2D:** Type 2 diabetic patient blood culture cells; **$\beta 1$, $\beta 3$, $\beta 6$, $\beta 2$, and $\beta 4$:** β -conglutin challenge in T2D blood culture cells.

$p < 0.05$ LPS *versus* C; $p < 0.05$ β -conglutin *versus* C; $p < 0.05$ LPS+ β -conglutin *versus* LPS; $p < 0.05$ LPS *versus* C; $p < 0.05$ LPS *versus* β -conglutin; $p < 0.05$ LPS+mix $p < 0.05$ mix *versus* β -conglutin; $p < 0.05$ mix *versus* β -conglutin.

Figure 3. Effect of NLL $\beta 1$, $\beta 3$ $\beta 6$, $\beta 2$, $\beta 4$ on the mRNA expression of cytokines TNF α , INF γ , IL-1 β , IL-2, IL-6, IL-8, IL-12, and IL-17.

PANC-1-pancreatic cells were incubated for 24h with LPS alone, β -conglutin proteins ($\beta 1$, $\beta 3$, $\beta 6$, $\beta 2$, $\beta 4$) alone or in combination with LPS. The bar graph shows PANC-1-pancreatic cells protein levels of TNF α (A), INF γ (B), IL-1 β (C), IL-2 (D), IL-6 (E), IL-8 (F), IL-12 (G), and IL-17 (H). Data represent three independent experiments.

C: Untreated control culture cells; **LPS:** LPS-treated culture cells; **$\beta 1$, $\beta 3$, $\beta 6$, $\beta 2$, and $\beta 4$:** β -conglutin challenge culture cells; **$\beta 1$ +LPS, $\beta 3$ +LPS, $\beta 6$ +LPS, $\beta 2$ +LPS, $\beta 4$ +LPS:** β -conglutin+LPS challenge; **LPS+mix:** LPS treated culture cells+ $\beta 1$ + $\beta 3$ + $\beta 6$ + $\beta 2$ + $\beta 4$ challenge culture cells; **mix:** $\beta 1$ + $\beta 3$ + $\beta 6$ + $\beta 2$ + $\beta 4$ challenge culture cells. $p < 0.05$ represent statistically significant differences associated with each figure.

$p^* < 0.05$ LPS *versus* C, $p^{**} < 0.05$ β -conglutin *versus* C, $p^\# < 0.05$ LPS+ β -conglutin *versus* LPS, $p^\$ < 0.05$ LPS *versus* C, $p^\heartsuit < 0.05$ LPS *versus* β -conglutin, $p^{***} < 0.05$ mix *versus* β -conglutin, $p^{*\heartsuit} < 0.05$ mix *versus* conglutin.

Figure 4. Effect of NLL conglutins $\beta 1$, $\beta 3$ $\beta 6$, $\beta 2$, and $\beta 4$ on NO production in T2D blood culture.

Whole blood cells were incubated for 24 h with each purified β -conglutin protein ($\beta 1$, $\beta 3$, $\beta 6$, $\beta 2$, $\beta 4$). NO levels were determined using a commercial assay kit. The bar graph shows NO production in blood culture supernatants from T2D and C (A). Data represent three independent experiments.

C: Untreated healthy control subject supernatant blood culture cells; **T2D:** Type 2 diabetic patient supernatant blood culture cells; **$\beta 1$, $\beta 3$, $\beta 6$, $\beta 2$, and $\beta 4$:** β -conglutin challenge T2D supernatant blood culture cells; **mix:** $\beta 1$ + $\beta 3$ + $\beta 6$ + $\beta 2$ + $\beta 4$ challenge T2D supernatant blood culture cells. $p < 0.05$ represent statistically significant differences associated with each figure.

1 $p^* < 0.05$ T2D *versus* C, $p^{**} < 0.05$ β -conglutin *versus* T2D, $p^{##} < 0.05$ β -conglutin *versus* C.

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Table 1. Cell viability and dose effects of each purified NLL β -conglutin proteins (β 1, β 3 β 6, β 2, β 4).

Cytotoxicity of β 1, β 3, β 6, β 2, and β 4 on PANC-1-pancreatic cells. Cells were treated with 10 μ g and 20 μ g of sample proteins for 24h. Data represent three independent experiments.

Samples	10 μ g	20 μ g
Control	100 $\pm$ 3.8	100 $\pm$ 4.5
β 1	102.0 $\pm$ 15.8	97.2 $\pm$ 1.1
β 2	97.0 $\pm$ 2.2	98.3 $\pm$ 1.5
β 3	93.0 $\pm$ 3.1	98.0 $\pm$ 12.4
β 4	103.0 $\pm$ 6.2	99.7 $\pm$ 2.0
β 6	99.0 $\pm$ 23.6	104.0 $\pm$ 3.1
Mix (β 1+ β 3+ β 6+ β 2+ β 4)	88.8 $\pm$ 3.1	100.0 $\pm$ 3.4
LPS	90.1 $\pm$ 15.6	99.0 $\pm$ 1.3
LPS+ β 1	114.0 $\pm$ 3.0	88.0 $\pm$ 0.1
LPS+ β 2	114.0 $\pm$ 0.3	88.0 $\pm$ 2.2
LPS+ β 3	116.0 $\pm$ 5.1	92.0 $\pm$ 1.3
LPS+ β 4	113.0 $\pm$ 1.3	87.0 $\pm$ 0.1
LPS+ β 6	114.0 $\pm$ 3.2	97.2 $\pm$ 3.5
LPS+Mix	117.0 $\pm$ 3.4	116.0 $\pm$ 5.8

Table 2. Characteristics of the study groups.

Values represent the median (25th percentile, 75th percentile). To detect differences between groups, we used analysis of variance Significant *P*-values (<0.01) are marked with an asterisk. BMI, body mass index; HbA1c, glycosylated hemoglobin 1c; BPM, beats per minute; T2D, type 2 diabetes.

Patient characteristics	Control group	T2D	<i>P</i> -value
Male	14	14	—
Age	50 (40, 53)	48 (45, 58)	0.007
Fasting glycaemia (mg/dL)	83 (80, 91)	165 (132, 198)	<0.001
Blood pressure (mmHg)	12/7 (11/7, 12/7)	15/8 (14/8, 15/7)	<0.001
BMI (kg/m ²)	24.8 (23.0, 26.6)	33.3 (27.5, 46.7)	<0.001
Heart rate (bpm)	64 (65, 76)	84 (96, 80)	<0.001
HbA1c (%)	5.5 (5.4, 5.7)	6.7 (6.0, 7.4)	<0.001

Table 3. Effect of NLL conglutin proteins β 1, β 3 β 6, β 2, and β 4 on inflammation diabetes-related genes in isolated PBMC of type 2 diabetic patients.

Numbers represent fold-change obtained from real-time PCR array data analysis of inflammation diabetes-related genes CCL5, CCR2, ICAM-1, IFN- γ , TNF- α , NF- κ B1, IL-6, and IL-12. Treatments with β 1, β 3, β 6, β 2, and β 4, resulted in a significant up- or down-regulation of these eight pro-inflammatory genes compared to control patients. Data represent three independent experiments. The p -value ($P<0.05$) represents statistically significant differences, and they are included in italic.

Gene name	Gene acronym	T2D	β 1	β 3	β 6	β 2	β 4
Chemokine (C-C motif) ligand 5	CCL5	23.940 <i>0.031</i>	7.900 <i>0.032</i>	5.940 <i>0.036</i>	3.990 <i>0.035</i>	31.940 <i>0.031</i>	29.970 <i>0.032</i>
Chemokine (C-C motif) receptor 2	CCR2	34.650 <i>0.030</i>	1.010 <i>0.033</i>	0.010 <i>0.031</i>	1.060 <i>0.032</i>	34.760 <i>0.031</i>	33.980 <i>0.034</i>
Intercellular adhesion molecule 1	ICAM-1	28.860 <i>0.010</i>	0.100 <i>0.012</i>	6.800 <i>0.011</i>	3.197 <i>0.009</i>	28.950 <i>0.012</i>	32.510 <i>0.010</i>
Interferon, gamma	IFN γ	2.360 <i>0.018</i>	-3.730 <i>0.017</i>	3.430 <i>0.019</i>	-3.440 <i>0.017</i>	37.70 <i>0.018</i>	29.970 <i>0.019</i>
Tumor necrosis factor	TNF- α	34.390 <i>0.019</i>	5.940 <i>0.018</i>	2.400 <i>0.019</i>	2.710 <i>0.020</i>	32.810 <i>0.019</i>	23.700 <i>0.019</i>
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NF κ B1 (p50)	27.930 <i>0.030</i>	7.370 <i>0.0320</i>	2.950 <i>0.031</i>	1.600 <i>0.032</i>	23.190 <i>0.033</i>	21.940 <i>0.033</i>
Interleukin 6	IL-6	29.990 <i>0.001</i>	2.490 <i>0.002</i>	1.840 <i>0.003</i>	2.000 <i>0.001</i>	27.970 <i>0.001</i>	24.970 <i>0.002</i>
Interleukin 12	IL-12	29.970 <i>0.021</i>	4.260 <i>0.023</i>	2.540 <i>0.027</i>	2.990 <i>0.027</i>	30.750 <i>0.027</i>	34.970 <i>0.027</i>

SUPPLEMENTARY MATERIAL

Supplementary Table 1. Fold-change in mRNA expression level of pro-inflammatory cytokines.

Numbers represent fold-changes calculated *versus* control from data in Figure 3. Positive values mean gene up-regulation.

Cytokines	LPS	β2	β4	β1+LPS	β3+LPS	β6+LPS	β2+LPS	β4+LPS	Mix+LPS
TNF-α	36	39	19	1.2	1.5	0.4	29	29	1.2
INF-γ	9	6	6	0	0	0	6	6	1
IL-1β	21	24	21	2	0.4	1.9	23	28	0.9
IL-2	6	8.4	5.7	1.6	2.03	0.9	6.2	3.8	0.9
IL-6	71	78	39	0	1	0	59	59	0
IL-8	12	12	12	0.2	1	0.9	12	12	0
IL-12	70	70	70	0.5	0.2	0.2	94	62	3
IL-17	32	38	40	2	4	3	33	32	1

Supplementary Table 2. Fold-change in mRNA expression level of pro-inflammatory cytokines.

Numbers represent fold-changes calculated *versus* LPS from data in Figure 3. Negative values mean gene down-regulation.

Cytokines	β1+LPS	β3+LPS	β6+LPS	Mix+LPS
TNF-α	−28	−22	−72	−19
INF-γ	−100	−400	−122	−48
IL-1 β	−8	−9	−11	−18
IL-2	−2	−3	−3	−5
IL-6	−49	−33	−2000	−45
IL-8	−45	−10	−13	−1.8
IL-12	−127	−2.54	−338	−21
IL-17	−13	−2	−9	−10

Figure 1
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Figure 3_Continued
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