International Dairy Journal xxx (2013) 1–9

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Immunomodulatory activities of whey β-lactoglobulin tryptic-digested fractions

Q2 Javier Rodríguez-Carrio [a], Ayoa Fernández [b], Francisco A. Riera [b], Ana Suárez [a,*]

[a] Area of Immunology, Department of Functional Biology, University of Oviedo, 33006 Asturias, Spain
[b] Department of Chemical Engineering and Environmental Technology, University of Oviedo, 33006 Asturias, Spain

ARTICLE INFO

Article history:
Received 26 March 2013
Received in revised form
31 May 2013
Accepted 1 July 2013

ABSTRACT

Although bioactive peptides can be obtained from whey protein hydrolysis, data about their immune effects are inconclusive. The in vitro effects on the human immune system of different β-lactoglobulin tryptic-digested fractions separated by membrane filtration were evaluated, analysing the proliferation, cytokine secretion and differentiation of human peripheral blood cells. This procedure allowed the enrichment of peptide sets with specific features, suggesting that the differences in the peptidic composition were related to their immune function. The fraction enriched in large and acid peptides was able to induce Th1 responses, whereas fractions containing short peptides were involved in monocyte activation increasing TNFα secretion. Moreover, one of these fractions was able to induce TGFβ secretion and regulatory T cell differentiation, key mechanisms by which whey-derived peptides could impair allergic and inflammatory responses. No impact on immune cells was triggered by the unfractionated hydrolysate, supporting the use of membrane-fractionating techniques for obtaining immune bioactive-enriched fractions.

1. Introduction

Milk whey was formerly considered as a waste product from the cheese industry. However, new evidence on its nutritional and potential bioactive functions has led to the consideration of this product as a source of valuable ingredients for the food industry. In fact, it has been reported that peptides with several health-promoting activities may be obtained from bovine whey (Riera, Fernández, & Muro, 2012; Smithers, 2007).

It is known that primary sequences of whey proteins may contain many biologically active peptides, specific short protein fragments that have positive effects on human health apart from their nutritional characteristics (Kitts & Weiler, 2003). These peptides show no effects when contained within the whole protein sequence, but they can be released by enzymatic proteolysis. Since the levels of bioactive peptides released during digestion of bovine whey proteins in adults is probably too low to develop effects on human health in vivo (Gauthier, Pouliot, & Saint-Sauveur, 2006), regularly consumed dairy products could be supplemented with specific bioactive peptide-enriched fractions. In this sense, enzymatic hydrolysis followed by membrane fractionation (Pihlanto & Korhonen, 2003) could supply enriched fractions with different biological effects.

Bioactive peptides from whey proteins, mainly β-lactoglobulin (β-LG), are reported to have opioid (Antila et al., 1991), antimicrobial (Pellegrini, Dettling, Thomas, & Hunziker, 2001), antioxidative (Pena-Ramos & Xiong, 2001), antithrombotic (Pihlanto & Korhonen, 2003), hypocholesterolaemic (Madureira, Tavares, Gomes, Pintado, & Malcata, 2010), antihypertensive (Mullally, Meisel, & FitzGerald, 1997; Pihlanto-Leppälä & Korhonen, 1998), mineral-binding (Pihlanto & Korhonen, 2003) as well as immune effects (Pihlanto & Korhonen, 2003).

Recently, several studies have focused on the immunomodulatory activity of whey protein hydrolysates by assessing their effects on lymphocyte activation and proliferation, cytokine secretion, antibody production and promoting phagocytic activity (Gauthier et al., 2006). However, different and even opposite immunomodulatory effects have been described, probably due to the involvement of different bioactive peptides. Among the reported immunomodulatory effects, there are suppressive activities. These effects are of growing interest since they could have a role in preventing or improving common diseases whose prevalence have increased in recent decades, such as atopic allergies or inflammatory pathologies (von Mutius, Fritzsch, Weiland, Roll, & Magnussen, 1992). In this scenario, anti-inflammatory cytokines and regulatory T cells (Treg) may be important mediators, since they are

* Corresponding author. Tel.: +34 98510 2789.
E-mail address: anasua@uniovi.es (A. Suárez).

0958-6946/$ – see front matter © 2013 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.idairyj.2013.07.004

competent suppressors of effector immune responses, mainly mediated by T helper (Th) cells (Elkord, 2008). Accordingly, some nutraceuticals have been shown to impact on Treg numbers or function both in vivo and in vitro (Issazadeh-Navikas, Teimer, & Bockermann, 2012; Wang et al., 2012).

In line with this immunoregulatory effect, the hydrolysis of whey protein has been considered an effective way to produce hypoallergenic milk formulae, adequate to prevent the development of allergy to cows' milk proteins in children, as well as in adults at risk (Szajewska & Horvath, 2010; von Berg et al., 2008). In this sense, enzymatic cleavage of whey proteins has been considered the main mechanism for avoiding immune adverse reactions, since it was assumed that allergenic epitopes were mostly diminished by the enzymatic treatment (Knipping et al., 2012). However, several studies highlight the possibility that specific immunoregulatory mechanisms may also have a role in the hypoallergenic profile (Pecquet, Bovetto, Maynard, & Fritsche, 2000). That is, as occurs with peripheral immune tolerance, hypoallergenicity could not only be maintained by a passive mechanism (hydrolysis of the allergenic proteins), but also by an active immunoregulatory effect, such as the generation of suppressive cytokines and/or regulatory cells after the interaction of immune cells with specific peptides.

The main aim of the present study was to evaluate the potential immunomodulatory activities of different peptidic fractions obtained after membrane separation of a β-LG tryptic hydrolysate, analysing the effect on human peripheral blood immune cell proliferation, cytokine production and lymphocyte differentiation.

2. Material and methods

2.1. Preparation of different bovine β-LG fractions

The total hydrolysate (TH) sample was obtained after the enzymatic hydrolysis of bovine β-LG (Davisco Food Co., city, MN, USA) with modified trypsin (T1426.1G, Sigma, St. Louis, MO, USA). The protein content of the commercial substrate was 97.9% (*w*/*w*), representing 91.5% β-LG, and the trypsin was treated with L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) to reduce chymotrypsin activity. The hydrolysis was carried out under the optimum temperature and pH conditions for enzyme activity, i.e., 37 °C and 8.0, respectively, and at a final enzyme/substrate ratio (E/S) of 1/250. A 2.5 L solution containing 15 g L^{-1} of the substrate was used for hydrolysate preparation as previously described by Fernández Suárez, Zhu, FitzGerald, and Riera (2013) with few modifications. The TH sample was taken after 24 h of reaction.

The tryptic hydrolysate was filtered using a Pellicon 2 mini holder (Millipore, city/state, USA) equipped with three different tangential flow membrane cassettes: two polyethersulfone membranes having cut offs of 1 kDa (Sartorius; Göttingen, Germany) and 5 kDa (Millipore; city, MA, USA), respectively, and a stabilised cellulose membrane of 2 kDa cut off (Sartorius; Göttingen, Germany). Each membrane has a filtration area of 0.1 m^2. The fractionation experiments were carried out with no salt addition and at pH 8.0 according to Fernández et al. (2013), Fernández and Riera (2013a). Transmembrane pressure and the temperature of the hydrolysate were set to 7.5 × 10^4 Pa and 37 °C, respectively. Fractions denoted by PES-1, PES-5 and HYD-2 correspond with the permeate streams obtained using the 1 kDa polyethersulfone membrane, 5 kDa polyethersulfone membrane and 2 kDa Hydrosart membrane, respectively. Finally, the fraction composed of the peptides rejected by the 5 kDa polyethersulfone membrane, after a continuous diafiltration process, was termed the DR fraction. Distilled water was added to the hydrolysate during the diafiltration process at the same rate as the permeate was generated until a diafiltration volume of 7.5 L was reached. All the samples were then freeze-dried and stored at −20 °C prior their use.

2.2. Analysis of peptide composition

All the freeze-dried samples were analysed using a reversed phase-high performance liquid chromatography (RP-HPLC) system (Agilent 1200 series, Agilent Technologies; city, CA, USA) and the peptides present in the TH chromatographic profile (Fig. 1A) were identified by mass spectrometry (MS) analysis as previously described (Fernández & Riera, 2013b).

Table 1 shows the physico-chemical characteristics of the identified peptides, which are responsible for membrane–peptide interactions in this fractionation process. The molar concentration of the peptides was calculated from the peak area in the chromatograms according to Eq. (1) (Muñoz-Tamayo et al., 2012).

$$x_j = 1 \times 10^6 \left(\frac{A_j}{\varepsilon_j \cdot l \cdot v} \right) \cdot f \qquad (1)$$

where x_j (μM) is the concentration of the peptide *j*, A_j (AU define AU × min) the area of the HPLC peak of peptide *j* at 214 nm, *l* (0.6 cm) the path length of the UV cell, *v* (20 μL) the injection volume, *f* (1000 μL min^{-1}) the flow rate and ε_j (AU M^{-1} cm^{-1}) the molar extinction coefficient of peptide *j* at 214 nm calculated according to Kuipers and Gruppen (2007).

2.3. Peripheral blood mononuclear cell isolation

Human peripheral blood mononuclear cells (PBMC) were obtained from healthy donors buffy coat preparations (Blood Transfusion Centre, Oviedo) by centrifugation over Ficoll-Hyplaque gradient (Lymphoprep™, PAA Laboratories GmbH, Cölbe, Germany). After washing twice with sterile phosphate buffered saline (PBS), PBMC were resuspended at 2 × 10^6 cells mL^{-1} in RPMI 1640 medium (BioWhittaker; Verviers, Belgium) supplemented with 10% heat-inactivated foetal calf serum (FCS; PAA Laboratories GmbH) and 100 μg mL^{-1} streptomycin and ampicillin (Sigma). Blood donors (n = 12) were healthy adults, regular consumers of dairy products, with neither pathology nor treatment at the time of donation. Approval for this study was obtained from the Regional Ethics Committee for Clinical Investigation (Asturias, Spain).

2.4. PBMC proliferation assay

Peptide fractions were dissolved in sterile complete RPMI 1640 (BioWhittaker) and were kept at 4 °C for 1–2 h before culture assays. The solubility of the samples for the cell cultures was checked prior to the assays and it as always complete. Then, peptide solutions were added to 2 × 10^6 PBMC cells in 96-well round-bottom plates and a range of concentrations of 0.1–10^4 μg mL^{-1} were tested (each concentration was assayed in quadruplicate). No significant differences in the pH of the culture medium were detected at the studied concentrations. The assay was performed under resting and phytohaemagglutinin (PHA)-stimulating (2.5 μg mL^{-1}; Sigma) conditions. The plates were incubated at 37 °C in a 5% CO_2 atmosphere. On day 4 of culture, the cells were pulsed with 1 μCi ^{3}H-thymidine (Perkin Elmer; Boston, MA, USA) per well and, after 16 h, the cells were harvested onto glass microfibre filters and proliferation was quantified using a Wallac Scintillation Liquid Counter (Perkin Elmer). Results were determined as a stimulation index (SI), which was calculated as the ratio between the mean counts per minute (cpm) values measured in each culture condition and the mean cpm values measured in unstimulated wells.

Please cite this article in press as: Rodríguez-Carrio, J., et al., Immunomodulatory activities of whey β-lactoglobulin tryptic-digested fractions, *International Dairy Journal* (2013), http://dx.doi.org/10.1016/j.idairyj.2013.07.004

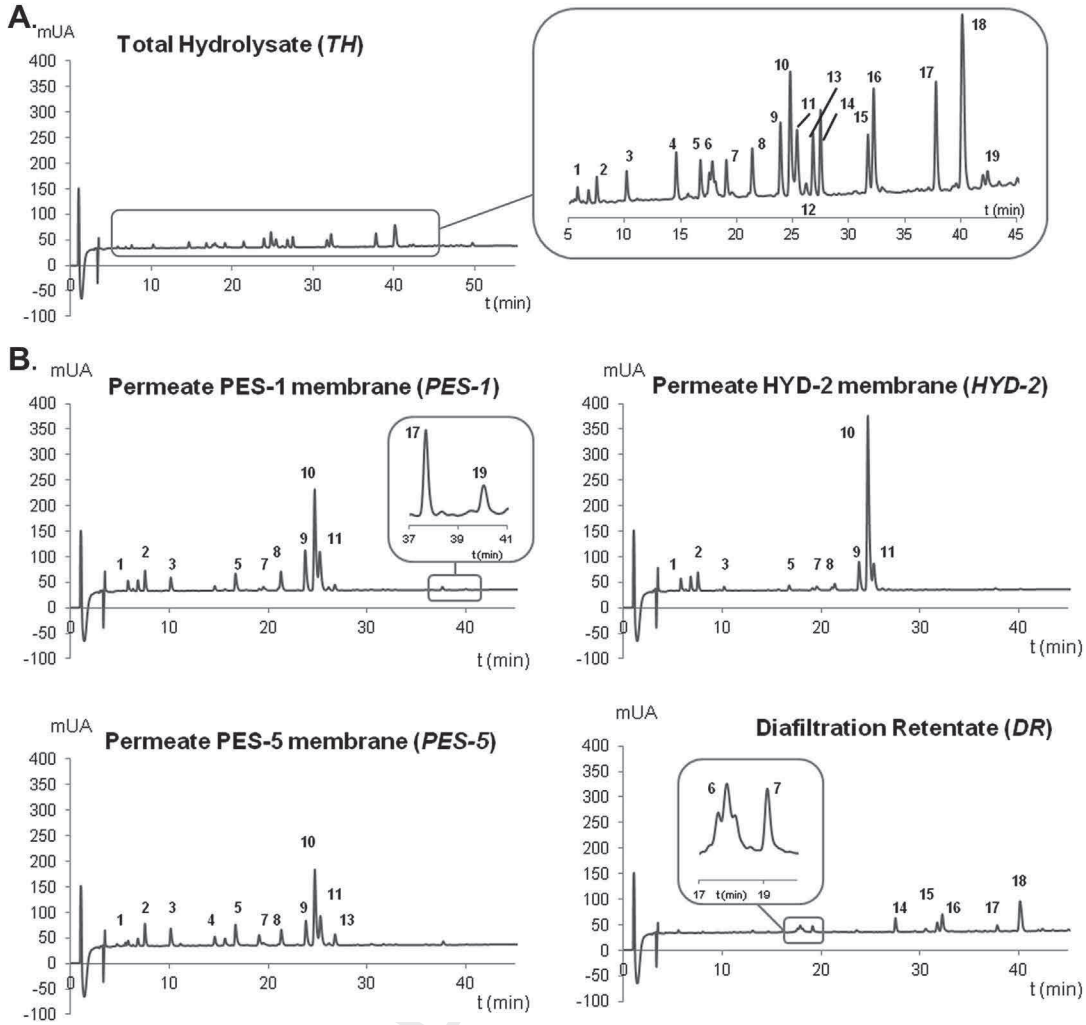

Fig. 1. Chromatographic profiles of the β-lactoglobulin fractions studied. Panel A: chromatographic profile of the total hydrolysate before membrane separation; peptides are identified in Table 1. Panel B: chromatographic profiles of the fractions obtained after permeation experiments with different membranes.

2.5. Cytokine analyses

The supernatants from 5 day cultured PBMC were collected and stored at −20 °C until cytokine measurement. Levels of human tumour necrosis factor alpha (TNFα), interferon gamma (IFNγ) and transforming growth factor beta (TGFβ) were measured using commercial OptEIA kits (BD Biosciences; Heidelberg, Germany), according to the manufacturer's instructions. Detection limits for TNFα, IFNγ and TGFβ1 were 1.95 pg mL^{-1}, 1.17 pg mL^{-1} and 31.5 pg mL^{-1}, respectively. All assays have a within-run imprecision (coefficient of variation) of <7%, and a between-run coefficient of variation of <10%. Results are the mean of four different donors. All the peptidic fractions at the different assayed concentrations showed undetectable levels of these cytokines, thus excluding the possibility of direct binding of cytokines to sample peptides or cross-reactivity in the cited immunoassays.

2.6. Flow cytometry analysis

To determine regulatory T cell population (Treg), phenotype of PBMC after culture with the different peptidic fractions was studied by flow cytometry. Cells were cultured as previously described and, after 5 days, they were collected and washed twice with PBS. Then, cells were incubated for 30 min at 4 °C in the presence of a viability dye (Fixable Viability Dye eFluor 450, eBioscience, San Diego, CA, USA), so as to exclude non-viable cells from the flow cytometry analysis. After that, extracellular staining with anti-CD4 APC (BD Biosciences), anti-CD25 FITC (BD Biosciences) and anti-CD127 PE-Cy7 (eBioscience) were performed for 30 min at 4 °C and then cells were washed again. Next, cells were fixed and permeabilised (FOXP3/transcription factor staining buffer set, eBioscience), followed by an intracellular staining with anti-FOXP3 PE (clone PCH101, eBioscience), or a PE isotype-conjugated irrelevant antibody (BD Biosciences Pharmingen, San Diego, CA, USA) as a negative control. Finally, cells were washed again and analysed in a FACS Canto II cytometer (BD Biosciences), equipped with three lasers (excitation 405 nm, 488 nm and 633 nm) using the FACS Diva v.2.4 software (BD Biosciences).

To quantify Treg population, lymphocytes were gated using forward- and side-scatter parameters. Pacific Blue-stained cells were excluded, since they were non-viable. $CD4^+$ cells were then gated and CD25, FOXP3 and CD127 expression was established according to isotype control fluorescence. A minimum of 30,000 $CD4^+$ T lymphocytes was acquired. Data were expressed as a percentage of viable $CD4^+$ T cells.

Please cite this article in press as: Rodríguez-Carrio, J., et al., Immunomodulatory activities of whey β-lactoglobulin tryptic-digested fractions, *International Dairy Journal* (2013), http://dx.doi.org/10.1016/j.idairyj.2013.07.004

Table 1
Physico-chemical characteristics of the peptides contained in the β-lactoglobulin total hydrolysate sample.

Peak number[a]	Peptidic sequence (MS analysis)	Sequence[b]	Molecular mass (Da)	pI[c]	A/B/N[d]	Charge[e] pH 8	$H\varphi_{av}$ (kcal res^{-1})[f]
1	ALK	f(139–141)	330.43	8.8	B	1	1.55
2	FDK	f(136–138)	408.45	5.84	N	0 (−)	1.38
3	IIAEK	f(71–75)	572.7	6	N	0 (−)	1.63
4	IDALNENK	f(84–91)	916	4.37	A	−1	0.95
5	GLDIQK	f(9–14)	672.78	5.84	N	0 (−)	1.14
6	WEND(G)ECAQK + WEND(G)ECAQKK	f(61–69) + f(61–70)	2125.65–2183.65–2311.76		A		
7	TPEVDDEALEK	f(125–135)	1245.31	3.83	A	−4	0.85
8	LIVTQTMK	f(1–8)	933.17	8.75	B	1	1.34
9	ALPMHIR	f(142–148)	837.05	9.8	B	1	1.54
10	VAGTWY	f(15–20)	695.77	5.49	N	0 (−)	1.46
11	IPAVFK	f(79–83)	673.85	8.75	B	0 (+)	2.02
12	TKIPAVFK	f(77–83)	903.13	10.00	B	2	1.76
13	VLVLDTDYK	f(92–100)	1065.23	4.21	A	−1	1.44
14	TPEVDDEALEKFDK	f(125–138)	1635.74	4.02	A	−4	0.97
15	WEND(G)ECAQKK + LSFNPTQLEEQCHI	f(61–70) + f(149–162)	2833.14 (2891.18)	4.91 (4.57)	A	−3 (−4)	0.85
16	WEND(G)ECAQK + LSFNPTQLEEQCHI	f(61–69) + f(149–162)	2704.97 (2763.01)	4.48 (4.25)	A	−2 (−3)	0.82
17	SLAMASSDISSLLDAQSAPLR	f(21–40)	2117.4	4.21	A	−1	1.01
18	VYVEELKPTPEGDLEILLQK	f(41–60)	2313.67	4.25	A	−3	1.37
19	VAGTWYSLAMAASDISLLDAQSAPLR	f(15–40)	2708.08	4.21	A	−1	1.11

[a] Peak numbers according to the chromatogram in Fig. 1A.
[b] Amino acid interval within the sequence of the parent protein.
[c] Isoelectric points (pI) were calculated using the ExPASy Molecular Biology Server (http://www.expasy.org/).
[d] Classified as A: acidic (pI < 5), B: basic (pI > 7) and N: neutral (5 < pI < 7).
[e] Charge of the most abundant ion at pH 8.
[f] Average hydrophobicity calculated according to Bigelow (1967).

2.7. Statistical analyses

The Kolmogorov–Smirnov test was used to study normal distribution of the data. Since data were normally distributed, parametric methods were performed. Differences between samples and controls in stimulation indices, cytokine levels and Treg frequencies were determined using one-way analysis of variance (ANOVA) and *t*-Dunnet multiple comparisons test. A p-value <0.05 was considered statistically significant. SPSS 15.0 and GraphPad Prism 5.00 (GraphPad Software, Inc., La Jolla, CA, USA) were used for statistical analysis.

3. Results and discussion

3.1. Peptidic composition of the β-LG fractions

The evaluation of the chromatographic profile of TH sample (Fig. 1A) and the different fractions (Fig. 1B), and their peptidic composition, presented in Table 2, showed that most larger peptides were rejected by PES-1, HYD-2 and PES-5 membranes, whereas those having a molecular mass lower than 1 kDa were mainly transmitted to the permeate stream. In contrast, the chromatographic profile corresponding to the DR sample showed an

Table 2
Peptidic composition of the freeze-dried fractions after membrane separation.

Peak number[a]	Peptide	Sequence[b]	Peptide mass distribution in fractions[c]				
			TH	DR	PES-5	HYD-2	PES-1
1	ALK	f(139–141)	0.70	1.29	2.48	3.35	4.40
2	FDK	f(136–138)	0.51	0.00	3.54	1.71	3.05
3	IIAEK	f(71–75)	1.72	0.00	8.54	0.89	5.79
4	IDALNENK	f(84–91)	2.53	0.00	4.24	0.00	1.68
5	GLDIQK	f(9–14)	2.10	0.00	10.72	1.36	7.93
6	WEND(G)ECAQK + WEND(G)ECAQKK	f(61–69) + f(61–70)	1.02	1.70	0.00	0.00	0.00
7	TPEVDDEALEK	f(125–135)	1.40	2.16	4.10	0.49	1.79
8	LIVTQTMK	f(1–8)	2.54	0.00	6.98	1.74	8.64
9	ALPMHIR	f(142–148)	1.76	0.52	5.28	3.61	8.50
10	VAGTWY	f(15–20)	1.07	0.00	5.38	7.49	6.68
11	IPAVFK	f(79–83)	1.87	0.00	7.25	3.83	9.28
12	TKIPAVFK	f(77–83)	0.33	0.00	0.47	0.26	0.71
13	VLVLDTDYK	f(92–100)	2.12	0.00	3.13	0.18	1.48
14	TPEVDDEALEKFDK	f(125–138)	2.55	3.72	0.00	0.00	0.00
15	WEND(G)ECAQKK + LSFNPTQLEEQCHI	f(61–70) + f(149–162)	1.14	1.61	0.00	0.00	0.00
16	WEND(G)ECAQK + LSFNPTQLEEQCHI	f(61–69) + f(149–162)	2.18	3.50	0.00	0.00	0.00
17	SLAMASSDISSLLDAQSAPLR	f(21–40)	4.58	2.32	1.27	0.00	1.03
18	VYVEELKPTPEGDLEILLQK	f(41–60)	8.90	13.37	0.00	0.00	0.00
19	VAGTWYSLAMASSDISSLLDAQSAPLR	f(15–40)	0.52	0.41	0.00	0.00	0.21
Mass percentage of the sample attributed to the identified peptides			39.53	30.60	63.39	24.93	61.16

[a] Peak numbers according to the chromatogram in Fig. 1A.
[b] Amino acid interval within the sequence of the parent protein.
[c] The value for each peptide represents the mass percentage of this peptide in the different fractions after freeze drying. Fractions are: TH, total hydrolysate; DR, diafiltration retentate; PES-5, 5 kDa polyethersulfone membrane permeate; HYD-2, 2 kDa Hydrosart membrane permeate; PES-1, 1 kDa polyethersulfone membrane permeate.

Please cite this article in press as: Rodríguez-Carrio, J., et al., Immunomodulatory activities of whey β-lactoglobulin tryptic-digested fractions, *International Dairy Journal* (2013), http://dx.doi.org/10.1016/j.idairyj.2013.07.004

almost complete absence of the smaller peptides such as f(136–138), f(71–75) or f(84–91). As observed in Table 2, the sum of all the identified peptides in the samples did not correspond with the total amount of freeze-dried fraction used to perform the analysis. These differences may be due to the presence of salts, unidentified peptides and single amino acids. Partial solubilisation was observed before the HPLC analysis (using Milli Q water as solvent), especially in the case of the HYD-2 fraction. However, solubilisation was checked as complete before the cell assays.

The differences in the peptidic composition of the isolated fractions indicated that the membrane filtration procedure used here allowed the isolation of whey fractions containing peptides with similar physico-chemical characteristics. Thus, the main peptides present in PES-1 fraction, f(1–8), f(79–83) and f(142–148), show a basic pI, positive charge and slightly higher values of hydrophobicity than those present in the fractions HYD-2 and PES-5, f(15–20) and f(9–14), f(71–75), respectively, which had lower pI values, were neutral and uncharged. In contrast, the main peptides in DR fraction, f(41–60) and f(125–138) had the lowest pI and were negatively charged. Finally, it is interesting to note that the peptide f(15–40), despite its charge and size, was unexpectedly present in the PES-1 fraction, although it was completely absent in PES-5 and HYD-2. Probably, peptide–peptide interactions could be a potential explanation of why this peptide is surprisingly present in this fraction, since aggregating properties have been reported (Groleau, Morin, Gauthier, & Pouliot, 2003). In fact, this peptide has been shown to interact with other peptides and this could impair the separation of peptide mixtures (Groleau et al., 2003). This peptide was also present in DR and TH samples, but accompanied in this case by a number of other larger, acid and negatively charged peptides. Thus, the large differences in peptide size, charge and hydrophobicity detected among fractions, suggest that they could induce different immune responses.

3.2. β-LG isolated fractions influence PBMC proliferation

To investigate the interaction between bovine β-LG peptides and the human immune system in vitro, PBMCs were used as a responder population to determine cellular proliferation and cytokine production post-exposure with the different peptidic fractions. Thus, PBMCs isolated from healthy individuals (resting cells) were cultured in medium alone, as a negative control, or with TH, PES-1, HYD-2, PES-5 and DR fractions or PHA, as a positive control (Table 3). In addition, PHA-stimulated PBMCs were also cultured with the different β-LG fractions to determine potential additive or inhibitory effects (Table 4).

Table 3 shows that the DR fraction increased the proliferation of resting cells in a dose-dependent manner within the range (10–10^3 μg mL^{-1}), while PES-1, HYD-2 and PES-5 were also able to induce a significant proliferation at intermediate doses (10^2–10^3 μg mL^{-1}), but exhibited a cytotoxic effect at the highest concentrations. Accordingly, microscopic observation revealed an important loss of cell integrity in these cultures, with cells surrounded by debris aggregates. Moreover, flow cytometric analysis showed a significant decrease of viable cells at the highest concentration (76.68 ± 3.64%, *t*-Dunnet post-hoc test: $p < 0.001$), compared with both control cells (94.92 ± 0.92%) and lower concentrations (10^3:93.73 ± 1.30% and 10^2: 94.03 ± 0.73%). Interestingly, no significant differences in cell proliferation were observed with the TH fraction, probably due to the presence of a mixture of heterogeneous peptides that could induce different and opposite effects, thus leading to overall null effects. This finding is in accordance with the idea that whey enzymatic cleavage by itself is not enough to get significant immune properties (Pihlanto & Korhonen, 2003).

On the other hand, only the highest concentrations of PES-1, HYD-2 or PES-5 provided an additive stimulation above the proliferation rate of PHA-stimulated PBMCs (Table 4), suggesting that the strong effect of PHA on PBMC proliferation (mean cpm value of 58,754 ± 5869 versus 3645 ± 319 cpm in resting cells) could mask the slight effects induced by the peptide fractions. Interestingly, at low doses (0.1–10 μg mL^{-1}), cells cultured with PES-1 showed a slight suppression of proliferation compared with other fractions (PES-1 versus HYD-2 and TH, $p < 0.05$ in all cases). DR fraction, however, which was the strongest stimulant for resting cells, also increased the proliferation index of PHA-stimulated cells at intermediate concentrations, but it was cytotoxic at the highest concentration. Curiously, microscopic observation of DR cultures revealed characteristic aggregates that did not appear in other fractions. This finding may be related to the presence of the peptide f(41–60), which has been reported as an aggregating agent (Groleau et al., 2003). Similar to resting cells, no significant proliferative effect was observed with the TH fraction.

At this point, our results strongly suggested that all the isolated bovine β-LG fractions, but not the TH, could exert effects on the immune system, in particular PES-1, PES-5 and DR at intermediate doses. However, the target cell population and the type of immune response achieved by each fraction could be different; therefore, functional experiments needed to be performed to understand comparative effects among fractions.

3.3. Cytokine profile depends on β-LG peptidic fraction

Subsequently, to determine the cytokine expression profile induced by the different β-LG fractions, the concentration of IFNγ, TNFα and TGFβ cytokines was determined in PBMC culture supernatants after 5 days of treatment. In view of previous results, cytokine analyses were carried out in the supernatants from PES-1, PES-5, DR and TH cultures at concentrations that led to optimal or sub-optimal effects on PBMC proliferation (10–10^3 μg mL^{-1}), in

Table 3
Effects of different peptide fractions on resting human peripheral blood mononuclear cells proliferation analysing stimulation indices after 96 h culture.[a]

Fraction[b]	Concentration (μg mL^{-1})					
	0.1	1	10	10^2	10^3	10^4
TH	1.20 (0.17)	1.17 (0.25)	1.19 (0.15)	1.23 (0.15)	1.03 (0.28)	0.83 (0.20)
PES-1	1.35 (0.03)	1.04 (0.15)	1.07 (0.30)	1.50 (0.30)***	1.47 (0.03)**	0.21 (0.05)***
HYD-2	1.26 (0.05)	1.17 (0.18)	1.13 (0.21)	1.34 (0.22)*	1.21 (0.14)	0.85 (0.48)
PES-5	1.30 (0.24)	1.06 (0.15)	1.02 (0.22)	0.93 (0.23)	1.28 (0.20)*	0.61 (0.50)***
DR	1.22 (0.15)	0.94 (0.23)	0.95 (0.08)	1.52 (0.34)	3.64 (1.00)***	3.73 (1.24)***

[a] Data are expressed as mean, with standard deviation in parentheses, of 3 independent donors and each fraction was assayed by quadruplicate. Statistical differences between negative control (unstimulated cells) and the cells cultured with the indicated fraction at the indicated concentration are given (*$p < 0.050$, **$p < 0.010$, ***$p < 0.001$); differences were assessed by one-way ANOVA and *t*-Dunnet post-hoc test.

[b] Fractions are: TH, total hydrolysate; PES-1, 1 kDa polyethersulfone membrane permeate; HYD-2, 2 kDa Hydrosart membrane permeate; PES-5, 5 kDa polyethersulfone membrane permeate; DR, diafiltration retentate.

Please cite this article in press as: Rodríguez-Carrio, J., et al., Immunomodulatory activities of whey β-lactoglobulin tryptic-digested fractions, *International Dairy Journal* (2013), http://dx.doi.org/10.1016/j.idairyj.2013.07.004

Table 4
Effects of different peptide fractions on phytohaemagglutinin (PHA)-stimulated human peripheral blood mononuclear cells proliferation analysing stimulation indices (SI) after 96 h culture.[a]

Fraction[b]	Concentration (μg mL^{-1})					
	0.1	1	10	10^2	10^3	10^4
TH	19.60 (0.07)	21.11 (3.18)	18.92 (2.89)	19.78 (2.02)	19.40 (0.93)	15.62 (10.54)
PES-1	12.99 (1.81)	12.55 (8.49)	15.55 (1.20)	13.20 (9.05)	13.17 (9.11)	33.28 (1.79)***
HYD-2	24.29 (2.98)	27.71 (3.85)	25.92 (4.29)	20.26 (5.18)	16.45 (2.09)	30.69 (1.87)*
PES-5	16.90 (0.06)	18.25 (2.42)	14.24 (4.00)	20.46 (2.05)	18.86 (0.11)	29.33 (7.54)***
DR	16.70 (0.15)	16.05 (1.70)	20.00 (2.78)*	30.02 (4.92)***	17.34 (1.14)	4.59 (0.11)***

[a] Data are expressed as mean, with standard deviation in parentheses, of 3 independent donors and each fraction was assayed by quadruplicate. PHA-stimulated cells without peptides provided a SI of 15.59 (1.55) cpm. Statistical differences between negative control (PHA-stimulated cells) and the cells cultured with the indicated fraction at the indicated concentration are given (*$p < 0.050$, **$p < 0.010$, ***$p < 0.001$). Differences were assessed by one-way ANOVA and *t*-Dunnet post-hoc test.
[b] Fractions are: TH, total hydrolysate; PES-1, 1 kDa polyethersulfone membrane permeate; HYD-2, 2 kDa Hydrosart membrane permeate; PES-5, 5 kDa polyethersulfone membrane permeate; DR, diafiltration retentate.

both resting and PHA-stimulated cells (Fig. 2). PBMCs cultured in medium alone and in the presence of PHA were used as controls.

As observed in proliferation assays, the most interesting results were obtained with resting cells. Fig. 2A shows that, among the studied fractions, only DR induced an increase in the production of IFNγ. The upregulation of this Th1 cytokine, which was accompanied by an increase in PBMC proliferation, suggests the activation of antigen specific memory/effector Th1 lymphocytes, present in the peripheral blood of healthy individuals. This effect could be related to the presence of large and acid peptides in this fraction, features suggestive of an immunogenic ability, as has been previously reported (Saint-Sauveur, Gauthier, Boutin, & Montoni, 2008). The absence of significant results observed in TH cultures was in accordance with previous data reporting that complex mixtures of whey protein hydrolysates did not have any impact on IFNγ secretion (Cross & Gill, 1999; Saint-Sauveur et al., 2008).

Unlike the DR fraction, PES-1 and PES-5 did not produce a Th1 stimulatory ability, probably because they were enriched in short peptides, a feature related to low T cell immunogenicity. These fractions, however, significantly increased the secretion of TNFα, a proinflammatory cytokine mostly produced by activated monocytes, supporting that they may also influence immune responses. Moreover, the most surprising result was the ability of PES-1 to induce the production of TGFβ, a strong tolerogenic and immunosuppressor cytokine also secreted by activated monocytes. Furthermore, this induction was only observed at low PES-1 concentrations since, in a dose-dependent manner, this fraction increased TNFα production while it decreased TGFβ secretion. Thus, in spite of the inability of PES-1 and PES-5 to stimulate Th cells, they may influence immune responses through the activation of monocytes. This finding is in line with previous studies reporting the activation of macrophage function by whey protein hydrolysates (Gattegno, Migliore-Samour, Saffar, & Jolles, 1988; Li & Mine, 2004).

Monocytes are vital components of innate immunity, mediating diverse biological functions that are determined by their activation states. They respond to microbial infection or physiological changes in the microenvironment differentiating into M1 or M2 macrophages. Classical activation by bacterial lipopolysaccharide or inflammatory signals leads to the generation of M1 macrophages, producers of TNFα, while alternative activation leads to the generation of M2 macrophages, which are anti-inflammatory and produce immunosuppressor cytokines, including TGFβ.

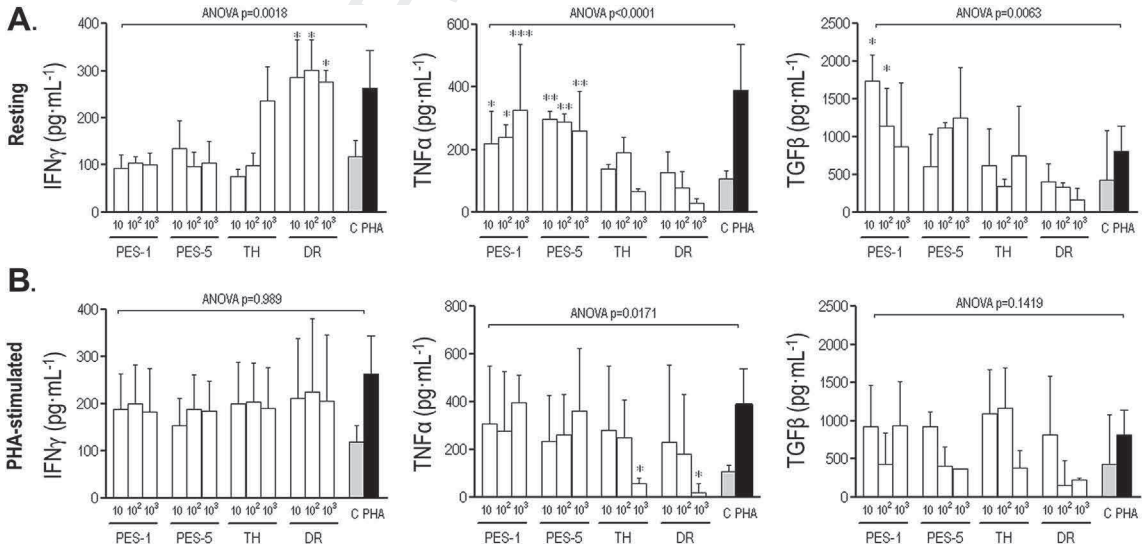

Fig. 2. Cytokine stimulation by different β-lactoglobulin fractions. Interferon gamma (IFNγ), tumour necrosis factor alpha (TNFα) and transforming growth factor beta (TGFβ) were measured by ELISA techniques in the supernatants from PBMC after culture with different peptidic fractions. In panel A cells were cultured in the presence of different fractions under resting conditions, whereas in panel B cells were under phytohaemagglutinin (PHA)-stimulating conditions. Bars represent mean and standard deviation from four different experiments performed with different blood donors ($n = 12$). Fraction concentrations are indicated at the bottom of the bars (10–10^3 μg mL^{-1}). Negative control (resting cells, ■) and positive control (PHA-stimulated cells, ■) values are indicated in each graph. Differences among all the assayed samples were evaluated by one-way ANOVA and *p*-values are indicated at the top of the histogram. Differences between each sample and the control (unstimulated cells in A, PHA-stimulated cells in B) were evaluated by *t*-Dunnet post-hoc test and are indicated as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Please cite this article in press as: Rodríguez-Carrio, J., et al., Immunomodulatory activities of whey β-lactoglobulin tryptic-digested fractions, *International Dairy Journal* (2013), http://dx.doi.org/10.1016/j.idairyj.2013.07.004

Although activation usually arises after exposure to pathogens or tissue injury, several short peptides, like natural host defence peptides (HDPs), can also stimulate macrophage differentiation. Therefore, in recent years there has been an increased effort to design and develop synthetic peptides, termed innate defence regulators (IDRs), with enhanced immunomodulatory activities that depend on uptake into eukaryotic cells, making them an attractive new anti-infective strategy for their potential value in the therapy and prophylaxis of infection (Nijnik et al., 2010; Wieczorek et al., 2010). Therefore, specific whey-derived peptides could act as natural IDRs.

On the other hand, the addition of β-LG peptidic fractions to PHA-stimulated cells did not significantly affect cytokine production (Fig. 2B), except for the TNFα inhibition observed with high doses of TH and DR, which seems to be due to a cytotoxic effect on monocytes. Accordingly, decreased cell viability at these concentrations was suggested by the proliferation assays (Table 4).

To summarise, the apparent lack of immune activity displayed by the TH may be explained by the diverse peptide–cell interactions arising in the context of a complex mixture of peptides, since our results strongly suggested that each particular β-LG fraction could trigger a characteristic immune ability, which could lead to opposite effects on one another. In this sense, the TGFβ-inducer potential of PES-1 fraction may be of special interest, due to the key regulatory effects of this cytokine on many cell types. TGFβ promotes immunoglobulin class switch to IgA, the isotype predominant in mucosal epithelia, and a crucial mechanism for the defence against pathogens in the gut. In addition, this cytokine is required to generate induced Treg cells, especially in peripheral tissues associated with mucosal surfaces, a population involved in both the immunological tolerance and the control of excessive inflammation, thus pointing to the relevance of this finding in the field of allergic and inflammatory conditions.

3.4. PES-1 fraction can promote regulatory T cell responses

In view of the previously observed upregulation of TGFβ, we aimed to evaluate whether the PES-1 fraction could influence the generation of Treg cells in vitro. Thus, PBMCs were cultured in medium alone or in the presence of different concentrations (10–10^4 μg mL^{-1}) of PES-1 fraction or the unfractionated TH. The proportion of Treg cells after 5 days of culture was quantified by flow

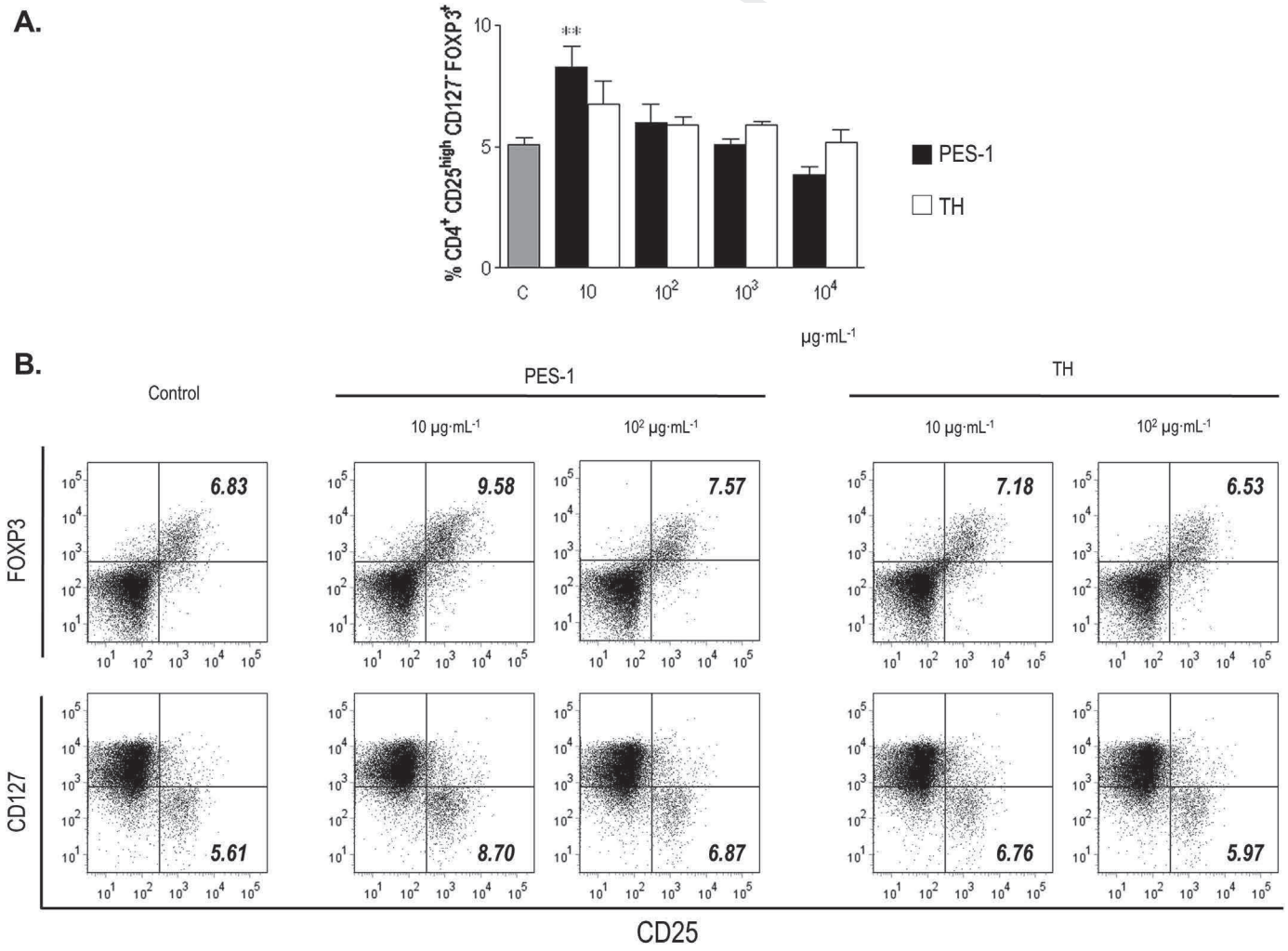

Fig. 3. Enrichment of regulatory T cell (Treg) population by the permeate stream obtained using the 1 kDa polyethersulfone membrane (PES-1 fraction). Human peripheral blood mononuclear cell (PBMC) phenotype was analysed by flow cytometry after culture with PES-1 fraction or the unfractionated total hydrolysate (TH). For each condition, 30,000 $CD4^+$ events were analysed. Panel A: Treg frequency ($CD4^+CD25^{high}CD127^-FOXP3^+$ cells) on PBMC after 5-days culture in the presence of PES-1 (■) or TH (□). Bars represent mean and standard deviation of 5 independent experiments performed with different blood donors ($n = 12$). Differences between each sample and the negative control were assessed by *t*-Dunnet post hoc test. Panel B: flow cytometry analysis of Treg population by their CD25, CD127 and FOXP3 expression. Frequencies of a representative experiment are shown for the $CD4^+CD25^{high}FOXP3^+$ and $CD4^+CD25^{high}CD127^-$ populations. $^{**}p < 0.01$.

Please cite this article in press as: Rodríguez-Carrio, J., et al., Immunomodulatory activities of whey β-lactoglobulin tryptic-digested fractions, *International Dairy Journal* (2013), http://dx.doi.org/10.1016/j.idairyj.2013.07.004

cytometry, analysing CD4, CD25, FOXP3 and CD127 expression in viable lymphocytes (Fig. 3A). We noticed that the highest dose (10^4 μg mL^{-1}) caused a decrease in the amount of total viable cells (76.47 ± 4.90%), supporting the reported cytotoxic effect of this concentration. However, no differences were observed in the total $CD4^+$ T lymphocyte population among the different culture conditions ($p > 0.05$). Fig. 3A shows that PES-1 fraction at 10 μg mL^{-1} significantly increased the percentage of Treg cells ($CD4^+CD25^{high}FOXP3^+CD127^-$), but they decreased at higher concentrations in a dose-dependent manner. Similar results were obtained quantifying separately $CD4^+CD25^{high}FOXP3^+$ and $CD4^+CD25^{high}CD127^-$ populations ($p < 0.01$) (Fig. 3B). The TH fraction had no significant effect on these populations at any of the studied concentrations.

These data are in line with TGFβ production and proliferation results in PES-1 cultures at low concentrations, suggesting the ability of specific peptide(s) included in this fraction to modulate effector immune responses in vitro through the enrichment of Treg cells, a TGFβ-dependent population. Although induction of TGFβ production by whey hydrolysated fractions has been reported in mice (Saint-Sauveur, Gauthier, Boutin, Montoni, & Fliss, 2009), this is the first study showing a significant effect on human cells, and not only on TGFβ, but also on the Treg population.

Our results, however, suggest a dose-dependent effect on monocyte differentiation. Relatively low doses of the short peptides included in PES-1 fraction could generate anti-inflammatory M2 macrophages by an alternative monocyte activation pathway, leading to TGFβ secretion, whereas higher amounts of these peptides could drive a classic M1 differentiation and TNFα production. Other hypotheses, however, could also explain these results. Given that PES-5 fraction was unable to induce TGFβ, a specific peptide(s) included in PES-1 could be responsible for the effect. Thus, the peptide f(15–40) was present, at low concentrations, in PES-1 but not in PES-5. Given that it is 4–7 times larger than most of the other peptides included in this fraction, the absolute number of molecules present at lower PES-1 concentrations could have a greater functional impact than in higher ones, where it may be masked by the large amount of short peptides, since the ratio between the number of short and long peptides changed. Analysing the effect of this peptide by itself (i.e., by means of synthesis) would be needed to confirm our hypothesis.

In line with this, it has been shown that several IDRs suppress proinflammatory responses modulating M1–M2 macrophage differentiation (Pena et al., 2013), suppressing potentially harmful excessive inflammatory responses (Scott et al., 2007) and offering protection by enhancing the innate immune defences of the host, thus suggesting again that whey-derived peptides could be used as natural IDRs.

Induction of Treg population, probably through TGFβ production, as we reported here, could be one of the potentially active mechanisms by which certain whey peptides exert their suppressive effects. Prevention of type I hypersensitivity reactions is an area in which milk protein hydrolysis has become increasing relevant (Gauthier et al., 2006), and some immunosuppressive mechanisms have been identified (Prioult, Pecquet, & Fliss, 2004). In fact, a previous study reported that a β-LG hydrolysate was able to induce oral tolerance in an animal model of allergy (Fritsche, Pahud, Pecquet, & Pfeifer, 1997).

4. Conclusions

This study showed diverse ex vivo immune effects triggered by β-LG separated fractions that were absent in the unfractionated total hydrolysate. The fraction enriched in acidic and large peptides increased the secretion of IFNγ, a Th1 cytokine implicated in the control of intracellular pathogens. Conversely, fractions including short peptides were better stimulants of monocytes than T cells, leading to TNFα production. However, low doses of one of these fractions were able to increase TGFβ secretion and Treg cells, key mechanisms for immunological tolerance and the control of inflammatory conditions in gut mucosa. This fraction unexpectedly included a large peptide, probably due to peptide–peptide interactions.

RTherefore, our results support the use of membrane isolated whey-derived products in the food, nutraceutical or pharmacological industries, not only because of their nutritional value but also for their immunological activities. In fact, it could be an easy and valuable process to obtain bioactive-enriched food products, although the potential interactions with the background diet could modify their immune effects, so this limitation must be taken into account.

References

Antila, P., Paakkari, I., Järvinen, A., Mattila, M. J., Laukkanen, M., Pihlanto-Leppälä, A., et al. (1991). Opioid peptides derived from in vitro proteolysis of bovine whey proteins. *International Dairy Journal, 1*, 215–229.

von Berg, A., Filipiak-Pittroff, B., Kramer, U., Link, E., Bollrath, C., Brockow, I., et al. (2008). Preventive effect of hydrolyzed infant formulas persists until age 6 years: long-term results from the German Infant Nutritional Intervention Study (GINI). *Journal of Allergy and Clinical Immunology, 121*, 1442–1447.

Bigelow, C. C. (1967). On the average hydrophobicity of proteins and the relation between it and protein structure. *Journal of Theoretical Biology, 16*, 187–211.

Cross, M. L., & Gill, H. S. (1999). Modulation of immune function by a modified bovine whey protein concentrate. *Immunology and Cell Biology, 77*, 345–350.

Elkord, E. (2008). Novel therapeutic strategies by regulatory T cells in allergy. *Chemical Immunology and Allergy, 94*, 150–157.

~~Fernández, A., & Riera, F. (2013a). Membrane fractionation of a β-lactoglobulin tryptic digest: effect of the ionic strength.~~

Fernández, A., & Riera, F. (2013b). β-lactoglobulin tryptic-digestion: a model approach for peptide release. *Biochemical Engineering Journal, 70*, 88–96.

Fernández, A., Suárez, A., Zhu, Y., FitzGerald, R., & Riera, F. (2013). Membrane fractionation of a β-lactoglobulin tryptic digest: effect of the pH. *Journal of Food Engineering, 114*, 83–89.

Fritsche, R., Pahud, J. J., Pecquet, S., & Pfeifer, A. (1997). Induction of systemic immunologic tolerance to β-lactoglobulin by oral administration of a whey protein hydrolysate. *Journal of Allergy and Clinical Immunology, 100*, 266–273.

Gattegno, L., Migliore-Samour, D., Saffar, L., & Jolles, P. (1988). Enhancement of phagocytic activity of human monocytic-macrophagic cells by immunostimulating peptides from human casein. *Immunology Letters, 18*, 27–31.

Gauthier, S. F., Pouliot, Y., & Saint-Sauveur, D. (2006). Immunomodulatory peptides obtained by the enzymatic hydrolysis of whey proteins. *International Dairy Journal, 16*, 1315–1323.

Groleau, P. E., Morin, P., Gauthier, S. F., & Pouliot, Y. (2003). Effect of physicochemical conditions on peptide-peptide interactions in a tryptic hydrolysate of β-lactoglobulin and identification of aggregating peptides. *Journal of Agricultural and Food Chemistry, 51*, 4370–4375.

Issazadeh-Navikas, S., Teimer, R., & Bockermann, R. (2012). Influence of dietary components on regulatory T cells. *Molecular Medicine, 18*, 95–110.

Kitts, D. D., & Weiler, K. (2003). Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. *Current Pharmaceutical Design, 9*, 1309–1323.

Knipping, K., van Esch, B. C., van Ieperen-van Dijk, A. G., van Hoffen, E., van Baalen, T., Knippels, L. M. J., et al. (2012). Enzymatic treatment of whey proteins in cow's milk results in differential inhibition of IgE-mediated mast cell activation compared to T-cell activation. *International Archives of Allergy and Immunology, 159*, 263–270.

Kuipers, B. J., & Gruppen, H. (2007). Prediction of molar extinction coefficients of proteins and peptides using UV absorption of the constituent amino acids at 214 nm to enable quantitative reverse phase high-performance liquid chromatography-mass spectrometry analysis. *Journal of Agricultural and Food Chemistry, 55*, 5445–5451.

Li, E. W., & Mine, Y. (2004). Immunoenhancing effects of bovine glycomacropeptide and its derivatives on the proliferative response and phagocytic activities of human macrophagelike cells, U937. *Journal of Agricultural and Food Chemistry, 52*, 2704–2708.

Madureira, A. R., Tavares, T., Gomes, A. M., Pintado, M. E., & Malcata, F. X. (2010). Invited review: physiological properties of bioactive peptides obtained from whey proteins. *Journal of Dairy Science, 93*, 437–455.

Mullally, M. M., Meisel, H., & FitzGerald, R. J. (1997). Identification of a novel angiotensin-I-converting enzyme inhibitory peptide corresponding to a tryptic fragment of bovine beta-lactoglobulin. *FEBS Letters, 402*, 99–101.

Muñoz-Tamayo, R., de Groot, J., Wierenga, P. A., Gruppen, H., Zwietering, M. H., & Sijtsma, L. (2012). Modeling peptide formation during the hydrolysis of β-casein by *Lactococcus lactis*. *Process Biochemistry, 47*, 83–93.

Please cite this article in press as: Rodríguez-Carrio, J., et al., Immunomodulatory activities of whey β-lactoglobulin tryptic-digested fractions, *International Dairy Journal* (2013), http://dx.doi.org/10.1016/j.idairyj.2013.07.004

von Mutius, E., Fritzsch, C., Weiland, S., Roll, G., & Magnussen, H. (1992). Prevalence of asthma and allergic disorders among children in united Germany: a descriptive comparison. *British Medical Journal, 305*, 1395–1399.

Nijnik, A., Madera, L., Ma, S., Waldbrook, M., Elliott, M. R., Easton, D. M., et al. (2010). Synthetic cationic peptide IDR-1002 provides protection against bacterial infections through chemokine induction and enhanced leukocyte recruitment. *Journal of Immunology, 184*, 2539–2550.

Pecquet, S., Bovetto, L., Maynard, F., & Fritsche, R. (2000). Peptides obtained by tryptic hydrolysis of bovine beta-lactoglobulin induce specific oral tolerance in mice. *Journal of Allergy and Clinical Immunology, 105*, 514–521.

Pellegrini, A., Dettling, C., Thomas, U., & Hunziker, P. (2001). Isolation and characterization of four bactericidal domains in the bovine β-lactoglobulin. *Biochimica et Biophysica Acta, 1526*, 131–140.

Pena-Ramos, E. A., & Xiong, Y. L. (2001). Antioxidative activity of whey protein hydrolysates in a liposomal system. *Journal of Dairy Science, 84*, 2577–2583.

Pena, O. M., Afacan, N., Pistolic, J., Chen, C., Madera, L., Falsafi, R., et al. (2013). Synthetic cationic peptide IDR-1018 modulates human macrophage differentiation. *PLoS One, 8*, e52449.

Pihlanto-Leppälä, A., & Korhonen, H. (1998). Angiotensin I converting enzyme inhibitory peptides derived from bovine milk proteins. *International Dairy Journal, 8*, 325–331.

Pihlanto, A., & Korhonen, H. (2003). Bioactive peptides and proteins. *Advances in Food and Nutrition Research, 47*, 175–276.

Prioult, G., Pecquet, S., & Fliss, I. (2004). Stimulation of interleukin-10 production by acidic β-lactoglobulin-derived peptides hydrolyzed with *Lactobacillus paracasei* NCC2461 peptidases. *Clinical and Diagnostic Laboratory Immunology, 11*, 266–271.

Riera, F., Fernández, A., & Muro, C. (2012). Cheese whey as a source of bioactive peptides: production, analysis, purification and animal and human trials. In R. Mauro, & G. Ortera (Eds.), *Whey: Types, compositions and health implications* (pp. 1–52). New York, NY, USA: Nova Science Publishers.

Saint-Sauveur, D., Gauthier, S. F., Boutin, Y., & Montoni, A. (2008). Immunomodulating properties of a whey protein isolate, its enzymatic digest and peptide fractions. *International Dairy Journal, 18*, 260–270.

Saint-Sauveur, D., Gauthier, S., Boutin, Y., Montoni, A., & Fliss, I. (2009). Effect of feeding whey peptide fractions on the immune response in healthy and *Escherichia coli* infected mice. *International Dairy Journal, 19*, 537–544.

Scott, M. G., Dullaghan, E., Mookherjee, N., Glavas, N., Waldbrook, M., Thompson, A., et al. (2007). An anti-infective peptide that selectively modulates the innate immune response. *Nature Biotechnology, 25*, 465–472.

Smithers, G. W. (2007). Whey and whey proteins – from 'gutter-to-gold'. *International Dairy Journal, 18*, 695–704.

Szajewska, H., & Horvath, A. (2010). Meta-analysis of the evidence for a partially hydrolyzed 100% whey formula for the prevention of allergic diseases. *Current Medical Research and Opinion, 26*, 423–437.

Wang, J., Ren, Z., Xu, Y., Xiao, S., Meydani, S. N., & Wu, D. (2012). Epigallocatechin-3-gallate ameliorates experimental autoimmune encephalomyelitis by altering balance among $CD4^+$ T-cell subsets. *American Journal of Pathology, 180*, 221–234.

Wieczorek, M., Jenssen, H., Kindrachuk, J., Scott, W. R., Elliott, M., Hilpert, K., et al. (2010). Structural studies of a peptide with immune modulating and direct antimicrobial activity. *Chemistry and Biology, 17*, 970–980.

Please cite this article in press as: Rodríguez-Carrio, J., et al., Immunomodulatory activities of whey β-lactoglobulin tryptic-digested fractions, *International Dairy Journal* (2013), http://dx.doi.org/10.1016/j.idairyj.2013.07.004