Journal of Functional Foods 70 (2020) 103969

Contents lists available at ScienceDirect

Journal of Functional Foods

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

# In silico and functional analyses of immunomodulatory peptides encrypted in the human gut metaproteome

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ARTICLE INFO

*Keywords:*
Extracellular proteins
Tolerance
Anti-inflammatory
Treg
Innate immune system

ABSTRACT

This work supports the massive presence of potential immunomodulatory peptides in the human gut metaproteome. These peptides were identified through the MAHMI database as potentially anti-inflammatory, and sixteen of them synthesized for characterize their mechanism of action. From them, peptide HM14 was encrypted in an extracellular protein produced by *Bifidobacterium longum*, a common member of the human microbiota, and displayed the highest anti-inflammatory capability. Molecular mechanism of action of HM14 pointed to a specific interaction between this immunomodulatory peptide and antigen presenting cells, which resulted in a higher formation of iTreg cells. Moreover, HM14 was effective in decreasing pro-inflammatory parameters in PBMCs isolated from a cohort of Crohn's patients. Finally, non-targeted metabolomics confirmed the ability of HM14 to modulate the metabolic activity of PBMCs to fulfil its energy and biosynthetic requirements. Overall, our combined *in silico*/multiomics approach supports the human gut metaproteome as a source for immunomodulatory peptides.

## 1. Introduction

The human gastrointestinal tract (GIT) is quite a complex ecosystem characterized by a continuous interaction between food, host cells and billions of microorganisms that are known collectively as the gut microbiota (Qin et al., 2010; Sonnenburg, 2015). A healthy individual harbors thousands of microbial species in his/her gut, but surprisingly a reduced number of Phyla predominate: Bacteroidetes, Firmicutes and Actinobacteria (Donaldson, Lee, & Mazmanian, 2015). Gut microorganisms and humans have coevolved to keep mutually beneficial relationships, but we are still far from understanding the molecular mechanisms underlying this mutualism. The ensemble of genes provided by our gut microbiota is denominated gut metegenome, and sometimes the term human microbiome (which can refer also to the gut environment) is used as synonym (Qin et al., 2010). Accounting for near 10.000.000 unique genes, clearly outnumbering the human 21.306 of protein-coding genes (Salzberg, 2018), our gut bacteria complement metabolic attributes that are absent in our organism such us processing of non-digestible nutrients, production of short-chain fatty acids (SCFAs), vitamins or xenobiotic degradation (Brestoff &

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https://doi.org/10.1016/j.jff.2020.103969
Received 7 January 2020; Received in revised form 8 April 2020; Accepted 12 April 2020
Available online 25 April 2020

Artis, 2013). Other beneficial effects attributed to our gut microbiota include immunomodulation, production of antimicrobial substances, enhancement of the mucosal barrier function and competition with enteropathogens for adhesion sites, limiting potential microbial invasions and teaching our immune system to differentiate between beneficial and harmful bacteria. Therefore, and in a healthy situation, mutualism predominates in the human gastrointestinal tract as the main ecological interaction (Bäckhed, Ley, Sonnenburg, Peterson, & Gordon, 2005).

The issue here is how this catalogue of mutualistic interactions is achieved and maintained over time. During the last years, it is becoming more and more evident that the relative proportions of the whole collection of microbes colonizing our GIT are critical in determining health or disease (Franzosa et al., 2019). For instance, the proportion between Firmicutes and Bacteroides appears to be related to most autoimmune and inflammatory diseases, being lower values of this ratio observed for instance in Lupus Systemic Erythematosus or Inflammatory Bowel Disease (IBD) (Kamada, Seo, Chen, & Núñez, 2013; Manichanh, Borruel, Casellas, & Guarner, 2012). Unravelling what type of molecular mechanisms explains the beneficial effects promoted by our gut microbiota is a challenging field for researchers worldwide (Kamada et al., 2013). Among the different molecules mediating molecular interactions between microbiota and host, bacterial proteins are noteworthy (Sánchez, Urdaci, & Margolles, 2010). Extracellular proteins, those that are secreted and released to the bacterial surroundings, may be able to interact directly with mucosal cells (Ruiz, Hevia, Bernardo, Margolles, & Sánchez, 2014; Sánchez et al., 2011). Extracellular proteins from intestinal bacteria are known to regulate not only signaling pathways, but secretion of chemokines, cytokines, anti-bactericidal peptides (defensins), mucus secretion, production of pseudopods by dendritic cells (DCs), rearrangement of the tight-junctions in epithelial cells, modulation of the immune function and the response of the gut-associated lymphoid tissue (GALT) cells (Hevia, Delgado, Sánchez, & Margolles, 2015; Sánchez, Hevia, González, & Margolles, 2015).

Few years ago, our research group demonstrated how a peptide encrypted in an extracellular protein from *Lactobacillus plantarum* was able to drive the immune response of DCs isolated from Ulcerative Colitis patients towards more regulatory configurations (Bernardo et al., 2012). In general, extracellular proteins secreted by our gut microbiota may be cleaved by intestinal proteases, rending peptides that can present a bioactivity different from that observed in the native protein, although this point has not been shown in vivo so far. As proposed by Karelin and colleagues, generation of encrypted peptides would define a gut-specific peptide pool with key roles for the maintenance of gut homeostasis (Karelin, Blishchenko, & Ivanov, 1998). With this hypothesis in mind, we designed the Mechanism of Action of the Human Microbiome database (MAHMI. http://www.mahmi.org) in which a catalog of near 10.000.000 unique proteins generated by the MetaHit project was digested with the main intestinal proteases and crossed against a curated database of known immunomodulatory peptides/proteins (Blanco-Míguez, Gutiérrez-Jácome, Fdez-Riverola, Lourenço, & Sánchez, 2017; Li et al., 2014). Some of these peptides were synthesized and incubated with peripheral blood mononuclear cells (PBMCs) in order to understand if they were or not able to induce a downstream response in the immune cells. Analysis of an 18 cytokine array revealed that all peptides induced different changes in the cytokine profiles with respect to basal conditions, being two of them able to induce production of IL-17 and IL-22, the latter related to healing in the framework of chronic inflammatory settings (Hidalgo-Cantabrana et al., 2017). Moreover, a combined metatranscriptomics (RNASeq) and metabolomics (GC–MS) approach, based on previous knowledge of our group (Cambeiro-Pérez et al., 2018), revealed both molecular and metabolic changes induced by one of these peptides (termed HM14) that would support an anti-inflammatory molecular mechanism of action. Main results are discussed next.

## 2. Material and methods

### 2.1. Ethics

Ethics approval for this study (reference code AGL2013-44039-R) was obtained from the Regional Ethics Committee for Clinical Research in compliance with the Declaration of Helsinki. Samples used in this study were obtained from anonymous donors of the regional blood donation system and from a cohort of Crohn's Disease patients.

### 2.2. Peptide identification and bioactivity prediction

Bacterial peptides used in this study were identified through the MAHMI pipeline (http://www.mahmi.org). Briefly, this pipeline allows peptide comparison against a knowledge database of anti-proliferative and immunomodulatory peptides by sequence similarity analysis. In this case, the protein catalogue created by the METAHIT project (Li et al., 2014) (http://gigadb.org/dataset/100064), which represents unique proteins of the whole human gut metaproteome, was digested *in silico* and queried against the reference immunomodulatory and anti-proliferative peptides of the MAHMI database. Those 133 peptides encrypted in larger proteins synthesised by intestinal microorganisms from L. 134 plantarum and B. longum species which bioactive prediction according to the MAHMI 135 score was equal or higher than 75 were retained (De Jesus Oliveira, De Oliveira, & Da Silva Junior, 2019; Hidalgo-Cantabrana et al., 2017; Wang et al., 2011) (Table 2). Sixteen peptides were finally selected and synthesised at the GeneCust facilities (Ellange. Luxemburg).

### 2.3. Peripheral blood mononuclear cells (PBMCs) isolation

The capability of synthetic bacterial peptides to induce immune modulation in vitro was assessed using a PBMCs model. PBMCs were isolated from the buffy coat of 5 healthy donors from the Community Center for Blood and Tissues of Asturias (Oviedo. Spain) as previously described (Hidalgo-Cantabrana et al., 2017).

### 2.4. Isolation of $CD4^+$ T cells and naïve T cells

$CD4^+$ T cells were isolated from the buffy coat of healthy donors obtained from the Community Center for Blood and Tissues of Asturias (Oviedo. Spain). In short, 5.0 mL of buffy were diluted with equal volume of phosphate buffered saline (PBS) and 25 μL $mL^{-1}$ of RosetteSep™ Human $CD4^+$ T cell Enrichment Cocktail (Stemcell Technologies) was added and incubated for 20 min. Then 10 mL of PBS with 2.0% FBS was added to the buffy mixture. The 20 mL buffy solution, containing one part of blood and 3 parts of PBS, was added on top Ficoll-Hypaque for gradient separation to isolate $CD4^+$ T cells at 3.100 rpm, 20 min. The $CD4^+$ enrichment cocktail induced the precipitation of all the cells unless the $CD4^+$ T cells that stay on top when gradient separation is performed. After gradient separation, $CD4^+$ T cells were washed twice with PBS at 1.500 rpm, 5 min. $CD4^+$ cells were resuspended in 1.0 mL of PBS and counted in Neubauer chamber.

Naïve T cells ($CD4^+CD25^-$) were isolated from the $CD4^+$ T cells using the CD45RA MicroBeads (Milteny Biotec) following manufacturer instructions.

### 2.5. In vitro differentiation of induced Treg (iTreg)

The in vitro differentiation of iTreg was performed as previously described by Fantini, Dominitzki, Rizzo, Neurath, and Becker (2007)). Briefly, 24 well plate was coated with 1.0 μg $mL^{-1}$ anti-CD3 antibody in PBS for 2.0 h at 37 °C. Naïve $CD4^+CD25^-$ cells were resuspended $2x10^6$ in X-Vivo15 serum free medium with antibiotics and 500 μL were added to each well, after washing the plate from the anti-CD3 not adhered. Then, anti-CD28 antibody (2.0 μg $mL^{-1}$), TGFβ (5.0 ng $mL^{-1}$) and IL-2

(100 U $mL^{-1}$) were used for iTreg differentiation in the presence or absent of bacterial extracts (50 μg $mL^{-1}$). Cells were incubated for 5 days at 37 °C with 5.0% $CO_2$.

### 2.6. Generation of monocyte-derived DCs

PBMCs were obtained from standard buffy-coat preparations from 12 healthy blood donors (Asturian Blood Transfusion Center, Oviedo, Spain) by centrifugation over Ficoll-Hypaque gradients (Lymphoprep, Nycomed, Oslo, Norway). Monocytes ($CD14^+ \geq 95\%$) were isolated from previously obtained PBMCs by positive selection using magnetic CD14 MicroBeads and MACS system (Miltenyi). Immature DCs were obtained by culturing the PBMC fraction in Dutch modified RPMI 1640 (Sigma-Aldrich, Dorset, UK) containing 100 μg/mL penicillin/streptomycin, 2.0 mM L-glutamine, 50 mg $mL^{-1}$ gentamycin (Sigma-Aldrich) and 10% fetal calf serum (TCS cellworks, Buckingham. UK)] in 48 well culture dishes for 6 days (37 °C, 5.0% $CO_2$, high humidity), 200 IU $mL^{-1}$ GM-CSF and 200 IU $mL^{-1}$ IL-4 were used as differentiation factors. Half of the medium volume was replaced at days 3 and 5. MoDC differentiation was assessed by flow cytometry by comparing the percentage of population expressing CD14 and HLA-DR at days 0 and 6 using monoclonal, FITC and PE conjugated antibodies, respectively.

### 2.7. Co-cultivation of extracellular proteins and PBMCs

PBMCs were cultivated in round bottom 96 wells microplates using 200 μL of the cell suspension described above. Extracellular proteins were added to a final concentration of 0.1, 1.0 or 10 μg $mL^{-1}$, based on previous studies of the immunomodulatory peptide STp (Bernardo et al., 2012). PBMCs were activated with 100 ng $mL^{-1}$ of anti-CD3 antibody added to the RPMI medium. For each donor positive (LPS, 1.0 μg $mL^{-1}$) and negative controls of PBMCs stimulation were included. Microplates were incubated for 5 days at 37 °C with 5.0% $CO_2$. For metabolomics, PBMCs were cultivated in flat bottom 12 wells microplates ($2.5 \times 10^7$ cells $mL^{-1}$ per well) accordingly to previous studies (Cambeiro-Pérez et al., 2018).

### 2.8. Hematological analysis and immunological phenotyping

For flow cytometry analysis, PBMCs were surface-stained with Multiset CD3-FITC/$CD16^+$56-PE/CD45-PerCP/CD19-APC Reagent (BD Biosciences, San Jose, CA, USA), anti-CD4 (PerCP), anti-CD8 (PE or PerCP), anti-CD45RA (FITC) (Immunostep, Salamanca, Spain), anti-CCR7 (Alexa Fluor 647), anti-CD28 (APC-H7), anti-CD45RA (APC-H7), anti-CD4 (PE-Cy), anti-CD27 (PE-Cy), and anti-CD3 (FITC) (BD Biosciences). To analyze an activated phenotype, cells were stained with anti-CD3 (APC), anti-CD8 (FITC or PE), anti-CD4 (PerCP), anti-CD69 (FITC), anti-CD127 (PE), anti-HLA-DR (FITC), and anti-CD25 (APC) (BD Bioscience). One hundred μL of whole blood from each volunteer was stained with different combinations of labelled monoclonal antibodies for 20 min at room temperature. Samples were red-blood lysed with FACS Lysing Solution (BD Biosciences), washed in PBS, and analyzed using CellQuest software in a FACSCanto Cytometer (BD Biosciences). Appropriate isotype control mAbs were used for marker settings.

### 2.9. Activation and proliferation assays

To analyze the activated phenotype, PBMCs ($4.0 \times 10^6$ cells $mL^{-1}$) were cultured in the presence and absence of anti-CD3 (1.0 μg $mL^{-1}$) (eBioscience), extracellular protein extract from the 20,079 strain (DSM) and anti-$CD3^+$DSM. Cells were cultured for 18 h and then stained with anti-CD69 (FITC), anti-CD3 (PerCP), and anti-CD4 or anti-CD8 (APC) (Immunostep) and analyzed on the cytometer.

For the proliferation assay, PBMCs were resuspended in PBS at a final concentration of 5–10 × $10^6$ cells $mL^{-1}$ and incubated with 1.5 μM CFSE (Invitrogen, Paisley, Scotland. UK) for 10 min at 37 °C before being washed twice with RPMI 1640 medium containing $2.0 \times 10^{-3}$ M L-glutamine and HEPES. Cells were then cultured at $2.0 \times 10^6$ cells $mL^{-1}$ in medium, anti-CD3 (1.0 μg $mL^{-1}$), Pext or anti-$CD3^+$Pext. The proliferative responses of $CD4^+$ and $CD8^+$ T-lymphocytes were analyzed on day 7 after staining with anti-CD3 (PerCP), anti-CD8 (PE), and anti-CD4 (APC). The cells were analyzed on a BD FACSCanto flow cytometer.

### 2.10. Intracytoplasmic staining

PBMCs ($4.0 \times 10^6$ cells $mL^{-1}$) were cultured in the presence and absence of anti-CD3 (1.0 μg $mL^{-1}$), DSM and anti-$CD3^+$DSM. Frequencies of T cells with intracytoplasmic production of IFN-γ, TNF, IL-17 and IL-10 after 18 h of culture were measured. Cells were surface-stained with anti-CD3 (PerCP), anti-CD8 (PE), and anti-CD4 (APC) for 30 min at room temperature, lysed and fixed with FACS lysing solution, permeabilized with BD FACS Permeabilizing Solution 2 (Perm II) (BD Bioscience), and stained with anti-IFN-γ (FITC) (eBiosciences), anti-TNF (PE) (BD Bioscience), anti-IL-17 (APC) (Biolegend) or anti-IL-10 (Biolegend) for 30 min at room temperature. Cells were washed and resuspended in 1% paraformaldehyde until FACS analysis.

### 2.11. Cytokine quantification

Cytokines were measured both in plasma and in the supernatant of cell cultures. After 5 days, supernatant was collected and stored at −80 °C for multiplexed cytokine analyses. In the case of humans, the production of 18 different cytokines were quantified using the Th1/Th2/Th9/Th17/Th22/Treg Cytokine 18-Plex Human ProcartaPlex™ Panel (Affymetrix eBioscience, San Diego, USA) and the Luminex® xMap Technology equipment following manufacturer's settings. The results for each cytokine were represented using box plot diagrams and differences between peptides were statistically analysed.

### 2.12. RNA-seq

Fifteen micrograms of total RNA were extracted from 24 different moDC samples exposed or not to 1.0 μg $mL^{-1}$ LPS or 10 μg $mL^{-1}$ HM14, and also from $CD4^+$ cells isolated using magnetic-conjugated antibodies from PBMCs previously incubated with anti-CD3 or anti-$CD3^+$ 10 μg $mL^{-1}$ DSM. In both cases, the RNeasy Mini Kit (QIAGEN) was used following the manufacturer's instructions. Total mRNA was sequenced in the facilities of GenProbio SRL (http://www.genprobio.com/) in a HiSeq Illumina System (Illumina, Inc). Preparation of the libraries, ligation of required adaptors and sequencing was performed according to the manufacturer indications. Briefly, about 25 million paired-end reads of 100 nucleotides were obtained for each sample. Roughly this represented about $2.5 \times 10^9$ clean bases after application of quality filtering suggested by Illumina. Filtered RNA data was exported in FASTq format and was used as input for DEWE (http://www.sing-group.org/dewe/) (López-Fernández, Blanco-Míguez, Fdez-Riverola, Sánchez, & Lourenço, 2019). Files corresponding to this study are available at the European Nucleotide Archive under accession PRJEB33568.

### 2.13. Metabolomics

#### 2.13.1. Chemicals, solutions and materials

Standards of DL-norvaline, succinic acid-2.2.3.3-$d_4$, *trans*-cinnamic-$d_7$ acid and D-glucose-$_{13}C^6$ used as surrogates, were purchased from Sigma Aldrich (Madrid, Spain). Standards for Quality Control mix of external reference standards, D-fructose and glycine, were purchased from Panreac (Barcelona, Spain); urea from Scharlau (Barcelona, Spain); L-phenylalanine. nicotinic acid. succinic acid, DL-malic acid, myo-inositol and L-cysteine from Sigma Aldrich. Each standard was

prepared at 100 mM in water or methanol depending on the solubility of the chemical. Surrogates and mix of standards working solutions were prepared in *tert*-butylhydroquinone, purchased from Sigma Aldrich, 2.0 g $L^{-1}$ in methanol at 1.0 mM. These solutions were stored in amber flasks at −20 °C. In order to correct injection disturbances. two polychlorinated biphenyls (PCB30 and PCB204), purchased from Accustandard (New Haven,USA), were used as internal standard (IS). Saturated Alkane Mixture (C7–C40) and FAME mix solution were acquired from Supelco (Bellefonte, USA) to support the metabolite identification. Working solutions of n-alkanes and FAMEs mixture was prepared in acetone at 1.0 mg $L^{-1}$. For derivatization. N-Methyl-N-trimethylsilyl trifluoroacetamide (MSTFA), chlorotrimethylsilane (TMCS) and pyridine were purchased from Sigma Aldrich. Methoxylamine hydrochloride (MeOX) was from Supelco.

#### 2.13.2. PBMCs co-cultivation and quenching extraction

According to previous metabolomics study (Cambeiro-Pérez et al., 2018), PBMCs were cultivated in flat bottom 12-wells microplates ($2.5 \times 10^7$ cells $mL^{-1}$ per well) and subsequently stimulated in culture with the peptide HM14 (50 μg $mL^{-1}$) and LPS (1.0 μg $mL^{-1}$) as positive control. Negative controls (no stimulus) besides blank samples (with no cells) were also included for each donor. The microplates were incubated for 48 h at 37 °C with 5.0% $CO_2$. Quenching samples were obtained as was described before and stored to −80 °C until derivatization process prior to injection by GC-QqQ-MS.

#### 2.13.3. Derivatization

Quenching extracts (400 μL) and standard solutions were reduced to dryness under a gentle nitrogen stream and then derivatized in two steps, in order to make compounds with higher boiling points volatilize enough to be analysed by GC–MS. Firstly, methoxymation was performed by dissolving the dried samples in 100 μL of MeOX (20 mg $mL^{-1}$ in pyridine), then vortexed for 1.0 min and incubated at room temperature for 1 h. Secondly, the samples were silylated by adding 100 μL MSTFA with 1.0% TMCS and subsequent incubated at 70 °C for 30 min. Finally, derivatized extracts were transferred to vials and spiked with 2.0 μL of IS mix solution (100 mg $L^{-1}$) for GC-QqQ-MS analysis.

#### 2.13.4. Quality control samples

Three different types of Quality Control (QC) samples have been employed (QC, QC surrogate and QC standard). Biological QC sample, named as QC, was made by pooling 45 μL from each of analysed samples. Surrogate standard QC sample, named as QC surrogate, was made by spiking with surrogate standards to the QC sample. Reference standard QC sample was prepared with a mix of standards to evaluate the derivatization process.

#### 2.13.5. GC–MS analysis

Derivatized samples were analysed with an Agilent 7890 gas chromatograph coupled to an Agilent 7000C triple quadrupole detector and to an Agilent 7650C autosampler (Agilent. California. USA) interfaced to a PC computer running the software Agilent Mass Hunter Data Acquisition Software (B.08.00 version). Chromatographic separations were done by using an Agilent HP 5MS capillary column (30 m × 0.25 mm × 0.25 μm). Aliquots of the derivatized samples (0.5 μL) were injected using a multimode inlet (MMI) equipped with an Ultra Inert liner with glass wool from Agilent, in the splitless mode and the injection temperature set to 280 °C. In order to avoid sample cross-contamination, acetone washing steps were done before and after each injection as well as sample washes to prevent air bubble formation. The initial GC oven temperature was 100 °C and was held for 4.0 min, after which the temperature was increased with 4.0 °C $min^{-1}$ to 300 °C and held then for 1.56 min. Helium was used as a carrier gas with a flow rate of 1.0 mL $min^{-1}$. The MS was operated in electron impact ionization mode at 70 Ev and full scan monitoring mode from $m/z$ 35 to 600. The transfer line and ionization source temperatures were set to 280 °C.

#### 2.13.6. Metabolomics data processing, metabolite identification and statistical analysis

GC–MS raw data files (.d files) were evaluated through visual inspection of Total ion chromatograms (TICs) in terms of quality of the analytical batch (sensitivity loss, retention time precision, IS signal) using the MassHunter Qualitative Analysis B.07.00 (Agilent Technologies). QC raw data file was imported to MassHunter Unknowns Analysis Tool B.07.01 (Agilent Technologies) for the generation of an in-house spectral library for this experiment. The file was deconvoluted and metabolites putatively identification was performed by comparison of the mass spectra with NIST 11 (National Institute of Standards and Technology, 2011) and Fiehn RTL (Agilent Technology) libraries at 70% similarity index. To substantiate the identification, both n-alkanes and FAMEs mixtures were analysed during the analytical batch and retention indices were determined. Once the library was created, all the remaining samples were processed with Unknowns Analysis Tool for deconvolution and subsequent metabolites identification according to the QC in-house spectral library. Then, data were aligned and normalized prior to statistical analysis, using MassProfiler Professional B.14.0 (Agilent Technologies). A filter by frequency was applied to data, retaining those entities which appeared in more than 100% of samples in at least one condition. Statistical significance analysis using the one way ANOVA with Tukey's Honest Significance Difference (HSD) post Hoc test was performed to identify which entities were responsible for significant differences among groups with a p-value $< 0.05$. Fold change cut-off $> 2.0$ was also applied. Hierarchical clustering was performed by applying Pearson's uncentered-absolute distance metric and complete linkage to demonstrate relationships among groups. Unsupervised principal component analysis (PCA) was conducted to observe the distribution of samples and QCs; providing information about sample dispersion and the presence of outliers. Samples were classified into discrete classes also by supervised Partial Least Square Discriminant Analysis (PLS-DA).

### 2.14. Statistical analyses

All experiments were performed in independent biological quintuplicates. Data distribution did not follow normality. So initial comparisons were performed with the non-parametric Wilcoxon and Tukey pairwise tests. Differences in the value ranks between two conditions were assessed with the Mann-Whitney *U* and Wilcoxon tests for equal medians with Monte Carlo permutations (n = 99.999) implemented in the Past3 software v3.15 (Ryan, Hammer, & Harper, 2001). Comparisons with a p-value ≤ 0.05 were considered statistically significant. All the graphics showed in this paper were obtained in R environment.

## 3. Results

### 3.1. Cytokine profiling in healthy donors using an 18-plex cytokine array.

HM14 is encrypted in an extracellular glycosyl hydrolase family 43 protein (accession WP_013582931) from *Bifidobacterium longum*. In a previous study, we established how presence of this peptide modulated PBMCs immune response using a metabolomics, liquid-chromatography based approach (Cambeiro-Pérez et al., 2018). For this reason, we decided to deepen in the knowledge of this mechanism by cytokine profiling. For such purpose, we incubated this peptide with PBMCs isolated from healthy donors (n = 5) and tested the supernatants using a Luminex 18-plex cytokine array (Th1/Th2/Th9/Th17/Th22/Treg Cytokine 18-Plex Human Panel). When we analyzed the increments in key cytokines as affected by the presence of HM14 or the positive control LPS, we found that among those cytokines presenting statistical differences in their production and apart from the anti-inflammatory cytokine IL-10, HM14 induced statistically significant increments in IL-4, IL-6, GM-CSF and TNFα, but also in IL-2 (Fig. 1A) (Supplementary Fig. 1). Other peptides triggered pro-inflammatory settings or anti-

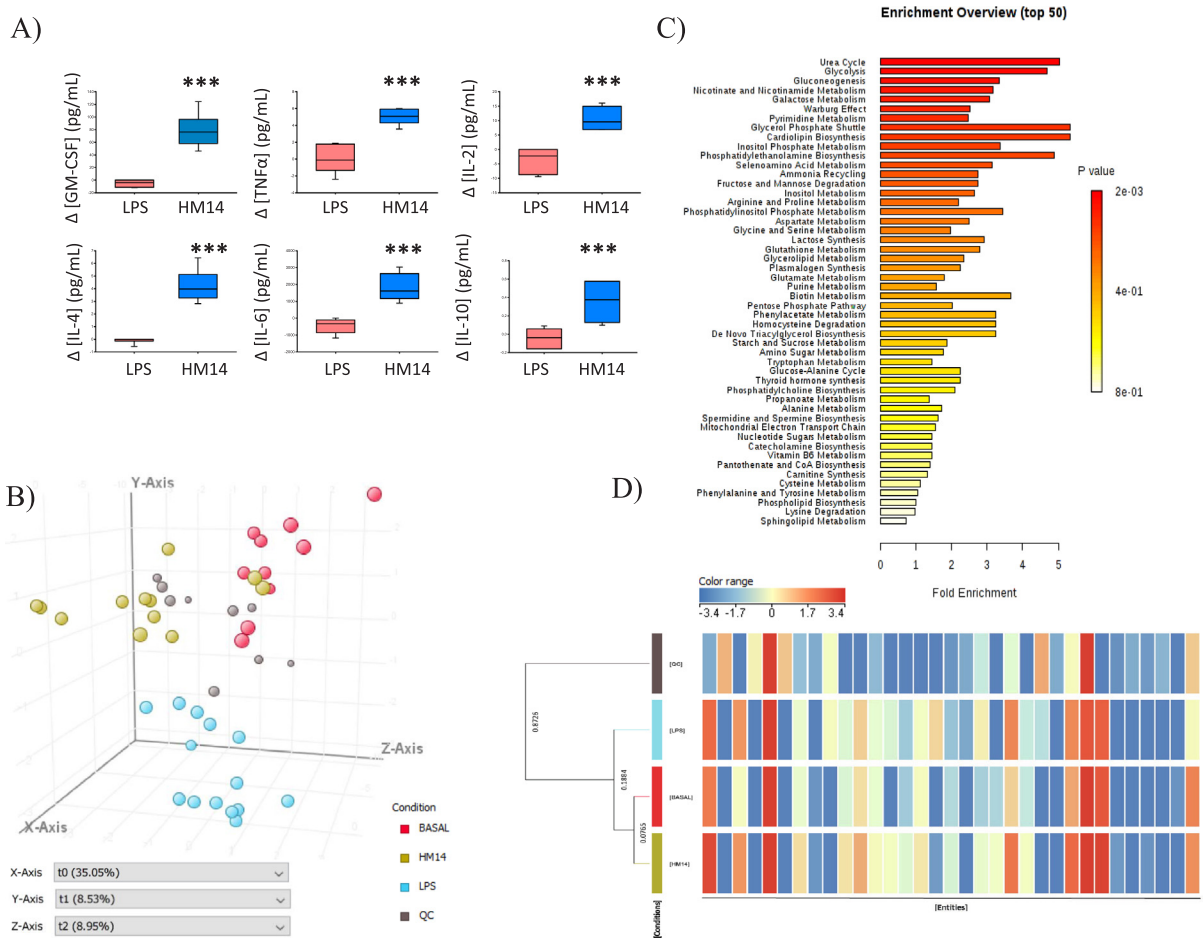


**Fig. 1.** (A) Increments (treatment *vs* basal conditions) in cytokine production by PBMCs incubated with HM14 or LPS. (B) PLS-DA 3D score plot, showing an overall accuracy of the model of 93.3%, $R^2 = 0.673$ and $Q^2 = 0.206$. (C) Enrichment analysis overview graph. (D) Hierarchical clustering analysis by applying Pearson's uncentered absolute similarity measure and complete linkage. Results are expressed as the median of 5 biological replicates. (*** $p < 0.05$).

inflammatory, but not in the extent of that of HM14.

### 3.2. Data quality assessment of untargeted GC/MS metabolomics

Firstly, we validated our untargeted GC/MS metabolomics approach to assess the performance of our method with the aim to complement previous data obtained by LC/MS (Cambeiro-Pérez et al., 2018). Due to the inexistence of official guidelines for validating untargeted metabolomics, we implemented previously published indications such as replicated samples, internal standards, Quality Control (QC) samples and blank samples (Gika, Zisi, Theodoridis, & Wilson, 2016; Naz, Vallejo, García, & Barbas, 2014; Sangster, Major, Plumb, Wilson, & Wilson, 2006).

Replicated sample measurement (including biological and technical replicates) was performed in order to reduce biological and analytical variability. Moreover, internal standards were added prior injection to each sample in order to correct instrumental variances.

Three different types of QC samples were employed (named as QC, QC surrogate and QC standard). QC standard was prepared with a mix of a representative set of compounds, belonging to different chemical classes, to evaluate the derivatization process. Derivatization precision was tested with four different concentrations of the reference standard mixture in quadruplicate in the same batch and across 7 days, showing RSDs in the most of cases below 10%. Recoveries of surrogate standards were measured in all samples ranged from 75 to 104% with RSDs < 20%. To determine the overall precision of the experiment, QC and QC surrogate samples were analysed intermittently during the analytical run every 5 samples, resulting calculated RSDs in all cases < 10%.

Finally, analytical blanks allowed controlling the correct analytical method development and identifying background features whilst method blanks (BQuench samples) allowed identifying the background related to the entire methodological process from quenching to injection.

### 3.3. GC/MS results

After data processing, 102 putatively identified entities were significantly differentiated (p-value < 0.050 and fold change > 2.0) among groups. *Escherichia coli* lipopolysaccharide (LPS) was used as reference of pro-inflammatory metabolism. Data natural distribution seemed to be slightly clustered with the QC partially centred between conditions, as it was reflected through a supervised Partial Least Square Discriminant Analysis (PLS-DA) 3D score plot (Fig. 1B). In this case, separation between LPS (light blue circles) and the rest of conditions is more emphasized. To confirm the possible relationships among sample groups, a hierarchical clustering analysis was performed by applying Pearson's uncentered absolute similarity measure and complete linkage (Fig. 1C). The dendrogram clusters the four experimental conditions into three classes, indicating close clustering between HM14 and Basal, which differed from LPS. These results are concordant with those previously reported by LC/MS approach (Cambeiro-Pérez et al., 2018).

Finally, 30 metabolites were found overproduced in PBMCs after incubation with HM14 comparing to basal condition whilst 6

metabolites were found underproduced by HM14. Metabolomic data interpretation was performed by MetaboAnalyst 4.0 (http://www.metaboanalysts.ca) through MSEA (metabolite set enrichment analysis) with over representation analysis (ORA) tool. MSEA allows identifying the most significant metabolic pathways, based on their p-values and fold enrichment, included in the SMPDB (Small Molecular Pathway Database) based on normal human metabolic pathways. In our case, 68 metabolite sets were found modulated in HM14 condition of which, 42 were represented by more than 2 metabolites in the pathway (Fig. 1D). Among them, the most enriched pathways (p-value $< 0.050$ and FDR $< 0.30$) were: urea cycle, Glycolysis, gluconeogenesis, nicotinate and nicotinamide metabolism, galactose metabolism, Warburg effect, pyrimidine metabolism, glycerol phosphate shuttle, inositol phosphate metabolism, cardiolipin biosynthesis, inositol phosphate metabolism and phosphatidylethanolamine biosynthesis. A complete list of up and downregulated entities are available in Suppl. File 1.

### 3.4. Anti-inflammatory effect of HM14 in PBMCs from a Crohńs disease cohort

Given that HM14 induced metabolic responses compatible with an anti-inflammatory effect, and given that it promoted IL-10 and IL-2 secretion in PBMCs from healthy donors, we tested the effect of the peptide in PBMCs isolated from a cohort of Crohn Disease patients ($n = 5$). The fact that all the patients were sampled at the time of diagnosis is very important because it means immune cells were obtained in the absence of any treatment for IBD. We used the same 18-plex cytokine array and included another microbiota peptide (LR17), which was previously reported as Th17/Th22 promoter in a previous study (Hidalgo-Cantabrana et al., 2017). Globally, HM14 increased production of IL-10 and prevented the increase in IL12p70 elicited by the T-cell activator anti-CD3 antibody, confirming the anti-inflammatory effect of this *Bifidobacterium*-derived peptide. LR17 peptide was unable to reproduce HM14 behavior (Fig. 2; Suppl. File 2).

### 3.5. Molecular mechanism of action of HM14 over on monocyte-derived dendritic cells

HM14 was identified through the MAHMI database web-service (http://www.mahmi.org) which was able to identify immunomodulatory peptides. Based on our previous results obtained by cytokine profiling of bacteria-derived peptides (Bernardo et al., 2012) and metabolomics (Cambeiro-Pérez et al., 2018) from PBMCs, we focused our interest in monitoring the influence of HM14 *vs.* other MAHMI selected peptides on monocyte-derived dendritic cells (MoDCs). To confirm the effect of HM14, 15 peptides encrypted in extracellular proteins *of Bifidobacterium longum* subsp. *longum* and *Lactobacillus plantarum*, representatives of both probiotic and intestinal bacteria, including HM14, were incubated with MoDCs from healthy donors, by cytokine profiling and transcriptomics measurements.

We evaluated the production of anti-inflammatory cytokine IL-10. Only 4 out of the 15 peptides assayed induced the production of IL-10 by MoDCs when assayed at 10 $\mu g\,mL^{-1}$, the HM14 peptide showing on of the higher capacities to induce IL-10 secretion (Fig. 3A). Peptides SP13 and AY14, obtained from the already known anti-inflammatory molecule STp, were also associated with high IL-10 production. A further transcriptomic profiling of MoDCs using RNASeq, revealed that each of the IL-10-inducing peptides imprinted a particular gene expression pattern, being that of HM14 the most distant regarding the pro-inflammatory LPS molecule (Fig. 3B; Suppl. File 3 and 4).

Enrichment analysis (ConsensusPathDB-human; http://cpdb.molgen.mpg.de/) originated from the transcriptomic data, suggested that HM14 peptide induced a molecular mechanism of action dependent on Toll-like receptor and NF-kB signaling (Suppl. Fig. 2). A complementary enrichment analysis using the DAVID web-service (https://david.ncifcrf.gov/), evidenced that many terms and pathways involved in immunological processes were enriched in MoDCs as affected by the presence of HM14. Among the genes over-expressed by HM14 we detected several coding for interleukins or interleukin receptors, as well as markers of dendritic cell maturation and functionality, such as CD83, CD33 and CD48 (Table 1).

### 3.6. Molecular mechanism of action of HM14 over CD4 T-cells

As IL-10 and IL-2, two cytokines involved in regulatory T cell (Treg) expansion, were overproduced in the presence of HM14, we characterized whether this peptide was able to generate Treg cells in vitro (iTreg) and whether those cells were functional. iTreg cells were routinely produced from $CD4^+$ naïve cells in the presence of anti-CD3, anti-CD28, IL-2 and TGF-β. As it can be seen in Fig. 4A, HM14 did not blocked iTreg cell expansion, and there was a net increase in the % of iTreg cells ($n = 9$) compared to basal conditions. These iTreg cells induced the production of IFNγ when co-incubated with naïve $CD4^+$ T-cells, confirming their functionality, although the proportion of IFNγ induced in the presence of HM14 was significantly lower than the positive control (Fig. 4A, B). Finally, HM14 promoted IL-10 secretion by iTreg in the presence of the polyclonal CD3 activator (Fig. 4C), and no changes in TGFβ production were noticed (Fig. 4D).

Regarding IL-22 production, we previously noticed a remarkable but not statistically significant increase in this cytokine in PBMCs from healthy donors (Suppl. Fig. 1). As IL-22 may also have a healing role in chronic inflammatory settings, we performed several flow cytometry experiments with a larger cohort. As previously, presence of HM14 did not resulted in significant IL-22 production increases (Fig. 5A), but intracellular IL-22 staining revealed a significant increase in IL-$22^+$ cells (Fig. 5B, C).

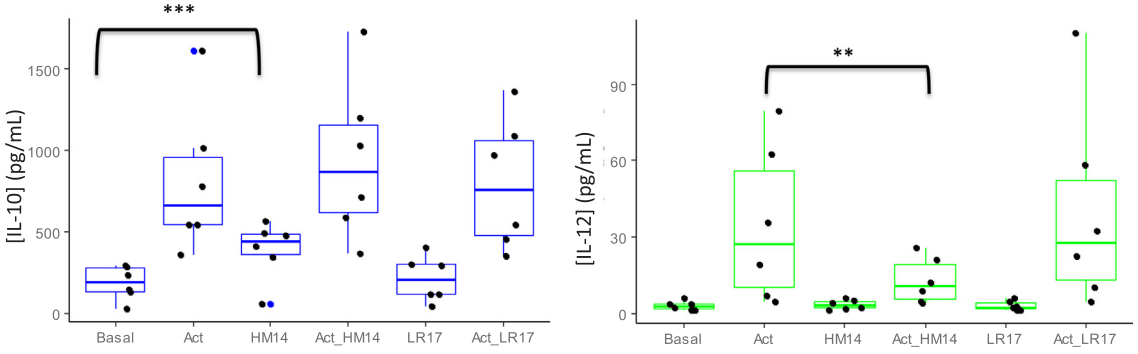


**Fig. 2.** Changes in key cytokines in PBMCs isolated from Crohn's Disease patients promoted by the incubation of different peptides. In the left panel, presence of HM14 is increasing IL10 production with respect to basal conditions, whereas in the right panel, the IL12-secreting effect of the polyclonal T-cell activator is attenuated by the presence of HM14. Both results are in agreement with the anti-inflammatory HM14 effect. Act: $CD4^+$ polyclonal activator. LR17: Th17 pro-inflammatory peptide.

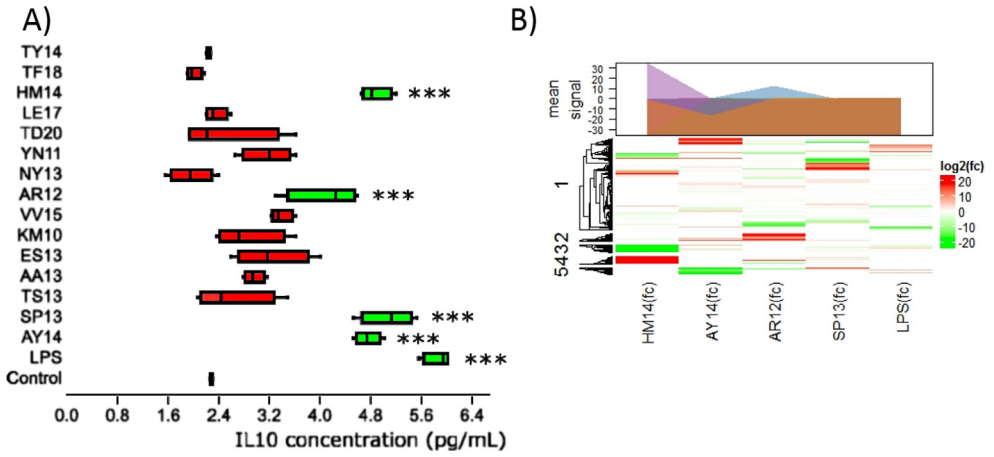


**Fig. 3.** (A) Levels of IL-10 (pg $mL^{-1}$) and (B) global patterns of gene expression profiles induced by different peptides over MoDCs.

## 4. Discussion

There is solid scientific evidence pointing that certain bacteria promote mucosal homeostasis favoring the expansion of Treg cells, increasing the production of anti-inflammatory cytokines and decreasing the release of pro-inflammatory cytokines (Ruiz et al., 2014). Given the anatomic structure of the intestinal mucus layer (Swidsinski et al., 2007), the existence of a molecular cross-talking between bacteria an immune cells through soluble mediators has been proposed (Sánchez et al., 2010). These soluble mediators derived from probiotic bacteria would be responsible for molecular cross-talking mechanisms, supporting beneficial effects over host health; the term postbiotics has been proposed for these compounds (Tsilingiri & Rescigno, 2013; Tsilingiri et al., 2012). Currently, we have extended evidence for the presence of a varied array of molecules and metabolites that, shed from our intestinal microbiota, alters the immune status after binding to specific receptors (Maslowski & Mackay, 2011). Therefore, a plausible hypothesis is that a continuous molecular cross-talking between all the molecules released by our intestinal microbiota and human host may determine the immune function during the whole life of an individual.

Ten years ago, we had one of the first evidences of the presence of an immunomodulatory peptide encoded in an extracellular protein secreted by the lactic acid bacterium *Lactobacillus plantarum*, denominated ST peptide (Al-Hassi et al., 2014; Bernardo et al., 2012). Notably, this peptide was patented and it has been approved in the EEUU market (US9340588B2, https://patents.google.com/patent/US9340588B2/en). Recently, we moved to the hypothesis of whether the occurrence of this kind of bioactive peptides was general in probiotic and gut microorganisms, always in the framework of finding those related to beneficial effects over the health status of the human host (Blanco-Míguez et al., 2017; Li et al., 2014). For this purpose, we have chosen peptides encrypted in the extracellular proteins of *Bifidobacterium longum* subsp. *longum* and *Lactobacillus plantarum*, as the first is a major component of the human gut microbiota and the second one a representative species with many probiotic strains, and also the species were the ST peptide was discovered, which was shown to modulate the immune status of both intestinal and MoDCs (Al-Hassi et al., 2014; Bernardo et al., 2012). We have already discovered several hundreds of peptides with potential immunomodulatory activity based on the MAHMI pipeline. Some promoted Th17- or Th22-like cell responses as deduced from the cytokine profiles induced in DCs, but others, such as a peptide encrypted in a *B. longum* subsp. *longum* extracellular protein, displayed many tolerogenic properties in the extracellular environment of human lamina propria mononuclear cells cultures (Fernández-Tomé et al., 2019; Hidalgo-Cantabrana et al., 2017). Further, we have *in silico* evidence for the presence of potential neuroactive peptides also encrypted in the intestinal metaproteome (Blanco-Míguez, Fdez-Riverola, Lourenço, & Sánchez, 2019).

HM14 peptide, which is present in this work, is a novel molecule able not only to increase IL-10 production in healthy donors and in a cohort of Crohńs patients, but also able to prevent the increase in the pro-inflammatory cytokine IL-12 as response to a clonal T-cell activator. HM14 peptide is therefore an example of the thousands of potential anti-inflammatory peptides that might be generated from our intestinal microbiota. The fact to have both pro, and anti-inflammatory peptides released from the gut microbiota is a reflect of the own human physiology, where many immunomodulatory proteins with opposite functions are present, such as IL-1β (pro-inflammatory) and IL-10 (regulatory) (Geranurimi et al., 2019). Presence of immunomodulatory peptides is not only a microbiota property, as several small peptides encrypted in other proteins such as the human annexin A1 reported anti-inflammatory activity in animal models of IBD (Cobos et al., 2018); presence of encrypted immunomodulatory peptides is also described in food proteins, with strong links related to the maintenance of gut homeostasis (Fernández-Tomé et al., 2019).

Additional experimental results supported the role of HM14 as an immunomodulatory peptide. HM14 was able to influence the function of iTreg cells that, when incubated with naïve T cells, induced them to produce significantly less IFNγ and a slightly higher amount of IL-10. The peptide also increased the production of IL-2 in healthy donors. IL-2 has essential roles in key functions of the immune system, tolerance and immunity, through a direct effect on T cells. For instance, IL-2 is central in the differentiation of naïve $CD4^+$T cells into Treg cells, an immune cell population which is precisely decreased in autoinflammatory diseases (Zelante, Fric, Wong, & Ricciardi-Castagnoli, 2012). Effect of HM14 on CD4 + T lymphocyte differentiation towards iTreg was clear. In PBMC cultures, HM14 could be acting on many cellular types and induces production of many different cytokines. This makes impossible to analyze its effect on iTregs in this conditions.

RNASeq experiments suggested that HM14 signaled through antigen presenting cells using innate immune pathways. In this sense *hla-dqb1*, *hla-dqa1*, *hla-dra* or *hla-drb1* genes were over-expressed. All these genes code for proteins that are part of the peptide-presenting machinery (usually peptides of 10–30 residues), which is generally referred as the major histocompatibility complex (MHC) class II. This complex is mainly expressed in antigen presenting cells, including macrophages and dendritic cells (Anders et al., 2011); however, epithelial cells from the gastrointestinal tract, have also been reported to express MHC class II molecules (Wosen, Mukhopadhyay, MacAubas, & Mellins, 2018); this is supposed to mediate a kind of non-professional antigen presentation, with potential implications in immune system-microbiota molecular crosstalk.

It was also noteworthy the over-expression of the *tlr1* gene, as representative of the Toll-like receptors, which are part of the innate

**Table 1**
Relevant gene expression changes induced by HM14 on monocyte-derived dendritic cells.

| Gene_name | Fold change | p-value | q-value |
|---|---|---|---|
| *Receptors* | | | |
| HLA-DQB1 | 3477.18 | 0.000 | 0.004 |
| HLA-DQA1 | 533.24 | 0.000 | 0.003 |
| HLA-DRA | 1.47 | 0.001 | 0.005 |
| HLA-DRB1 | 1.42 | 0.002 | 0.009 |
| HLA-DMA | 1.67 | 0.001 | 0.005 |
| HLA-DMB | 1.97 | 0.000 | 0.003 |
| HLA-DPB1 | 1.97 | 0.001 | 0.008 |
| TLR1 | 1.69 | 0.050 | 0.110 |
| CCL18 | 4796.71 | 0.000 | 0.004 |
| CCL2 | 842.23 | 0.001 | 0.006 |
| CCL13 | 220.93 | 0.001 | 0.005 |
| CXCL8 | 175.02 | 0.000 | 0.004 |
| CCL3 | 98.45 | 0.000 | 0.003 |
| CCL4 | 72.54 | 0.000 | 0.003 |
| CCL7 | 38.43 | 0.000 | 0.004 |
| CCL23 | 29.06 | 0.002 | 0.009 |
| CXCL2 | 15.97 | 0.008 | 0.028 |
| CCL17 | 5.15 | 0.000 | 0.001 |
| *Intracellular signal transduction* | | | |
| JAK3 | 13.05 | 0.000 | 0.002 |
| NFKB1 | 2.80 | 0.000 | 0.003 |
| NFKB2 | 3.70 | 0.000 | 0.003 |
| NFKBIA | 7.61 | 0.000 | 0.003 |
| NFKBID | 2.70 | 0.008 | 0.028 |
| *Interleukin receptors* | | | |
| IL17RB | 1350.71 | 0.000 | 0.004 |
| IL7R | 59.11 | 0.000 | 0.003 |
| IL3RA | 39.54 | 0.001 | 0.007 |
| IL36RN | 5.50 | 0.034 | 0.082 |
| IL18BP | 3.42 | 0.000 | 0.005 |
| IL1RAP | 1.71 | 0.004 | 0.016 |
| IL1RN | 2.39 | 0.002 | 0.009 |
| IL22RA2 | 12.90 | 0.000 | 0.004 |
| IL2RG | 2.90 | 0.001 | 0.008 |
| IL10RA | 1.29 | 0.002 | 0.011 |
| IL21R | 1.15 | 0.043 | 0.099 |
| IL3RA | 8.77 | 0.038 | 0.089 |
| *Interleukins* | | | |
| IL10 | 19.80 | 0.003 | 0.013 |
| TGFA | 5.58 | 0.000 | 0.003 |
| IL1A | 15.45 | 0.000 | 0.003 |
| IL1B | 13.00 | 0.002 | 0.012 |
| IL4I1 | 3.14 | 0.028 | 0.070 |
| *Dendritic cell maturation markers* | | | |
| CD274 (PD-L1) | 6.63 | 0.000 | 0.002 |
| CD80 | 6.44 | 0.000 | 0.004 |
| CD86 | 1.17 | 0.009 | 0.030 |
| CD83 | 3.10 | 0.000 | 0.002 |
| CD40 | 2.48 | 0.002 | 0.012 |

immune system where they recognize microbial associated molecular patters. Ligand to TLR1 is unknown, although it is known that the heterodimer with TLR2 recognizes di- and tryacylated lipopeptides (West et al., 2011). Finally, a number of C-C Motif Chemokine Ligand and related genes were also overexpressed, such as *ccl18* (4796.71-fold) or *ccl2* (842.23-fold). Usually, these ligands display chemotactic activity for T cells, but not for other immune cells such as monocytes. These chemokines participates in the attraction of naïve T cells toward antigen presenting cells, which is in agreement with the hypothesis that HM14 is recognized via innate immunity (Krohn, Garin, Gabay, & Proudfoot, 2013). This was reinforced with the upregulation of genes coding for dendritic cell maturation markers, such as *pdl1*, *cd40*, *cd83*, *cd80* and *cd86*. Moreover, genes coding for key intracellular signal transduction proteins such as *jak3*, *nfkb1* and *nfkb2* were also overexpressed. Finally, overexpression of genes coding for interleukin receptors involved in Treg cell expansion such as *il2rg* and *il10ra*, and

**Table 2**
Peptides used in this study.

| Sequence | Short name | Bacterial species |
|---|---|---|
| N-EVNGDSTTTTSTS-C | ES13 | *L. plantarum* |
| N-TQTTQQTTTTQSS-C | TS13 | *L. plantarum* |
| N-AQTSQTQAQPSQA-C | AA13 | *L. plantarum* |
| N-SQTQSSQTQTSKP-C | SP13 | *L. plantarum* |
| N-AAQTTQTSSSTSNY-C | AY14 | *L. plantarum* |
| N-TLAADPVMLTKPEY-C | TY14 | *B. longum* subsp. *infantis* |
| N-KPRRRRIAAM-C | KM10 | *B. longum* subsp. *infantis* |
| N-TQSTASGGEPAPADLQSF-C | TF18 | *B. longum* subsp. *infantis* |
| N-HTEGEAQAIDAPAM-C | HM14 | *B. longum* subsp. *infantis* |
| N-NTEGIDLTKVGDY-C | NY13 | *B. longum* subsp. *infantis* |
| N-AEVEGDNNAMLR-C | AR12 | *B. longum* subsp. *infantis* |
| N-LRFPGGCIVEGVTPGNE-C | LE17 | *B. longum* subsp. *infantis* |
| N-SEGMHVVDAG-C | SG10 | *B. longum* subsp. *infantis* |
| N-VSGGTVTLPDDATNV-C | VV15 | *B. longum* subsp. *infantis* |
| N-YGRSENTGTSN-C | YN11 | *B. longum* subsp. *infantis* |
| N-TVATGSEEPTAGLPSEGPAD-C | TD20 | *B. longum* subsp. *infantis* |

citokines such as *il10*. The rest of the in vitro experiments, including the generation of iTreg cells and the anti-inflammatory effect over PBMCs isolated from IBD patients strongly suggest that HM14 is recognized via innate immunity, presented to naïve T cells which are further differentiated to Treg.

Non-targeted metabolomics allowed us to understand the impact of HM14 on metabolic pathways and its implications on the development and function of PBMCs.

A previous metabolomics research from our group demonstrated a differential response in cells exposed to HM14 with respect to LPS and basal condition. Specifically, changes in nicotinate and nicotinamide metabolism as well as increments in palmitic acid and metanephrine were observed in HM14 treated-cells (Cambeiro-Pérez et al., 2018). However, since the analytical platform selected in that previous work was LC-QTOF-MS, predominant differences were observed in glycerophospholipids metabolism. That is why, in order to cover the largest number of compounds and their wide range of polarities, which allows us to know in depth the metabolic state of the cell, GC–MS metabolomics analysis was performed.

This platform not only reinforces the previous knowledge about changes in nicotinate and nicotinamide metabolism but also reveals important alterations in aminoacids biosynthesis in HM14 treated-cells, besides providing valuable information regarding carbohydrate, purine and pyrimidine metabolism.

Aminoacids are known to play an important role in immune function by regulating lymphocytes, natural killer cells and macrophages activation; antibodies and cytokines production; cellular redox state; gene expression and lymphocyte proliferation (Li, Yin, Li, Woo Kim, & Wu, 2007). Among them, arginine, glutamine, ornithine, citrulline, hydroxyproline, serine, tyrosine and alanine were found up-regulated by HM14 in PBMCs, whilst only proline was found down-regulated. Glutamine is known to be the major energy substrate for immune cells as well as to be responsible for regulate lymphocytes proliferation and glutathione production, crucial for preventing cell oxidative stress, besides being an essential precursor for the synthesis of purine and pyrimidine nucleotides (Cruzat, Rogero, Keane, Curi, & Newsholme, 2018). This regulation was also observed in RNASeq experiments were *gls* and *glul* genes, involved in glutamine and glutamate metabolism, were found overexpressed.

Glutamine is also an important precursor for de novo synthesis of arginine (Ligthart-melis et al., 2008), which was found up regulated in HM14 treated-cells as well as their downstream metabolic products, ornithine and citrulline. Moreover, serine supports de novo purine nucleotide biosynthesis (Ma et al., 2017), phosphatidylserine and sphingolipid synthesis and also contributes to nucleotide and redox metabolism, playing a major role in maintaining mitochondrial metabolism (Gao et al., 2018). Tyrosine is a precursor for catecholamines,

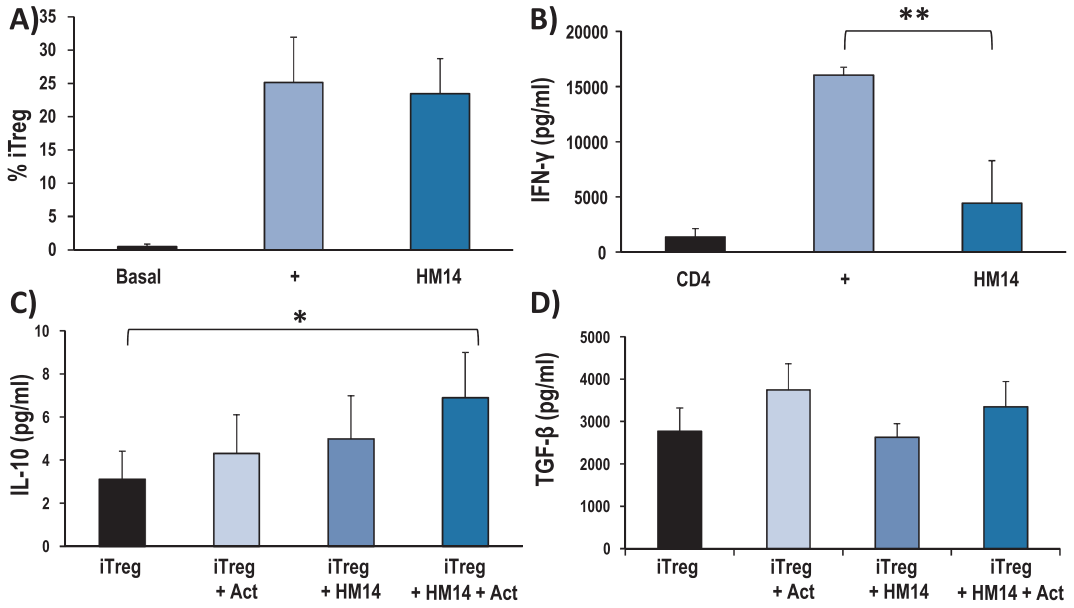


**Fig. 4.** iTreg generation and functionality. (A) Percentage of differentiation of iTreg modulated by the presence of HM14 with respect to normal protocols (+). (B) On-going production of IFN-γ in $CD4^+$ cells as affected by the presence of iTreg differentiated in the presence (HM14) or absence (+) of the HM14 peptide. (C) and (D), on-going production of IL-10 and TGF-β by iTreg cells treated with the polyclonal CD4 activator (Act), the peptide HM14 or a combination of both molecules. Results are expressed as the mean of 5 biological replicates (* $p < 0.05$** $p < 0.01$).

which regulate immune and inflammatory responses. There are evidence that PBMCs are able to synthesize and breakdown catecholamines (Marino et al., 1999). As was previously reported by our LC-MS metabolomics approach (Cambeiro-Pérez et al., 2018), two metabolites of catecholamine catabolism showed differences in HM14 treated-cells, specifically overproduction of metanephrine (breakdown product of epinephrine) and down regulation of homovanillic acid (break down product of dopamine). These results were supported by RNAseq data, that indicated an overexpression of *maoa* which encodes for the monoamine oxidase A, key enzyme in catecholamines metabolism. Tyrosine can be also incorporated into proteins. Phosphorylation and dephosphorylation of tyrosine residues, modulates protein's function by triggering an activation or inactivation mechanism of intracellular signalling molecules (Spalinger, Mccole, Rogler, & Scharl, 2015). The enzymes responsible for dephosphorylation are protein tyrosine phosphatases (PTPs). Among them, protein tyrosine phosphatase non-

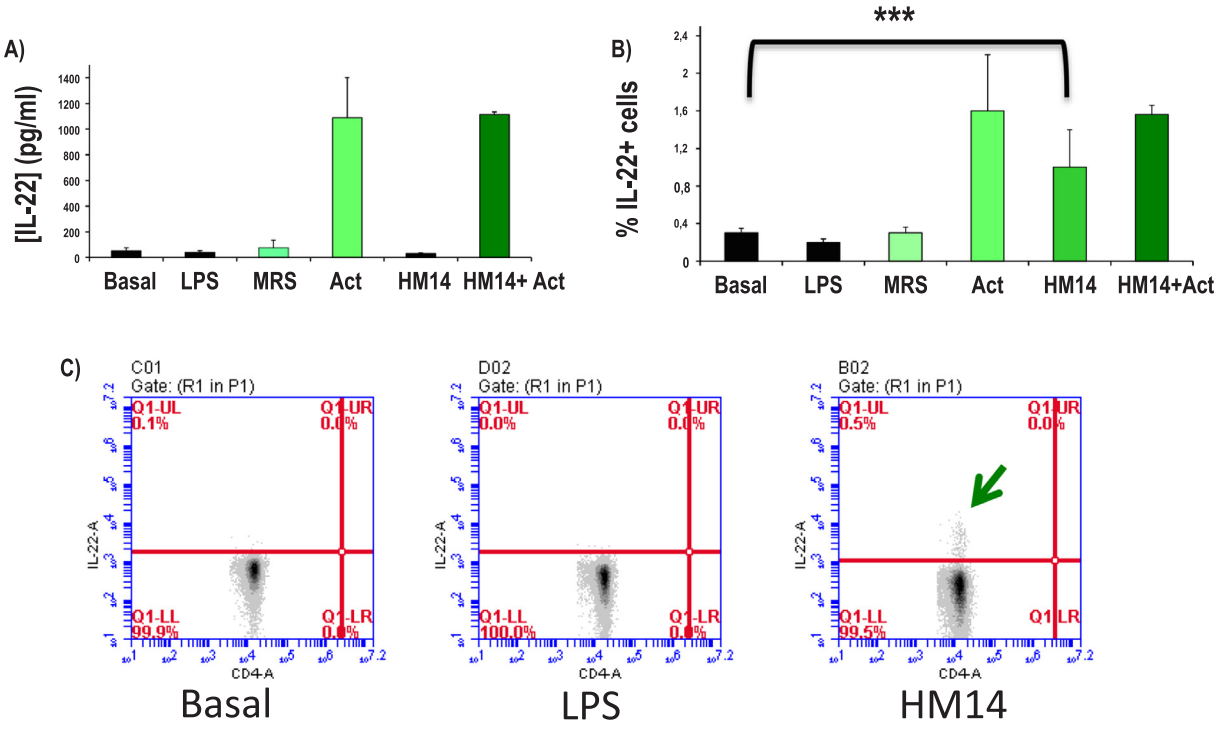


**Fig. 5.** (A) IL-22 production in PBMC fraction treated with LPS, MRS (Control protein extract), polyclonal CD4 activator (Act), the peptide HM14 or a combination of both. (B) IL-22 percentage of IL-22 positive cells in PBMC fraction treated with LPS, MRS (Control protein extract), polyclonal CD4 activator (Act), the peptide HM14 or a combination of both. (C) Intracitoplasmatic production of IL-22 in $CD4^+$ as affected by the exposure to LPS or HM14 peptide. Results are expressed as the mean of 5 biological replicates.

receptor (PTPN) type 2 and type 22 expression levels, are reported to be altered in actively inflamed intestinal tissue. In fact, PTPN2 knock-out mice suffer from severe intestinal and systemic inflammation and display alterations in innate and adaptive immune responses. PTN2 dysfunction increased secretion of pro-inflammatory cytokines, promotes disruption of the intestinal epithelial barrier, limit autophagosome formation and contributes to the manifestation of IBD (Spalinger et al., 2015, 2016). On the other hand, PTPN22 is also involved in controlling inflammatory signalling in lymphocytes and mononuclear cells, altering cytokine secretion pattern and autophagosome formation. Deficiencies in this enzyme, are more severe in colitis, revealing its importance for intestinal homeostasis in vivo (Spalinger, McCole, Rogler, & Scharl, 2015). Metatranscriptomics analysis suggested that HM14 enhanced the expression of *ptpn2* and *ptpn22* coding genes.

Several metabolites involved in nucleotide metabolism were found up regulated in presence of HM14 in PBMCs; adenosine, hypoxanthine, cytidine monophosphate, uridine monophosphate and glutamine. Purine and pyrimidine nucleotides are essential in DNA and RNA synthesis, crucial for tissue repair, protein synthesis and cell turnover. Moreover, nucleotides are involved in signal transduction, regulation of enzyme activity, membrane lipid biosynthesis and protein glycosylation (Lane & Fan, 2015; Quéméneur et al., 2003). RNAseq also supported the activation of these metabolic pathways both with the overexpression of the enzymatic machinery involved in the pathways themselves (*ak4*; *ak6*, *prps1*, *nt5c3a*, *dck*, *cmpk1*…), and the overexpression of several polymerases (*pole4*, *polk*, *polr2f*, *polr2k*, polr2l, *polr3f*, *polr3k*), essential for DNA and RNA biosynthesis. In this way, we are showing using two different omic technologies, metabolomics and transcriptomics, that presence of HM14 is triggering nucleic acid biosynthesis, likely related to an expansion of some of the populations integrating the PBMC fraction.

Among carbohydrates, glycolytic intermediaries were found up regulated in HM14 treated-cells such as 3-phosphoglyceric acid, glucose 6-phosphate and dihydroxyacetone phosphate; as it was also observed in metatranscriptomics analysis with the over-expression of key enzymes of glucose metabolism (*hk3*, *pgm2*, *fbp1*, *tpi1*, *bpgm*, *pgk1*, *eno1*, *dlat*). Due to cells rely on glucose for energy, an elevated glycolysis rate fuels the generation of the precursors required for cellular growth and proliferation (Shehata et al., 2017).

Generally, metabolomics results suggested that HM14 promotes protein synthesis through up-regulation of amino acid biosynthesis and nucleotide metabolism besides encouraging a glycolytic state to fulfil cell-type-specific metabolic requirements; as maintaining the enzymatic machinery, cell differentiation and expansion, cytokine production, signalling mechanisms, among others.

## 5. Conclusions

To conclude, the human gut metaproteome is source for a vast array of potential immunomodulatory peptides, that can be identified through an *in silico* pipeline. HM14 exerted its anti-inflammatory effect by acting on a yet uncharacterized innate immune receptor. Presentation of HM14 to naïve T cells, in vitro, stimulated iTreg expansion in healthy donors and reduced proinflamatory IL-12 in PBMCs isolated from Crohn's patients. In all in vitro settings, HM14 increased the production of anti-inflammatory IL-10 by immune cells. Metabolomics analysis also supported a differential behavior between immune cells exposed to HM14 and untreated cells; suggesting that HM14 was able to interact with cells and triggers an increase in protein biosynthesis rate and glycolytic intermediaries. This study opens new insights directed to the understanding on how these millions of immunomodulatory peptides originated from the gut metaproteome affect the way our microbiota interacts with our immune and potentially with our nervous system.

## 6. Ethics statement

Ethics approval for this study (reference code AGL2013-44039-R and PS2016) was obtained from the Regional Ethics Committee for Clinical Research in compliance with the Declaration of Helsinki. Samples used in this study were obtained from anonymous donors of the regional blood donation system and from a cohort of Crohn's Disease patients.

## CRediT authorship contribution statement

**Noelia Cambeiro-Pérez:** Investigation, Methodology. **Claudio Hidalgo-Cantabrana:** Investigation, Methodology, Supervision. **Marco Antonio Moro-García:** Investigation, Methodology, Supervision. **Aitor Blanco-Míguez:** Investigation, Methodology, Software. **Florentino Fdez-Riverola:** Writing - review & editing. **Sabino Riestra:** Writing - review & editing. **Anália Lourenço:** Writing - review & editing. **Rebeca Alonso-Arias:** Writing - review & editing, Supervision. **Abelardo Margolles:** Writing - review & editing, Supervision. **Elena Martínez-Carballo:** Writing - review & editing, Supervision. **Borja Sánchez:** Conceptualization, Funding acquisition, Supervision, Project administration, Writing - original draft, Writing - review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgements

Our work is supported by the Spanish "Programa Estatal de Investigación. Desarrollo e Innovación Orientada a los Retos de la Sociedad" (grants AGL2013-44761-P and AGL2016-78311-R); the Asociación Española Contra el Cancer ("Obtención de péptidos bioactivos contra el Cáncer Colo-Rectal a partir de secuencias genéticas de microbiomas intestinales", Grant PS-2016), by the Asturias Regional Plan I + D + i for research groups (FYCYT-IDI/2018/000236) and by the Autonomic "Investigadores Emerxentes do Sistema Universitario de Galicia" (Grant EM2014/046). This work was partially supported by the Consellería de Educación. Universidades e Formación Profesional (Xunta de Galicia) under the scope of the strategic funding of ED431C2018/55-GRC Competitive Reference Group. Finally, the authors wish to thank Jaume Morales and Rubén García form Agilent Technologies for technical support.

## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2020.103969.

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