Universitat Autònoma de Barcelona

INTESTINAL MUCOSAL HUMORAL RESPONSE AND NEURO-IMMUNE INTERACTION AS CONTRIBUTORS TO THE PATHOPHYSIOLOGY OF DIARRHEA-PREDOMINANT IRRITABLE BOWEL SYNDROME

CRISTINA PARDO CAMACHO

DOCTORAL THESIS

INTESTINAL MUCOSAL HUMORAL RESPONSE AND NEURO-IMMUNE INTERACTION AS CONTRIBUTORS TO THE PATHOPHYSIOLOGY OF DIARRHEA-PREDOMINANT IRRITABLE BOWEL SYNDROME

Presented by

CRISTINA PARDO CAMACHO

Directed by

María Vicario Pérez

Francisco Javier Santos Vicente

Tutor

Anna Maria Bassols Teixidó

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Dr. MARÍA VICARIO PÉREZ, Doctor of Pharmacy and

Dr. JAVIER SANTOS VICENTE, Doctor of Medicine,

Certify that the thesis entitled “Intestinal mucosal humoral response and neuro-immune interaction as contributors to the pathophysiology of diarrhea-predominant Irritable Bowel Syndrome” submitted by **Cristina Pardo Camacho**, was carried out under their supervision and tutorship of Dr. Anna Maria Bassols Teixidó, and authorize its presentation for the defense ahead of the corresponding tribunal.

In witness whereof, they hereby sign this document.

Barcelona, July 2020

Dr. María Vicario Pérez

(Director)

Dr. Javier Santos Vicente

(Director)

Dr. Anna Maria Bassols Teixidó

(Academic tutor)

The cover is a fluorescence microscope image of plasma cells (CD138 marker, red) and neurons (PGP9.5 marker, green) in the intestinal mucosa of a healthy donor (magnification 600x)

The back cover is a transmission electron microscope image showing the ultrastructure of plasma cell and nerves present in the intestinal mucosa of a diarrhea-predominant Irritable Bowel Syndrome patient (magnification 15,000x). Proximity between plasma cells and never endings can be observed.

Technical design assistance of the cover by Tània Manzal Cerdà.

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“Men love to wonder, and that is the seed of science”.

Ralph Waldo Emerson (1803-1882)

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A mis padres,

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ABBREVIATIONS

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ATP: Adenosine Triphosphate

BLIMP1: B lymphocyte-induced maturation protein-1

BSA: Bovine Serum Albumin

CD40L: CD40Ligand

cDNA: complementary Deoxyribonucleic acid

CLRs: C-type Lectin Receptors

CLS: Categorical Class

CpG ODN2006: Oligodeoxyribonucleotides containing CpG motifs

CRF: Corticotropin Realising Factor

Cv: Coefficient of variation

DAPI: 4 ',6-diamidino-2-fenilindol

DAMPs: Danger-Associated Molecular Patterns

DEG: Differentially Expressed Gene

DNA: Deoxyribonucleic acid

dsRNA: Double Stranded Ribonucleic acid

DTT: Dithiothreitol

DUOX2: Dual oxidase 2

EBV: Epstein-Barr Virus

ECACC: European Collection of Authenticated Cell Cultures

EDTA: Ethylenediamine tetraacetic acid

ELISA: Enzyme-Linked ImmunoSorbent Assay

FBS: Fetal Bovine Serum

FDR: False Discovery Rate

FcRn: neonatal Fc receptor

FSC-A: Forward Scatter Area

GALT: Gut-Associated Lymphoid Tissue

GEA: Gene Enrichment Analysis

GMT: Gene Matrix Transposed

GSEA: Gene Set Enrichment Analysis

GLM: Generalized Linear Model

GWA: Genome-Wide Association study

HBSS: Hanks Balanced Salt Solution

HEVs: High Endotelial Venules

HLA: Human Leukocyte Antigen

HPA: Hypothalamic Pituitary Adrenal

HV: Healthy Volunteers

IBS: Irritable Bowel Syndrome

IBS-C: Constipation-predominant Irritable Bowel Syndrome

IBS-D: Diarrhea-predominant Irritable Bowel Syndrome

IBS-M: Mixt pattern Irritable Bowel Syndrome

IBS-U: Unclassified Irritable Bowel Syndrome

ICR: Institute of Cancer Research

IECs: Intestinal Epithelial Cells

IELs: Intraepithelial Lympchocytes

IF: Immunofluorescence

Ig: Immunoglobulin

IL: Interleukine

KEGG: Kyoto Encyclopedia of Genes and Genomes

LPL: *lamina propria* lymphocytes

LPS: Lipopolysaccharide

MAMPs: Microbe-Associated Molecular Patterns

MAPK: Mitogen-Activated Protein Kinase

MHC: Major Histocompatibility Complex

NKR1: Neurokinin 1 Receptor

NLRs: Nucleotide-binding oligomerization domain (NOD)-Like Receptors

NOD: Nucleotide-binding Oligomerization Domain

O/N: Over-night incubation

PAMPS: Pathogen-Associated Molecular Patterns

PBS: Phosphate buffered-saline

PCA: Principal Component Analysis

PFA: Paraformaldehyde

PGP9.5: Protein Gene-Product 9.5

pIgR: polymeric Immunoglobulin Receptor

PRRs: Pattern Recognition Receptors

PWM: Lectin from Phytolacca Americana (pokeweed)

qPCR: quantitative Polymerase Chain Reaction

RIG-I: Retinoic Acid Inducible Gene-I

RIN: RNA Integrity Number

RLRs: Retinoic acid inducible gene-I (RIG-I)-Like Receptors

RNA: Ribonucleic acid

RNA-seq: RNA sequencing

RT-qPCR: Reverse Transcription quantitative Polymerase Chain Reaction

SAC: Protein A from *Staphylococcus aureus*

SNPs: Single-Nucleotide Polymorphisms

SP: Substance P

SP Vh: Substance P Vehicle

SPT: Skin Prick Test

ssRNA: Single Stranded Ribonucleic acid

SSC-A: Side Scatter Area

TACR1: Tachykinin Receptor 1

TCR: T Cell Receptor

TEM: Transmission Electron Microscopy

Th cells: T helper cells

TI: Thymus Independent

TLR: Toll-like receptors

VAS: Visual Analogue Scale

VIP: Vasoactive Intestinal Peptide

VST: Variance Stabilizing Transformation

XBP1: X-box binding protein 1

SUMMARY

SUMMARY

Irritable Bowel Syndrome (IBS) is a chronic and prevalent gastrointestinal disorder which curses with intestinal motility alterations and abdominal pain. IBS constitutes a relapsing and potentially disabling disorder and, currently, there is no specific diagnosis biomarker and only palliative treatments are available. The absence of a well-established pathophysiology highlights the need of identifying the underlying organic causes of motility alterations and the onset of symptoms. In the intestinal mucosa of these patients a certain degree of inflammation has been identified together with an increased intestinal permeability and a higher activity of the immune response. Previous studies from our group showed an increased humoral activity in the intestinal mucosa of diarrhea-predominant IBS (IBS-D) patients, associated with more severity of the symptoms. IBS patients often present anxiety and depression, and dysfunction of the gut-brain axis features IBS onset and outcome. Considering intestinal mucosa is highly innervated and the existence of a bidirectional modulation between nervous and immune systems, the main objective of this thesis was to characterize the activation humoral response by neuro-immune mechanisms in the intestinal mucosa of IBS-D patients.

To achieve our purpose, this project has been divided into three chapters. In chapter 1, we collected jejunal biopsies, blood and feces from IBS-D patients and healthy volunteers. We quantified immunoglobulins (Igs) in stool and observed higher levels of IgG in IBS-D group, more specifically IgG2 and IgG3, despite this last one did not reach statistical significance. The amount of total IgG positively correlated with the intensity of the abdominal pain reported by the patients. We conducted a phenotypical analysis in jejunal biopsies for the expression of CD38 and CD138 plasma cell marker, PGP9.5 neural marker and TACR1 (substance P receptor) expression, involved in nociceptive signaling. We observed that plasma cells and nerve endings are found in proximity and, when we performed a quantification of this distance by transmission electron microscopy (TEM), results showed plasma cells are significantly close to nerve endings in the IBS-D group. This distance inversely correlates with acute stress symptoms and depression score reported by patients, the later not reaching significance.

In Chapter 2, an RNA-seq analysis was conducted in RNA extracted from IBS-D patients and healthy volunteer jejunal biopsies. We performed a Gene Set Enrichment Analysis with all the genes in these samples; we observed the humoral response immunological phenotype is enriched and 60% of the genes with a highest enrichment score are involved in Ig structure. Pathways associated to intestinal barrier function are also overrepresented in IBS-D.

Finally, in Chapter 3, we evaluated several sources to obtain a B cell *in vitro* model to study the effect of neuropeptides on immune activity, more specifically substance P, in B cell activation/differentiation and Ig production. We confronted primary culture cells (B and plasma cells isolated from intestinal mucosa and blood) and an established B cell line (126BLCL). *In vitro* differentiated plasma cells obtained from blood were also analyzed. After conducting a deep phenotypic characterization, we concluded blood isolated B cells are the most suitable and feasible *in vitro* model for our purpose.

The results of this thesis reinforce the hypothesis of the neuro-immune crosstalk in the intestinal as playing a crucial role in IBS pathophysiology.

RESUMEN

El Síndrome del Intestino Irritable (SII) es un trastorno gastrointestinal crónico y prevalente que cursa con alteraciones en la motilidad intestinal y dolor abdominal. Constituye un trastorno recurrente y potencialmente incapacitante para el cual no existe un marcador específico diagnóstico ni tratamientos específicos. La ausencia de una patofisiología bien establecida subraya la necesidad identificar las causas orgánicas subyacentes a las alteraciones intestinales y a la generación de síntomas. En la mucosa intestinal de estos pacientes, se ha identificado un cierto grado de inflamación, un incremento de la permeabilidad intestinal y una mayor actividad de la respuesta inmunológica. Estudios previos de nuestro grupo revelaron un incremento de la actividad humoral en la mucosa intestinal de los pacientes con SII con predominio de diarrea (SII-D), asociados a síntomas más severos. Por otra parte, los pacientes que sufren de SII a menudo presentan ansiedad y depresión, sustentando que una disfunción del eje cerebro-intestino podría estar involucrada. Teniendo en cuenta la alta inervación de la mucosa intestinal, juntamente con la modulación bidireccional entre sistema nervioso e inmunitario, el objetivo de esta tesis doctoral es caracterizar la activación de la respuesta humoral por mecanismos neuro-inmunológicos en la mucosa intestinal de los pacientes con SII-D.

Para lograr nuestro objetivo, este proyecto se ha divido en tres capítulos. En el capítulo 1, obtuvimos biopsias yeyunales, sangre y heces de pacientes con SII-D y voluntarios sanos. Cuantificamos las Ig en muestras de heces y observamos que los niveles de inmunoglobulina (Ig) G son más altos en el grupo SII-D, concretamente IgG2 e IgG3, esta última sin alcanzar diferencias significativas. La cantidad de IgG total correlaciona de forma positiva con la intensidad del dolor abdominal reportada por los pacientes. Llevamos a cabo un análisis fenotípico en las biopsias de la expresión de los marcadores de célula plasmática CD38/CD138, el marcador neuronal PGP9.5 y la expresión de TACR1 (receptor de sustancia P), involucrado en la señalización nociceptiva. Observamos que las células plasmáticas y las terminaciones nerviosas se encuentran en proximidad y, cuando realizamos la cuantificación de esta distancia mediante microscopía electrónica de transmisión, los resultados mostraron que las células plasmáticas están significativamente más cerca de las terminaciones nerviosas en SII-D. Esta distancia correlaciona con los síntomas de estrés agudo y los niveles de depresión indicados por los pacientes, el segundo sin llegar a alcanzar la significación estadística.

En el capítulo 2, realizamos un análisis de RNA-seq con RNA extraído de biopsias yeyunales de pacientes con SII-D y voluntarios sanos. En estas muestras llevamos a cabo un análisis de enriquecimiento del conjunto de genes observamos que el fenotipo inmunológico de la respuesta humoral está enriquecido y 60% de los genes con mayor nivel de enriquecimiento

están involucrados en la estructura de las Igs. Las vías asociadas con la función barrera en el intestino también se encuentran sobrerrepresentadas en el SII-D.

Finalmente, en el capítulo 3, se evaluaron diferentes fuentes de obtención de célula B como modelo *in* vitro para estudiar el efecto de los neuropéptidos sobre la actividad inmunológica, más específicamente la sustancia P, en la activación/diferenciación de la célula B y la producción de Igs. Comparamos células de cultivo primario (células B y células plasmáticas aisladas de la mucosa intestinal y sangre) y una línea de célula B inmortalizada (126BLCL). También analizamos células plasmáticas diferenciadas *in vitro* obtenidas de las células aisladas de sangre. Tras realizar una detallada caracterización fenotípica, concluimos que las células B de sangre son el modelo *in* vitro más adecuado y factible para alcanzar nuestro objetivo.

Los resultados de esta tesis doctoral refuerzan la hipótesis de que la comunicación neuro-inmunológica juega un papel crucial en la patofisiología del SII.

RESUM

La Síndrome de l'Intestí Irritable (SII) és un trastorn gastrointestinal crònic i prevalent que cursa amb alteracions a la motilitat intestinal i amb dolor abdominal. La SII constitueix un trastorn recurrent i potencialment incapacitant pel qual, actualment, no existeix un marcador de diagnòstic específic i únicament hi ha disponibles tractaments pal·liatius. L'absència d'una patofisiologia ben establerta destaca la necessitat d'identificar les causes orgàniques subjacents a les alteracions intestinal i la generació de símptomes. A la mucosa intestinals d'aquests pacients s'ha identificat un cert grau d'inflamació així com un increment de la permeabilitat intestinal i una major activitat de la resposta immunitària. Estudis previs del nostre grup van revelar un de la activitat humoral a la mucosa intestinal dels pacients SII amb predomini de diarrea (SII-D), associats a símptomes més severs. Per una altra banda, els pacients que pateixen SII sovint presenten ansietat i depressió recolzant que una disfunció de l'eix cervell-intestí podria estar involucrada en l'aparició i el desenvolupament dels símptomes. Considerant l'alta innervació de l'intestí i la modulació bidireccional descrita entre el sistema nerviós i el sistema digestiu, l'objectiu d'aquesta tesi doctoral és caracteritzar l'activació de la resposta humoral per mecanismes neuro-immunològics a la mucosa intestinal dels pacients amb SII-D.

Per assolir el nostre objectiu, aquest projecte s'ha dividit en tres capítols. Al capítol 1, vam obtenir biòpsies jejunals, sang i femta de pacients amb SII-D i voluntaris sans. Vam quantificar les immunoglobulines (Igs) a les mostres de femta i es va observar que els nivells de IgG són més alts al grup SII-D, concretament IgG2 i IgG3, aquesta última sense diferències significatives. La quantitat de IgG total correlaciona de forma positiva amb la intensitat del dolor abdominal reportada pels pacients. Vam realitzar una anàlisis fenotípica a les biòpsies de l'expressió dels marcadors de cèl·lula plasmàtica CD38/CD138, el marcador neuronal PGP9.5 i TACR1 (receptor de substància P), involucrat a la senyalització nociceptiva. Vam observar que les cèl·lules plasmàtiques i las terminacions nervioses es troben en proximitat i en realitzar la quantificació d'aquesta distància mitjançant microscòpia electrònica de transmissió, els resultats van mostrar que les cèl·lules plasmàtiques estan significativament més properes a les terminacions nervioses al grup SII-D. Aquesta distància correlaciona amb els símptomes d'estrès agut i els nivells de depressió indicats pels pacients, l'últim sense assolir la significació estadística.

Al capítol 2, vam realitzar una anàlisi de RNA-seq amb RNA extret de biòpsies jejunals de pacients amb SII-D i voluntaris sans. Vam dur a terme en aquestes mostres, una anàlisi d'enriquiment del conjunt de gens, vam observar que el fenotip immunològic de la resposta humoral està enriquit i 60% dels gens amb un nivell major d'enriquiment estan involucrats a l'estructura de les Ig. Les vies associades amb la funció barrera a l'intestí també es troben sobrerepresentades a la SII-D.

Finalment, al capítol 3, es van avaluar diferents fonts d'obtenció de cèl·lula B com a model *in* vitro per estudiar l'efecte dels neuropèptids a l'activitat immunològica, més específicament la substància P, a l'activació/diferenciació de la cèl·lula B i la producció de Ig. Vam comparar cèl·lules de cultiu primari (cèl·lules B i cèl·lules plasmàtiques aïllades de la mucosa intestinal i sang) i una línia de cèl·lula B immortalitzada (126BLCL). També es va fer una anàlisis cèl·lules plasmàtiques diferenciades *in vitro* obtingudes a partir de les cèl·lules aïllades de sang. Rere una detallada caracterització fenotípica, vam concloure que les cèl·lules B de sang són el model *in* vitro més adequat i factible per assolir el nostre objectiu.

Els resultats d'aquesta tesi doctoral reforcen la hipòtesi de què la comunicació neuro-immunològica juga un paper crucial a la patofisiologia de la SII.

INTRODUCTION

1. THE INTESTINE AS A PROTECTIVE ORGAN

The intestine is a complex organ composed of different tissues where epithelial, endocrine, nervous and immune systems converge, enabling the development of absorption and energetic support functions together with immunological surveillance and defensive activity. The intestine is constantly challenged by a huge variety of luminal components including food, toxins and microbial particles. Epithelial cells separate the lumen from the internal milieu thanks to several structural and functional components, including tight junctions and extracellular and cellular components, such as mucus, antimicrobial peptides, and the microbiota. All constitute the intestinal barrier (1,2). In health, the intestinal barrier prevents direct interactions between most luminal contents and the immune system. The correct functioning of all the components of the small intestine is crucial for maintaining homeostasis. Therefore, when this barrier is damaged, the selective transport of external agents to the internal milieu is compromised (3,4), facilitating the access of harmful and potentially pathological elements, ultimately promoting the onset of tissue damage and function alteration (2,4). The loss of molecular and functional integrity of the epithelial barrier is associated with mucosal immune responses (1,2,5) and set in motion events that are closely related to the origin and clinical manifestations of several functional gastrointestinal disorders (FGID) (6). Within the gastrointestinal tract, the small intestine is the anatomical region exposed to higher amount of food and other ingested components and has, therefore, developed specific defensive mechanisms. The following information will focus on this particular intestinal segment.

1.1. ANATOMY OF THE SMALL INTESTINE

The small intestine has a characteristic anatomy organized in four concentric layers (Figure 1):

- **Mucosa:** This is the most external layer, directly interacting with the intestinal lumen, and is formed of a single cell line of epithelial cells of different types, which develop fundamental roles in digestion and absorption of nutrients, as well as defensive activities. The epithelium is folded all along the mucosa, forming the so-named intestinal villi to enlarge the absorptive surface. In the apical cell membrane of epithelial cells, the presence of microvilli accentuates this absorptive capacity, carried out by specialized cells, the enterocytes. The cellular components of the intestinal barrier include absorptive enterocytes, mucus-producing goblet cells, entero-endocrine cells that produce hormones and neurotransmitters such as ghrelin, somatostatin, cholecystokinin, gastric inhibitory polypeptide, glucagon-like peptides, peptide YY, and serotonin (7), M cells, and Paneth cells involved in the secretion of antimicrobial peptides such

as lactoferrin, hepcidin, bactericidal/permeability increasing protein, secretory phospholipase A2 type IIA, lysozyme as well as defensins and cathelicidins (8–10). Cells with secretory and immunological activity ensure the development of other important epithelial functions (11). The *lamina propria* is located underneath the epithelium and is a conjunctive tissue containing a high number of blood vessels, nerve cells and immune cells of both, adaptive and innate systems (T and B lymphocytes, IgA-secreting plasma cells, mast cells, dendritic cells and macrophages) (12).

Separating the submucosa and the *lamina propria*, a thin longitudinal smooth muscular coating involves a circular inner muscular layer, forming the *muscularis mucosae.* In all three layers of the mucosa, an abundant innervation is present, emanating mainly from the cell bodies of the enteric nervous system located in the Meissner plexus of the submucosal layer. Afferent connections from crypts and small intestine villi transmit chemoceptive and mechanoceptive information through parasympathetic nervous system (vagal pathways) to the central nervous system. Afferent pathways directed to thoracolumbar spinal cord (sympathetic nervous system) communicates nociceptive signaling from sensorial neurons in *lamina propria* (13). Efferent sympathetic signaling modulates cell bodies from enteric nervous system in the *muscularis mucosae*.

- **Submucosa:** Covering the *muscularis mucosae layer*, the submucosa is constituted by a network of connective tissue, lymphatic and blood vessels and nerve cells from the enteric nervous system (submucosal or Meissner plexus). Meissner plexus modulates the secretomotor function of digestive glands found in the mucosa. The activity of these ENS cells is regulated by efferent pathways of the sympathetic system.

- **Muscular layer:** An external longitudinal and an inner circular muscular layer are responsible for the peristaltic movements of the intestine. This role is possible thanks to the innervation of enteric nervous system cells found in this layer, the myenteric or Auerbach's plexus. In contrast to the submucosal plexus, only described in the small and the large bowel, myenteric plexus is present all along the digestive tract, from the esophagus to anal sphincter. Efferent connections from sympathetic system inhibits the excitatory effect of enteric nervous system neurons of myenteric ganglia, thereby modulates the fluid movement of the intestine. Afferents connections to thoracolumbar spinal cord and brain stem signal nociceptive and chemoceptive and mechanoceptive information, respectively, from Auerbach's plexus (13).

- **Serosa:** This layer is composed of loose connective tissue and flat epithelial cells, which constitute the furthest layer from the luminal light of the intestine.

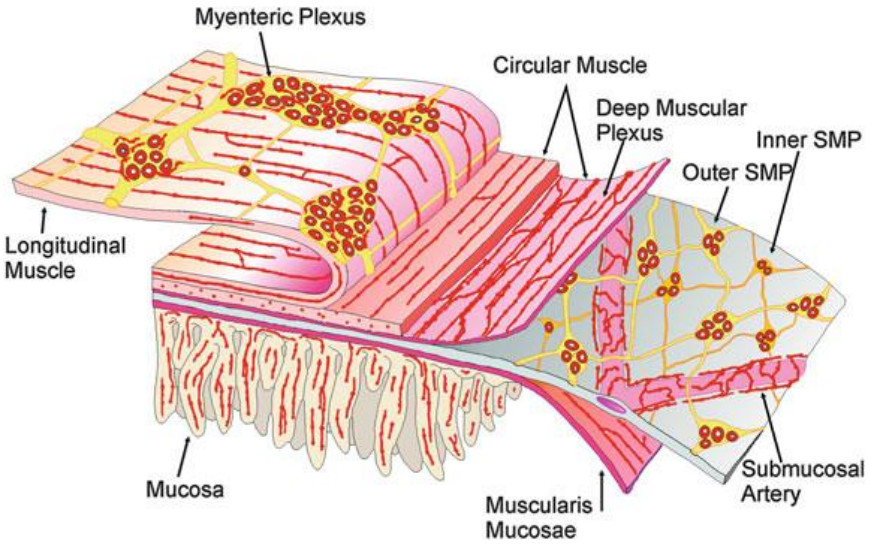

Figure 1: Schematic representation of the layers of the small intestine. Mucosa is the most external layer, which separates the intestinal lumen from the other layers of the intestine. Serosa (not represented) constitutes the most internal layer. SMP: Submucosal plexus. Extracted from (13).

1.2. THE INTESTINAL EPITHELIAL BARRIER

Dietary elements, microbial antigens and agents from the external environment threat constantly the intestinal epithelium, challenging the homeostatic balance of the whole intestine. Protecting the internal milieu, a great variety of components, both extracellular and cellular, impair the passage of luminal components, enabling the barrier function. Those elements present in the intestinal lumen face first a viscoelastic mucus gel layer with hydrophobic and surfactant properties, more than 50µm thick and secreted by goblet cells, covers the entire small intestinal mucosal surface (14). Components of mucus include water, phospholipids, the negatively charged mucins, and a variety of trefoil factors and other antimicrobials such as secretory immunoglobulin A (sIgA), cathelicidins (15) and defensins (16). Mucus helps to regulate gut permeability and protects the epithelial lining from lumenal shear forces, adhesion of and invasion by microorganisms, the toxic effects of dietary components, various chemicals and radiation as well as the impact of antigens present in the intestinal lumen (17). The mucus layer also contributes to the retention, close to the epithelial surface, of mucosal secretions containing antibacterial peptides and digestive enzyme and helps to sustain epithelial hydration (18). Mucus seems to enhance oral tolerance by imprinting dendritic cells with anti-inflammatory properties (13), it participates in epithelial renewal, differentiation and integrity, and interacts to other biological processes as well (19).

Mucins represent the most abundant component of the mucus gel. Up to twenty different mucin genes have been identified to date (MUC1 to MUC20) (20) with MUC1 to 4 being the most abundantly secreted mucins in the human intestine (21). The mucus layer is subdivided into an outer, much thicker, loosely adherent layer which contains large numbers of bacteria and bacterial products and a thinner but firmly adherent layer which lies on the glycocalyx along the luminal surface of the epithelium. The inner layer is impenetrable by bacteria because of its high density unlike the outer structure, where the intestinal flora resides. However, in the small bowel, bacteria can cross this layer and interact with the epithelial cells (22). The thickness of the inner mucus layer varies along the length of the intestine depending on the concentration of bacteria in the lumen; accordingly this component of the mucus layer is thicker in the highly colonized colon and thinner and more permeable in the less colonized small intestine (23). Therefore, it is crucial to have a system that ensures the homeostasis of the intestinal mucosa and impairs potential antigens to access to *lamina propria,* where most of the immune cell population of the intestinal mucosa resides. Contributing to this defensive response, together with the mucus, there is a whole set of elements, including pH and digestive enzymes, which act in a non-specific manner. Commensal microbiota compete with pathogenic bacteria for nutrients and an ecologic niche and their colonization and translocation are modulated by water and ion secretions from enterocytes (1). Paneth cells produce antimicrobial peptides targeting a large variety of microorganisms, including bacteria, yeasts, fungi, viruses and protozoa (16), with lytic activity against bacterial cell wall, increasing water secretion or modulating microbiota (defensins) (1). Inclusive the peristaltic movement antimicrobial products not only participates in the digestion but also reduces the retention time of luminal potential harmful microorganisms. Embedded among the mucus, secretory IgA (sIgA) from *lamina propria* resident plasma cells (15) opsonize antigens impairing they reach the epithelium.

Enterocytes constitute 80% of intestinal epithelial cells (IECs) (24) and together with Paneth, Goblet and enteroendocrine cells, intestinal epithelial lymphocytes, and stem cells within the crypts (5)) compose a polarized monolayer known as the intestinal epithelium. This cell population diversity enables the epithelium develop the digestive, barrier and defensive functions. IECs are connected to each other and to the connective matrix by protein complexes called cell junctions that modulate the physical integrity and therefore the transport across the space between cells (paracellular route)(1). These intercellular junctions can be divided into three groups according to their role: tight junctions, anchoring junctions and communicating junctions (Figure 2).

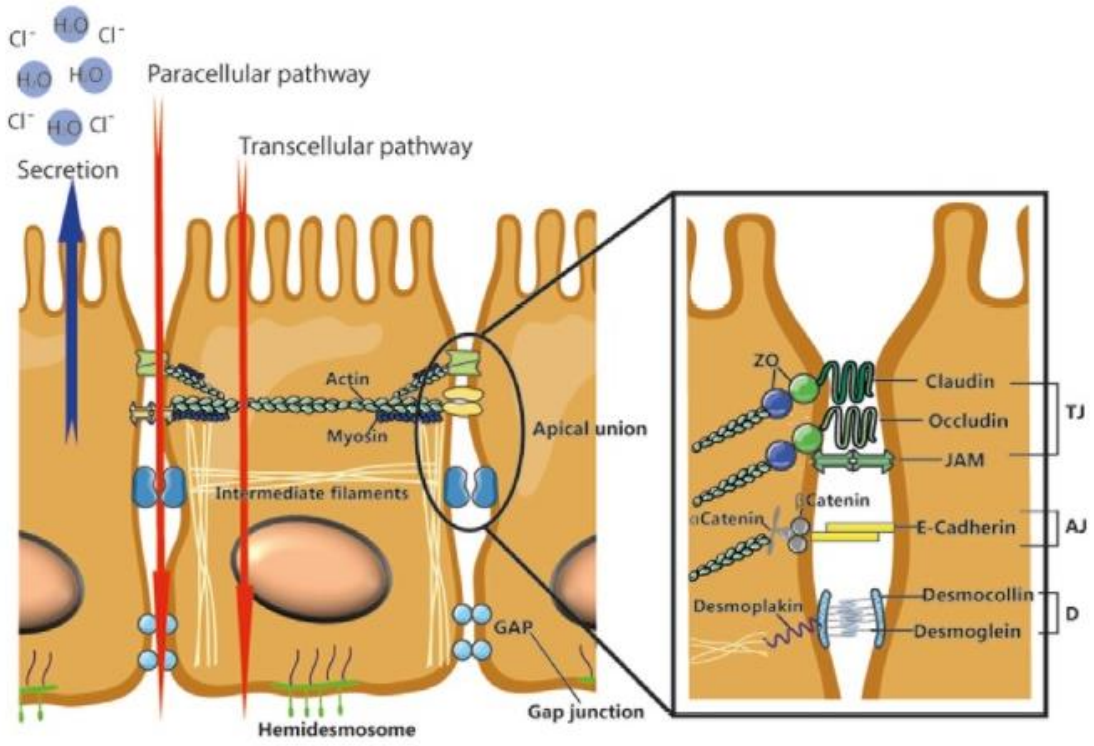

Figure 2: Representation of intercellular junctions. AJ: Adherens junction; D: Desmosome; TJ: Tight junction; ZO: Zonula occludens. Extracted from (1).

- **Tight junctions** are crucial for maintaining the polarity of the intestinal epithelium because their condition the most apical intercellular junction (25), sealing the gap between IECs and regulating the passage of water-soluble molecules (26). They are composed of transmembrane proteins (claudins, occludins, junctional adhesion molecules (JAM) and tricellulins) (1) that connect to *zonula occludens proteins,* and so modulate the tension of cytoskeletal actomyosin fibers (25,26).

- Anchoring junctions endure the structure of the epithelium by connecting the cytoskeleton of cells with each other and attaching them to the extracellular matrix (1). They can be divided into two categories, adherens junctions and desmosomes. Adherens junctions are formed by transmembrane proteins of cadherin and catenin superfamily and play a role in the assembly and maintenance of tight junctions thanks to their association to cytoskeleton (1,27). Desmosomes are the junctions between cadherin tails (desmoglein, desmocollin and desmoplakin) (1) and intermediary filaments of cell cytoskeleton, conferring a strong cell-to-cell adhesion and therefore resistance to mechanical stress (28). This transcellular network is dynamic and may change during processes such as embryo development and wound healing (29).

- Communicating junctions, also known as GAP junctions, are formed by hexameric assemblies of transmembrane proteins called connexins (30). Their function is allowing the interchange of small molecules and ions between neighboring cells through these intercellular channels (30,31). They are also involved in development, growth and differentiation of epithelial cells and in inducing and maintaining tight junctions and adherens junctions (1,31).

Changes in the junctions sealing the intercellular space between epithelial cells due to alterations of the physiological conditions (e.g. stress, non-steroidal anti-inflammatory drug intake, pathology, etc), can promote an increase in epithelial permeability and, consequently, the efficacy of the barrier function can be compromised. However, in order to maintain intestinal homeostasis, the epithelial layer is able to develop effective mechanisms, coordinated with the intestinal immune system.

1.3. PRIMING DEFENSES IN THE INTESTINAL EPITHELIUM

A great variety of external factors, including luminal microorganisms and dietary antigens, challenge constantly the intestinal epithelium. Therefore, an efficient first-line defensive response is needed to ensure normal bowel functioning. The response given by epithelium is carried out initially by IECs themselves, constituting a physicochemical barrier against invaders, but also by the immune system, recognizing in a non-specific manner luminal antigens (innate system) as well as activating and modulating a specific response (adaptive system).

1.3.1. Pathogen recognition by epithelial cells

Innate system antigen recognition is developed by pathogen-recognition receptors (PRRs) expressed in both intracellular and IECs membrane sites. PRRs detect evolutionary-conserved and repetitive structures found commonly in potential harmful antigens. PRR ligands can be classified into pathogen-associated molecular patterns (PAMPs), danger-associated molecular patterns (DAMPs) and microbe-associated molecular patterns (MAMPs) according to their origin (pathogens, cell-derived molecules or luminal microbes in general, respectively). Hence MAMPs include both commensal and pathogenic microorganisms, this classification may differ upon authors. Once PRRs are activated, mucosal protective mechanisms are triggered, specially mucus secretion, production of neuropeptides and antimicrobial peptides, modulators of the immune system (1,32), and epithelial shedding (32). PRRs can be divided depending on their function, ligand, location, induced responses, etc (Figure 3). Among this range of options, the most used PRR classification is based on structural properties: Toll-like receptors (TLRs)

nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs), cytosolic DNA sensors and C-type lectins (CLRs). Despite recently-discovered receptors have been added to these categories, it should be noted TLRs and NLRs are far the most extensively studied PRRs.

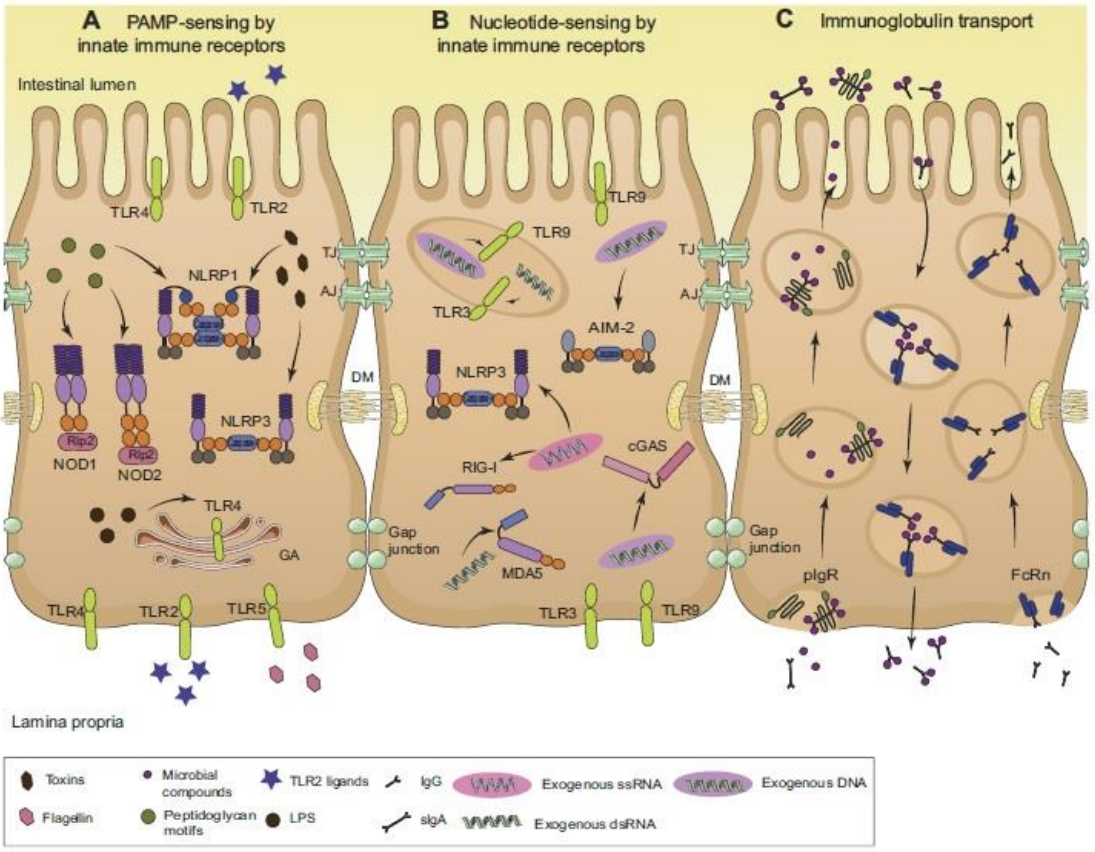

Figure 3: Representation of ligands and location of most notable receptors in human intestinal epithelial cells. A, B and C section are classified according to their function reacting against PAMP-nucleotide-sensing (A and B) or in Ig transport (C). AIM-2: absent-in-melanoma-2; AJ: Adherens junctions; c-GAS: cGMP-AMP synthase CLR: C-type lectin receptors; DM: Desmosome; FcRn: neonatal Fc receptor; GA: Golgi apparatus; Ig: Immunoglobulin; LPS: Lipopolysaccharide; MDA-5: Melanoma differentiation-associated gene 5; NLR: NOD-like receptors; NOD: Nucleotide-bindingoligomerization domain; PAMP: pathogen-associated molecular pattern; pIgR: Polymeric immunoglobulinreceptor; RIG-I: Retinoic acid-inducible gene-I; RLR: Retinoic acid-inducible gene I-like receptors; TJ: Tight junction; TLR: Toll-like receptors. Extracted from (32).

- **TLRs** are expressed by immune cells, both innate cells (mast cells, dendritic cells, monocytes/macrophages) and adaptive system (B cells). Their ligands are bacterial antigens (lipoproteins, lipoglycans, peptidoclycans, lipotichoic acid(33,34), LPS and flagellin (33,35)) and exogenous nucleotides (dsRNA (34) and DNA (36)). In IECs, TLRs play a crucial role triggering downstream cascades that modulate permeability, inflammation, immune response (cell recruitment, phagocytosis, trefoil factor production, maturation of dendritic cells, among others). Their location in the intestinal epithelium is mainly in the cell membrane (TLR2, -3, -4, -5, and -9) but some of them may also be found intracellular where ligand-recognition takes place (TLR3,-4 and -9) (32).

- **NLRs** are intracellular receptors involved in the recruitment of innate immune cells but also in reproduction and embryonic development. NLRs are expressed by several cell populations, but in the intestinal epithelium NOD1 and NOD2, two of the most known NLRs, play a fundamental role detecting bacterial peptidoglycans and evoking the formation of nodosome, a protein complex implicated in the control of bacterial infections and inflammation through activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kβ) and mitogen-activated protein kinase (MAPKs) pathways. NOD1 can be found in IECs, and their ligands are both gram-negative and gram-positive bacterial peptidoglycans; whereas NOD2, predominantly expressed in Paneth cells and monocytes, respond to muramyl dipeptide, a cell-wall component present in all bacteria. NOD2 have been associated with intestinal inflammation, Crohn's disease (37) and barrier function (38). The antibacterial host-defense developed by IECs is increased by inflammasome-forming NLRP1 and NLRP3 from IECs in response to toxins and bacterial compounds (cell-wall components and ssRNA) (39).

-**RLRs** are cytoplasmic PRRs from the RNA helicase family. Their ligands are long and short dsRNA and uncapped ssRNA from virus (32). Their function is often carried out together with DNA sensors, promoting the production of proinflammatory cytokines and ultimately modulating the host antiviral response (40).

-**Cytosolic DNA sensors** are intracellular PRRs, expressed in many cells types, which detect both bacterial and viral DNA. In spite of few cytosolic DNA sensors have been identified in intestinal epithelium, bacterial dsDNA detection by cyclic GMP-AMP synthase from IECs has been described; its activation promotes MHC calls I expression, CD8+ T cell proliferation and increase Natural Killer cell activity (41). The limited number of studies about this PRRs subtype and the variety of receptors capable of recognizing exogenous nucleotides make this classification unclear.

-**CLRs** are transmembrane PRRs activated by carbohydrates or present a similar structure to C-type lectin-like domains (42) commonly found in bacteria and fungi. According to their structure, they can be classified type-I (transmembrane receptors) and type-II (soluble receptors) (32). Upon bacterial or fungal infection, CLRs promote the production of pro-inflammatory mediators, fungal binding and phagocytosis, neutrophil influx, macrophage maturation and T-cell differentiation (42,43). The synergistic effect of CLRs and TLRs ensures optimal proinflammatory responses against fungus, bacteria, viruses, helminths and protozoa.

1.3.2. Contribution of epithelial cells to adaptive immune activity

As described above, one of the multiple ways the intestinal epithelium modulates the adaptive immune system is via PRR-induced responses. This innate-adaptive system connection makes possible an antigen-specific response through the activation of immune cells resident in the *lamina propria.* IECs transport of Ig produced by antibody-producing cells (plasmablast and plasma cells) of *lamina propria* across the epithelium through two Ig receptors expressed in their membrane: polymeric immunoglobulin (Ig) receptor (pIgR) and neonatal Fc receptor (FcRn) (Figure 3).

- **pIgR** binds secretory antibodies, specifically dimeric IgA and polymeric IgM, from the basolateral membrane of IECs and transport them to the intestinal lumen, where they are liberated thanks to the protease cleavage action. The transcytosis to the apical surface may occur with or without antigen bound to sIgA or sIgM and it is a unidirectional transport (44). The release of sIgA and sIgM play an important role excluding pathogens from the epithelial barrier by antibody opsonization of bacteria, thereby impairing their interaction with the epithelium.

-**FcRn** is responsible for the bidirectional transport of IgG and IgG immune complexes across the polarized epithelium. IgG from *lamina propria* is transported to the apical IEC surface, where it recognizes luminal antigens and forms immune complexes. Further, these immune complexes are internalized by IECs through FcRn and are released to the basolateral side of the epithelium. Dendritic cells residing in the *lamina propria* process these antigens and present them to T helper T cells of organized lymphoid aggregates, thereby activating the adaptive immune system (32,45). Besides cell signaling triggered by PRRs and Ig receptors expressed by IECs, the intestinal epithelium modulates the antibody-mediated response secreting APRIL (a proliferation and survival factor for plasma cells) and promotes cellular responses by recruiting T cells through IL-8 secretion.

2. THE GUT-ASSOCIATED LYMPHOID TISSUE (GALT)

In the intestine, the immune system shows characteristic features that enable the defensive and barrier function of the intestinal mucosa, constituting the gut-associated lymphoid tissue (GALT). GALT comprises organized lymphoid tissues and isolated immune cells that are tightly regulated (46) to ensure defensive responses against pathogens as well as to allow tolerance mechanisms towards food and microbial-derived antigens. Innate and adaptive immune processes collaborate to prevent maladaptive responses. Innate GALT recognizes repetitive structures commonly found in pathogens, evading their harmful effect. Adaptive GALT confers a specific response against antigens and generates immunological memory. Antimicrobial molecules secreted by the intestinal epithelium, together with phagocytic (macrophages) and destructive (Natural Killer) cells, are crucial factors in giving non-specific responses against those antigens which have reached the *lamina propia* (11). When these mechanisms are not efficient enough, the intervention of adaptive immune system, through cell and humoral responses, is necessary to avoid the onset of a potential pathological situation.

GALT is anatomically divided into two compartments:

- **Organized GALT:** it includes lymphoid aggregates forming Peyer Patches (PP), solitary lymphoid tissues and mesenteric lymph nodes (46). All along the intestine, the organized lymphoid structures are identified, being PPs specially frequent in the jejunum and mostly in the ileum, while in the colon lymphoid aggregates are present while PP are absent (5). PP contain several lymphoid follicles and are located immediately below the associated-follicular epithelium. The anatomy of PP can be divided into three main regions: the *subepithelial dome* or the antigen capture area, *interfollicular region* or T cell zone and *the B cell area*, where T/B cells and dendritic cells interact in germinal centers. Germinal centers presents a dark and a light zone, where B cells are distributed according their maturation state, dividing B cells (centroblasts) and resting B cells (centrocytes), respectively (47). A mantle zone, containing other immune cells, mostly T lymphocytes, plasma cells, dendritic cells and macrophages, covers the germinal center (Figure 4).

Solitary lymphoid tissues can be classified into cryptopatches and isolated lymphoid follicles. Cryptopatches are formed by a group of lymphoid aggregates constituted by immune cells which play a critical role in development of lymphoid organs and a coat of monophagocytic cells. When cryptopatches differentiate, they acquire a core of plasma cells, surrounded by this structure of lymphoid tissue inducer and monophagocytic cells (46). Despite their function is still not well-known, they may contribute to the induction of sIgA responses (48,49).

Mesenteric lymph nodes participate, together with PP, in oral tolerance processes by interacting with antigens presented by dendritic cells from *lamina propria.* Antigen-laden dendritic cells cannot access to peripheral circulation, preventing in this way the systemic spread of microorganisms (50).

Organized GALT is considered the inductor of GALT, as antigen-immune cells interaction takes place in the lymph aggregates (5).

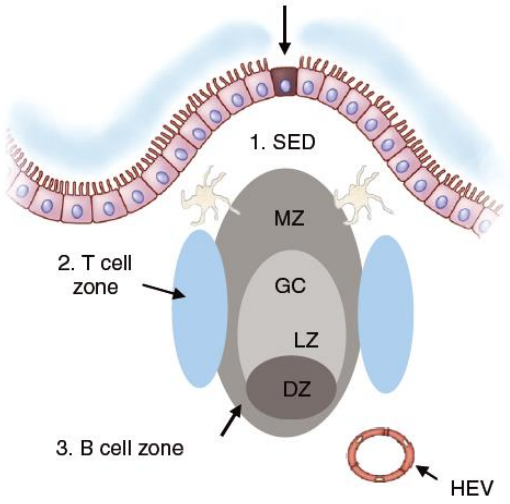

Figure 4: Representation of the structure of a germinal center from a lymph node. DZ: Dark zone; GC: Germinal Center; HEV: High endothelial venule; LZ: Light zone; MZ: Mantle zone; SED: Subepithelial dome. Extracted from (47)**.**

- Diffuse GALT is located above and below the basal membrane of the intestinal epithelium. It is composed by leukocyte cells: mast cells, eosinophils, macrophages, Natural Killer cells, neutrophils, *lamina propria* lymphocytes (LPL), including B and T cells, and intraepithelial lymphocytes (IELs), most of them T cells. Diffuse GALT acts as an effector lymphoid tissue due to its capacity to destroy pathogens (5).

3. DEVELOPMENT OF THE B CELL SYSTEM

3.1. B CELL ONTOGENY

B cells are continuously generated during the life span of an individual and suffer several rearrangements in genes related to antigen-recognition and Ig expression along their maturation process. In adults, B cells are originated in the bone marrow from hematopoietic stem cells, which will give rise to common lymphoid progenitor. Committed Natural Killers, Dendritic cells and B lymphocytes take at this point different routes within the lineage (51). From common lymphoid progenitors will follow a pre-pro B cell.

The sequential steps in the development of the committed B cell can be identified by the rearrangements of variable (V), diversity (D) and joining (J) region gene segments, responsible for the antigen-specificity of Igs (51,52). Stromal cells from bone marrow support this antigen independent process by secreting growth factors and interleukins that promote B cell proliferation and survival as well as adhesion molecules that retain B lymphocytes in the bone marrow, especially in the earlier steps of the development. The heavy chain of Ig is the first to be formed by the rearrangement of D-J and later the V-DJ gene segments, in early pro- and late pro- B cells, respectively. Once this process is completed, heavy chain is expressed mostly intracellularly but partly in the surface, paired with a surrogate light chain, generating a large pre-B cell with a high dividing ratio. During the next step of maturation, the heavy chain is only expressed in the cytoplasm and J-V gene segments of the light chain rearrange, as new small pre-B cell stop proliferating (52). Immature B cells display a complete IgM molecule in their membrane and migrate to the spleen, where they develop into naïve B cells (53).

In spleen, immature B cells undergo two transitional stages, T1 and T2. T2 are more proliferative and responsive to BCR-stimulation than T1 B cells (54) and are the precursors of long-lived follicular and marginal zone B cells (55). Naïve B cells that colonize germinal centers derive from follicular mature B cells, while B lymphocytes residing in the marginal zone do not circulate (51). There is a third type of B cell, called B1 phenotype, that resides in spleen, intestinal mucosa, peritoneal cavity and is also found in fetal liver. Contrary to immature B cells, mature B cells express both IgM and IgD on their surface. These lymphocytes migrate to secondary lymphoid tissues, such as GALT, where their differentiation process will continue.

The maturation stage of a B cell can be determined by the expression of proteins on their membrane that may be used as cell markers. CD20 and CD19 are commonly used to identify B

cells due to their continuous presence throughout development. Despite both CD20 and CD19 are considered pan-B cell markers, they do not co-exist at all maturation stages. CD19 is present on the surface of pro-B cell and is maintained until late plasmablast (even it may be found in low levels in plasma cells), while lymphocytes are positive for CD20 in pre-B cell and plasmablast framework (56–58). After activation in germinal centers, B cells display on their surface CD27 and increase its expression until they are terminally differentiated. Hence, memory B cells, plasmablasts and plasma cells are all positive for CD27. The expression of CD27 in memory B cells decreases the presence of CD38, used to identify antibody-secreting cells. Once B cells have completed the differentiation process, they become CD138+ and are, therefore, identified as plasma cells (58). Plasma cells may lose CD19 or even CD45 in terminal differentiation states and become long-lived plasma cells (59).

3.2. IMMUNOGLOBULINS: STRUCTURE AND FUNCTION

The intestine is the body organ with the highest number of immune cells and production of Igs (60). Igs are glycoproteins synthesized in response to antigen-B cell interaction, often with Th cell collaboration, in order to provide a specific immune defense. Its structure can be divided into two parts: fragment crystallizable (Fc) part, contains two heavy chains and allows plasma membrane anchoring, and fragment antigen-binding (Fab) part, formed by two light and two heavy chains. Each Ig is composed by a constant and a variable domain. In the latter, hypervariable regions, characterized by a high mutation rate, can be observed, enhancing the specificity for the antigen. Matching with the location of organized and diffuse GALT, several types of Igs, defined by their heavy chain, reside in the intestinal mucosa. B cells can change the isotype expressed/secreted by a process named class-switch recombination. Class-switching maintains the antigen specificity but provides a different effector function because the recombination process affects only the constant gene segments (C) of the heavy chain gene, not the variable domains (V, D and J segments) (Figure 5). Contrary to VDJ rearrangement, class-switching always leads to a productive recombination. C regions of Ig genes show different exons that encode for the heavy chains of IgM, IgD, IgG3, IgG1, IgA1, IgG2, IgG4, IgE and IgA2 (Cμ, Cδ, $C\gamma_3$, $C\gamma_1$, $C\alpha_1$, $C\gamma_2$, $C\gamma_4$, Cε and $C\alpha_2$, respectively and ranked by their natural order in the gene (61)). Switch recombination points interact with each other in the DNA strand, resulting in a region that is looped out and deleted. The upstream promotor leads the transcription of the resulting isotype-specific heavy chain gene. The location of C exons enables the class-switch from upstream to downstream isotypes but not inversely (e.g: IgM to IgG1 is possible but not IgG1 to IgM).

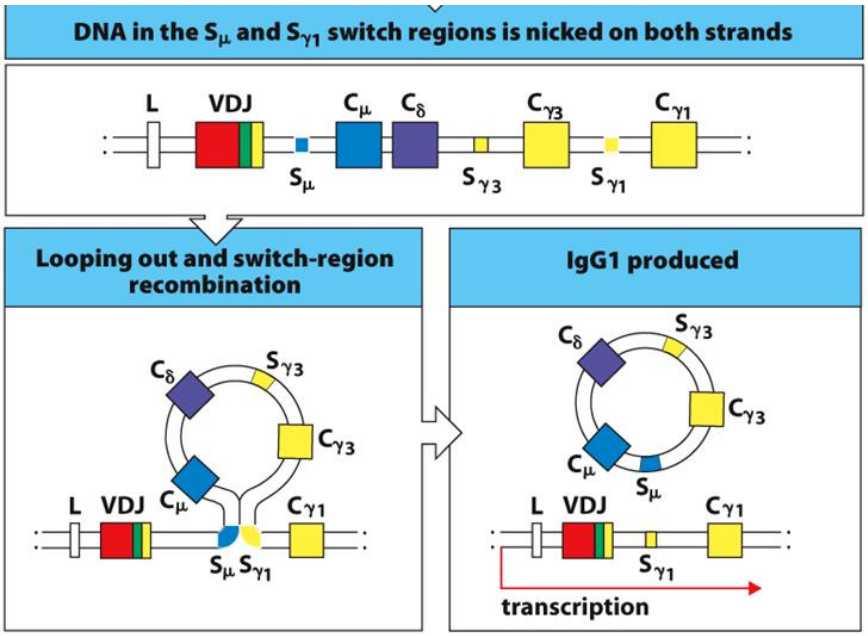

Figure 4.28 The Immune System, 4th ed. (© Garland Science 2015)

Figure 5: Immunoglobulin class-switch recombination. Extracted from (62).

The type of antigen and the site were B cells are primed determine the type of Ig to be produced. Each Ig has distinctive effector functions:

- IgA is the predominant Ig in the intestinal mucosa (63), where is released in dimers (occasionally even in trimers o tetramers) (64). When secreted, IgA is usually bound to the secretory component J, receiving the name of sIgA (65). Its main function is the direct neutralization of virus, bacteria and toxins. This Ig isotype hardly ever activates the complement system, therefore local inflammatory responses are not generated. Apart from sIgA, there are two subclasses: IgA1, prevalent in blood, and IgA2, abundant in different mucosal regions. IgA1 is more sensitive to bacterial proteases due to its longer hinge region (binding two domains of heavy chain) compared to IgA2 (66). However, in the duodenum and the jejunum, higher levels of IgA1 than IgA2 are observed (67). IgA, together with IgG, neutralize bacterial toxins by blocking their binding sites to the host cell receptor. High affinity IgA and IgG reduce viral infections by avoiding the access to the cytosolic compartment through a membrane receptor. Due to the effectiveness of neutralizing antibodies when not all the receptor binding sites are blocked, IgA and IgG may also change the conformational state of the virus, hampering the entrance into the cell (68).

- IgM is the second most abundant Ig in the intestinal mucosa. Its concentration is lower than IgA due to its less efficient transport into the intestinal lumen (via polymeric Ig receptor, shared

with IgA) and a more reduced number of IgM-secretory plasma cells in the *lamina propria*. In various mucosae, IgM is often found in its secretory form, acting as a pentamer bound in a non-covalent form by J components. This structural conformation makes it more susceptible to protease degradation (60). This isotype is the first antibody produced when the humoral response is triggered. When enteric antigens enter across the epithelial barrier, secretory IgM opsonize the antigens, agglutinating them and promoting the activation of the classical pathway of complement system (68,69).

-IgG can also be found in the intestinal mucosa at lower levels than sIgA and sIgM. The low concentration of IgG in the intestine is due to the reduced number of secretory cells and to its bidirectional transport across the epithelium, carried out by the neonatal Fc receptor (FcRn). Its effector activity is developed in a monomeric conformation and its function is subclass-dependent: IgG1 > IgG2 > IgG3 > IgG4 (ranked according to abundance in plasma of healthy). All subtypes of IgG develop neutralization functions, though activation of the complement system is limited to IgG1, IgG2 and, overall IgG3. To be recognized by a C1q molecule and initiate the classical via of the complement system, it is necessary that at least two IgG molecules are bound to the pathogen surface. Therefore, IgG is less effective triggering this cascade signaling as compared to IgM, which is able to activate the whole pathway with a single molecule thanks to the staple conformation it adopts when binds to the bacterium (68). Opsonization is mainly, but no exclusively, carried out by IgG1 (68), produced/secreted because of the presence of soluble or membrane antigenic proteins. IgG2 isotype is associated to the response against bacterial encapsulated polysaccharides (70). IgG3 is characterized by its pro-inflammatory effect, especially in viral infections (70). Finally, IgG4 responds against non-infectious agents (70) and plays a role in anti-inflammatory processes (71) .

-IgD levels are discrete compared to the other Igs. IgD is the Ig isotype with a longest hinge region, hereby with the shortest lifespan (72). The role of this antibody is not well-established, yet it may be involved in the maturation of B cells, considering its expression, together with IgM, on the cell membrane of naïve B lymphocytes (52,66).

- IgE is the less abundant isotype in blood (72). It participates in allergic and hypersensitivity reactions and infections caused by parasites. Its function is linked to mast cell, eosinophil and Langerhans cells activation by binding to their FcεRI receptor (66).

3.3. ANTIBODY GENERATION IN THE INTESTINAL MUCOSA

3.3.1. Induction of humoral response in the intestinal mucosa

The activation of resident B cells, dendritic cells and macrophages residing in the intestinal *lamina propia* only occurs when a luminal antigen crosses the epithelial barrier (Figure 6). There are two mechanisms by which intestinal humoral response can be initiated, depending on how antigens get access to the internal milieu:

- Classical pathway: the transit of those antigens with bacterial origin is regulated by enterocytes specialized in transport, called M cells (73), which lack the mucus layer and the glycocalyx in their apical membrane (74,75), facilitating antigens passage through the intestinal barrier by transcytosis (76). Once in the PP, antigens are processed by dendritic cells and presented to naïve B cells, which, in turn, will present them to $CD4^+$ T helper cells (Th cells). Th cells will initiate the differentiation process of B lymphocytes into antibody-secreting cells (77,78) by secreting cytokines in the germinal center. These activated B cells will migrate into mesenteric lymph nodes, reach system circulation via thoracic duct and will return to the intestinal mucosa through high endotelial venules (HEVs). These B cells will differentiate into a specific antibody-producing subtype of plasma cells according to the stimuli received in the *lamina propia*. This process will promote an efficient and distinct humoral response against the antigens (Figure) (5).

- Alternative pathway: Dendritic cells can extend their dendrites across the epithelium and grasp antigens from intestinal lumen, allowing them to access the *lamina propia*, where will endocyte and process them (79,80). Antigen-laden dendritic cells will migrate into mesenteric lymph nodes and return to the mucosa as in classical pathway. Free luminal antigens can be absorbed by blood vessels and be transported to the spleen. After they are processed by dendritic cells, antigens will regress to the intestine via HEVs (81).

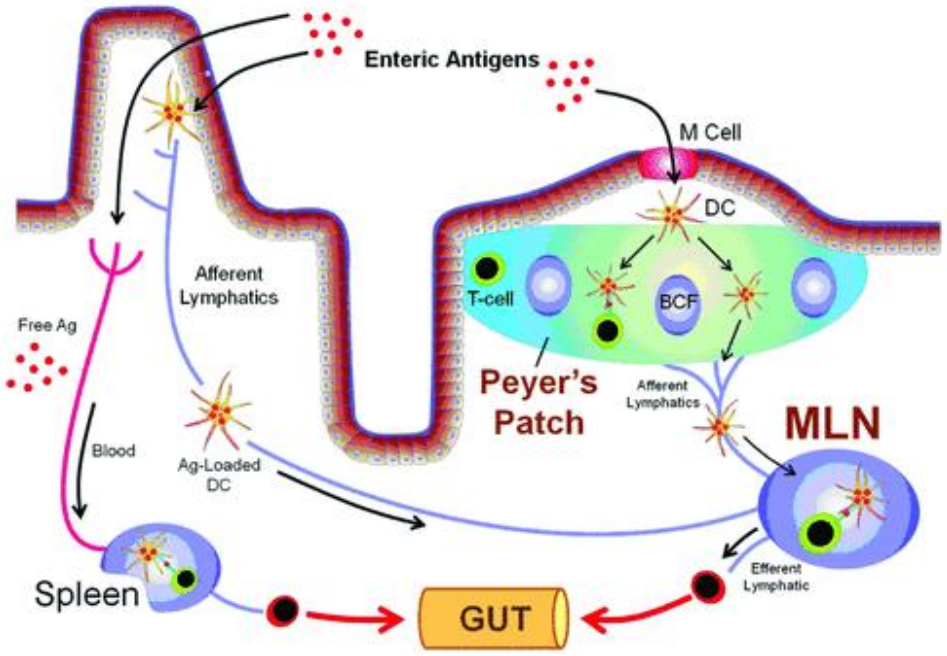

Figure 6: Representation of the elements forming the gut-assocciated lymphoid tissue. Ag: Antigen**;** BCF: B cell Follicle; DC: Dentritic cells; M Cell: Microfold Cell; MLN: Mesenteric lymph nodes. Extracted from (81).

3.3.2. T-cell dependent and independent mechanisms

Naïve B cells usually need the co-stimulatory signals from Th cells to be activated after the first interaction with an antigen through the B-cell antigen receptor (BCR) found in their membrane. Despite some antigens can initiate a humoral response by T-cell independent mechanisms, this activation is limited to those antigens with a mitogenic activity in B cells (thymus independent type 1 antigens (TI-1)) or those with highly repetitive structures commonly found in pathogenic microorganisms (thymus independent type 2 antigens (TI-2)). Most T-cell independent processes do not involve Ig isotype class-switching, resulting in a lower antigen-specific response.

- T-cell dependent mechanisms: Antigens bound to the BCR of naïve B cells are internalized and processed in lysosomes. Degraded derived-peptides are presented on the surface associated to major histocompatibility complex type-II (MHCII) molecules and recognized by Th cells. Only those Th cells specific for that antigen will get activated and produce cytokines to induce differentiation of B cells into antibody-secreting cells, process known as linked recognition. It is not necessary that both B and T cells recognize the same epitope of the antigen but belonging to the same molecular structure. Activation of naïve T cells is triggered by professional antigen

presenting cells, such as dendritic cells or macrophages. Antigen-specific activated Th cells recognize the appropriate antigen through their T Cell Receptor (TCR). The initiation of humoral response is facilitated by the close proximity of T and B cells in secondary lymphoid tissues. Once MHCII-TCR binding occurs, Th cells promote B cells to enter into the cell cycle though their membrane molecule CD40L (CD40L), also known as CD154. CD40L interacts with CD40, expressed in B cells to initiate the whole process (82). Together with CD40L, cytokines expressed by Th cell drive class-switching Ig isotype, such as IL-21 (crucial in first steps of the activation) (83) and IL-6 (important in B cell surviving) (84), among others.

- T-cell independent mechanisms: B cells can also be activated by both TI-1 and TI-2 antigens. The humoral response triggered by TI-1 is faster than the one dependent on T-cell stimuli because avoids initial steps of T cell priming by a specific antigen. However, these B cells do not produce such an efficient response (class-switching is induced by Th cells) and memory cells will not be generated. TI-1 antigens are considered to have mitogenic activity because their ability to act as polyclonal B cell activators at high concentrations. At concentrations of 10^3-10^5, only antigen-specific B cells are triggered (82). TI-2 antigens, mostly polysaccharides, contrary to TI-1 antigens which activate both mature and immature cells, induce antigen specific responses without needing T cell signaling. Despite TI-2 antigens are considered to activate T-cell independent pathways, Th cell cytokines induce these B cells to class-switching (cross-linking) and increase the production of antibodies (85). The most accepted theory is TI-2 antigens signals antigen-specific mature B cells by crosslinking B-cell receptor. Excessive receptor cross-linking turns B cell into anergic or unresponsive cells (82).

4. THE GUT-BRAIN AXIS

The neural control of the intestinal function, including motility, secretion, vascular activity, barrier function and interaction with immune and endocrine systems, are modulated by more than a 100 million of neurons resident in the intestine. These neurons form a complex network responsible for the bidirectional connection between the central nervous system and the digestive system, the so-named gut-brain axis (Figure 7). The great majority of the innervation of the digestive system comes from the enteric nervous system, an independent and intrinsic nervous system only found in the gastrointestinal tract. Similar to the central nervous system, enteric nervous system neurons secrete a great variety of different neurotransmitters (noradrenalin, corticotropin realising factor (CRF), substance P (SP), vasoactive intestinal peptide (VIP), acetycholine, serotonin, dopamine, etc) involved in the regulation of the gastrointestinal

function, innervating the longitudinal and circular muscular layers of the intestine as wells as the *lamina propria* and basolateral part of the epithelium in the intestinal mucosa. Neuronal bodies of enteric nervous system, located in the Auerbach and Meissner plexus, are in proximity to nerve fibers from parasympathetic and sympathetic systems, which have an excitatory or inhibitory effect, respectively, to the efferent signaling from enteric nervous system. Notably, 90% vagal fibres (main innervation from parasympathetic system in small bowel) are afferent (86) transmitting sensations of nausea, bloating or satiety and information of homeostatic status, which highlights the importance of the bidirectional connection between brain and gut. Recent scientific evidence is suggesting a close interaction of the central nervous system with the intestinal luminal content (such as microbiota), by identifying behavior modulation. This activity may occur not only through vagal pathways but also by afferent enteric nervous system fibres (87,88).

Most of the immune cells resident in the intestinal mucosa, including Peyer Patches (89), are innervated by the enteric nervous system (90). This connection represents a crucial communication between the nervous system and the immune system, as modulates immune activity depending whether detecting pathogenic factors or executing oral tolerance responses. The sympathetic nervous system plays a dual role in the control of inflammation, thanks to functionally different receptors, α-adrenoreceptors and β-adrenoreceptors, which respond at low or high concentrations of noradrenaline and ATP, respectively. The variability of the receptor affinity for their ligands allows opposing intracellular signaling, hence pro-inflammatory effects from α-adrenoreceptors take place and anti-inflammatory activity occurs when β-adrenoreceptors are activated (91). When sympathetic nervous system promotes inflammation, it induces also the migration of immune cells and amplifies the defensive response through the secretion of neuropeptides such as SP o VIP. The connection between nervous and immune system is also bidirectional. As example, the nociceptive neuropeptide SP is not only produced by neurons but by immune cells, contributing to inflammation (91,92). VIP may also be secreted by both the nervous and the immune system, in this case acting as an inhibitor of inflammation (92).

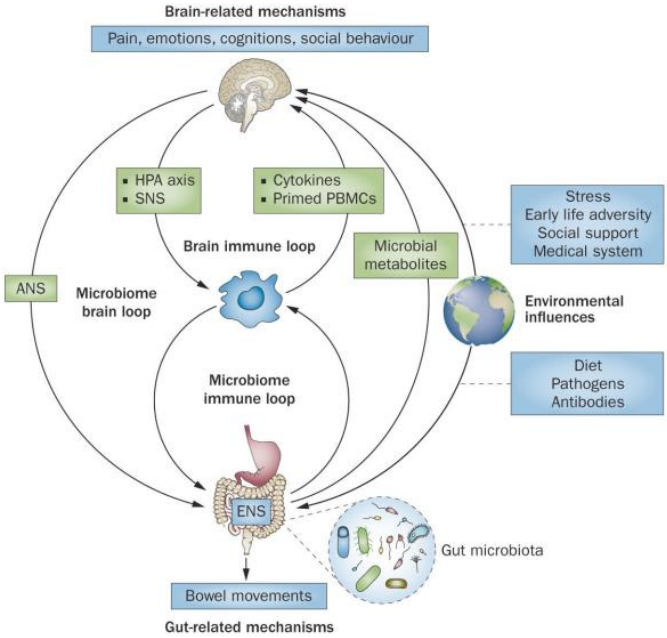

Figure 7: Gut-brain axis representation. ANS: Autonomous nervous system; ENS: Enteric nervous system; HPA axis: Hypothalamic-pituitary-adrenal axis; PBMC: Plasma blood mononuclear cells; SNS: Sympathetic nervous system. Extracted from (93).

However, in the intestinal mucosa, vagal innervation close to immune cells has not been described yet (90). The parasympathetic nervous system has anti-inflammatory activity playing an excitatory effect against cholinergic enteric nervous system neurons, which in turn will inhibit the release of pro-inflammatory cytokines of those macrophages in close proximity to nerve endings (90). The modulation of inflammation will alter in parallel barrier function as it will affect intestinal permeability through rearrangements of tight junctions (94). Inflammation has also been linked to contribute in pain generation through the transient receptor potential V1 (TRPV1), whose activation induce neurogenic inflammation (95). The association between inflammation and psychiatric symptoms have been described in the literature. Patients suffering from depression present higher levels of inflammatory biomarkers (96) as well as subjects suffering from motivational deficits (97,98) or suicide behaviors (99). Inflammation also seem to play a role in the ethiology of stress, anxiety and other metal health issues (100,101). More specifically, anxiety and depression are associated to digestive disorders which course with inflammation (or microinflammation) of the intestinal mucosa, such as Inflammatory Bowel Disease (IBD) (102) or Irritable Bowel Syndrome (IBS) (103,104). All the evidences suggest a narrow connection between nervous, immune and digestive system that influences directly or indirectly the other functions of the intestine (Figure 7).

5. IRRITABLE BOWEL SYNDROME

5.1. PREVALENCE, CLINICAL MANIFESTATIONS AND DIAGNOSIS CRITERIA

Irritable bowel syndrome (IBS) is a chronic digestive functional disorder with a high prevalence in industrialized societies, affecting up to 15% of the population (105) and being 1,67 times more prevalent in women than in men (106). IBS constitutes a relapsing and potentially disabling disorder which represents the main medical consultation of gastroenterology. Despite enormous technological advances and the intense research performed during the last decade, sensitive and specific biomarkers for positive diagnosis are not available yet. No macroscopic changes during endoscopy neither alterations in conventional histological analysis are observed. Besides, the etiology and physiopathology of this syndrome are not well-defined and satisfactory treatment options are not currently available. Therefore, IBS continues to be a disorder whose diagnosis is based on the presence of nonspecific symptoms in the absence of other gastrointestinal diseases. Patients must fulfill a set of symptoms, defined in the Rome criteria, to be diagnosed with IBS. The Rome criteria was designed to identify functional gastrointestinal disorders and is periodically actualized being, currently, the Rome IV criteria the last updated version (106). IBS is diagnosed when abdominal pain, recurrent and manifested at least 1 day per week in the last 3 months, is related to defecation or altered frequency/consistency of stools. Symptoms must have started at least 6 months before diagnosis (103). Patients often present dyspepsia, abdominal bloating and visceral hypersensitivity. According to the bowel motility pattern, IBS patients can be classified into four subtypes: diarrhea-predominant IBS (IBS-D), constipation-predominant IBS (IBS-C), mixed pattern IBS (IBS-M) and unclassified IBS (IBS-U) in those cases with altered frequency of depositions but no changes in stool consistency (107,108).

5.2. ETIOPATHOGENESIS

5.2.1. Risk factors

Despite intensive research, the pathophysiology of IBS is not completely understood but several physiopathological mechanisms, including complex interactions between the host and the environment, have been postulated to be involved in the development of this syndrome. These risk factors exposed below provide evidences to start considering IBS as organic disease and not a functional disorder.

- Genetic factors: IBS is a common gastrointestinal disorder with a multifactorial pathophysiology and familial clustering. Despite the aggregation of the syndrome may be explained in part by environmental factors, studies with twins demonstrate a predisposing genetic is necessary to develop the disorder (109). The heritability pattern of IBS is not known yet, but research focused on functional Single-Nucleotide Polymorphisms (SNPs) of candidate genes which constitute a risk factor is increasing. Unfortunately, only one GWA study has been performed so far, identifying susceptibility loci in IBS related to ion channels (110). However, since the causality of this results have not been fully explored, either confirmed by other authors, more research is needed to study the effect of genetic variants in this syndrome (111). Most of the research focus on genes involved in serotonin, corticotropin and cathecolaminergic signaling because their demonstrated importance in the pathophysiology of the syndrome. One of the limitations of studying SNPs of specific candidates is the distribution of these alleles usually depends on the studied population and may generate controversial results when larger studies are performed. Currently, there is no specific allele variants established to be linked to IBS and further research is needed.

- Environmental factors: A notable percentage of patients who suffered from a gastrointestinal infection (5-32%) develop IBS in the future (112), constituting what is known as post-infectious IBS subgroup. This fact suggests the importance of viral or bacterial intestinal infections in the development of IBS and are considered a risk factors to develop this syndrome. In fact, up to 78% of IBS patients present bacterial overgrowth whose symptoms ameliorated after its eradication (113). Changes in microbiota, compared to healthy volunteers, have also been described (114), showing a differential intestinal microbiota signature associated with the severity of IBS symptoms (115). Evidences demonstrating the role of microbiota in behavioral symptoms is growing (87,88) and together with gut-brain axis communication, it suggests external stressful stimuli the patients receive along their life-time may affect the outcome of this disorder. Epigenetic changes associated to candidate genes, especially those associated with

stress-response pathways or psychological distress (116,117), are potential targets for new IBS treatments. However, fewer studies have been carried out and no methylation and deacetylation differential patterns in IBS patient have been concluded.

The effect of diet also may influence the severity of IBS, not only because the worsening of symptoms reported by patients after meals, but also because a diet low in fermentable short-chain carbohydrates reduces symptoms in a portion of patients (50%) (118). Therefore, diet and its link with microbiota composition may impact clinical outcome.

- Intestinal permeability: structural abnormalities leading to an increased permeability of the epithelial barrier have been observed in the intestinal mucosa of IBS patients. The most accepted hypothesis is this increased permeability, potentially originated by tight junction disruption (119) and resulting in an increased paracellular flux, constitutes an early factor in IBS. This epithelial changes would be responsible for the uncontrolled passage of luminal antigens, and therefore the activation of immune cells residing in the *lamina propia*, originating a low-grade immune cell infiltration in the intestinal mucosa (120–122).

- Psychological features and Hypothalamic-Pituitary-Adrenal (HPA) axis: psychological stress related to mental disorders, early-life traumatic events, sleep disturbance and "over adjustment to the environment" seem to play a role in the onset and the severity of IBS (103,104), increasing susceptibility to develop IBS. The effect of these factors lead to a feedback circle of stress and pain due to the standing alertness against potential IBS signs (hypervigilance) (123), experimentation of psychological distress in the form of physical symptoms (somatization) and the irrational prediction of a negative outcome (catastrophizing) (124). The HPA axis generates glucocorticoid secretion upon stress stimuli and has been described to be altered in IBS. Glucocorticoids may affect the digestive system, modulating intestinal epithelial barrier (104), and also coordinates functions from central and peripheral nervous systems (121).

An aberrant communication between the central nervous system and the intestine, through the gut-brain axis, has also been postulated as one of the underlying mechanisms of IBS pathophysiology. In fact, a differential immune response observed in IBS patients seems to be associated with stress and depression (125) highlighting the connection between the two systems as key in IBS clinical severity. However, the intimate mechanisms of this interaction remain poorly described.

5.2.2. Immune response in IBS

An amplified immune response has been reported in about half of the patients with IBS (126). Despite this, the specific immune mechanisms involved in IBS onset are not established yet, which impairs finding specific biomarkers for IBS diagnosis. Several studies attempted to find a non-invasive biomarker; reporting an increased number of cytotoxic CD8+ T cells in blood, expressing gut homing integrins, and a higher number of IgG+ B cells (126) and increased levels of IL-6 in IBS patients (127).

The presence of an altered increased permeability together with microinflammation (T cells (128,129) and mast cell (130–136) infiltration, mostly) of the intestinal mucosa suggest there is a local defensive response. Several groups are consistent with the presence of a higher number of CD3+/CD4+ T cells in jejunum of IBS patients compared to healthy subjects. The number of T cells in colonic mucosa is controversial; some results show an increased density of cytotoxic CD8+ and CD3+/CD4+ T lymphocytes, others describe no differences with healthy controls. These inconsistencies are also applicable to studies about the number of infiltrated monocytes and macrophages (137), neutrophils, eosinophils and Natural Killer cells (138).

Most of groups describe a higher number of mast cell in the small bowel mucosa (138), especially in IBS-D patients (36,130,139). Interestingly, mast cells are found in close proximity to nerve endings (133). Upon mast cell activation, they secrete biological mediators, most notably tryptase, a mast cell marker activator, which mediates intestinal permeability, and therefore it modulates epithelial barrier function (137), during stress and inflammation (140). Tryptase has also associated to visceral hypersensivity in IBS-D (141). Interestinlgy, mast cell are in proximity to plasma cells in the jejunum of IBS-D (125). The number of studies focused on the role of B cells and plasma cells in IBS are limited, despite the evidences that suggest a key role of these cells in the clinical evolution of IBS. The jejunal mucosa, one of the most immunological active parts of the gastointestinal tract, harbors a significantly higher density and activation of B cells and plasma cells in IBS compared to controls. Besides, the intestinal content of IgG and the number of IgG+ cells is also increased compared to healthy volunteers. These changes are not detected in the peripheral blood, suggesting this immune response is local. Molecular markers of class-switching isotype from intestinal mucosa biopsies positively and strongly correlated with IBS symptoms at genic expression level (125).

IBS heterogeneous phenotype is an obstacle to research, leading occasionally to discrepancy of results depending on the target intestinal region of study and on the criteria for patient recruitment. Currently, there is no specific biomarker, despite risk factors for the development

of the disorder have been identified and the continuous research is unravelling little by little the pathophysiology of IBS.

5.2.3. Neuro-immune interactions in IBS

The evidences pointing at the dysregulation in gut-brain axis communication in IBS is increasing. Patients present comorbidity of psychiatric symptoms (anxiety, depression and stress) (142) and show a distinct brain activation to visceral stimuli, measured by image analysis (143). It is unclear whether the aberrant connection between central nervous system and the bowel is afferent or efferent, or both. Mucosal mediators from IBS subjects promote excitatory afferent signaling of nociceptive pathways and can also increase intestinal innervation (134,144). Regarding efferent pathways, it has been described the nervous system regulate the immune response by secreting neuropeptides that modulate their viability, proliferation and activity (90). The receptors expressed by the immune cells cover a great variety of neuropeptides secreted by both, peripheral and central nervous system. The signaling modulated by neuro-immune interaction may lead to pathological conditions when there is an overexpression of neuropeptides. The intestinal mucosa is highly innervated in physiological conditions and this innervation has been identified as increased in IBS patients (132). Several peptides associated to IBS symptoms have been postulated to play a role in IBS physiopathology. The amount of the stress-response hormone CRF in the granules of mucosal eosinophil is higher in patients compared to healthy volunteers and correlates with clinical symptoms (145). Another example is VIP, which regulates intestinal ion and water secretion and peristaltism and has been detected in higher amounts in plasma and sigmoid tissue in IBS patients compared to controls (146). Moreover, it is important to highlight changes observed for SP due to its role in nociceptive signaling and visceral hypersensitivity. SP is an essential neuropeptide in the interaction between neurons and immune cells and one of the most released neuropeptides in the intestinal mucosa. In fact, SP high affinity receptor, TACR1, is expressed in the entire gastrointestinal tract (147). In IBS patients, SP+ staining in rectum revealed a higher number of positive fibers compared to health (132).

The abnormal expression of some neuropeptides in this disorder must be considered together with the association between humoral immunity and psychiatric comorbidities as well as between stress episodes and the initiation/exacerbation described in functional gastrointestinal disorders (125,142). Some studies suggest that the proliferation and the secretion of Igs by antibody-producing cells is increased in response to norepinephrine, VIP or SP (90,148,149).

However, little information is available regarding the effect of neuropeptides in antibody-producing cells. Only few authors have approach this field and the lack of up-to-date results is a limitation for extracting conclusions.

Some of these neuropeptides may be also secreted by immune cells. Hence, the neuro-immune modulation may be bidirectional. Neuropeptides secreted by immune cells affect not only nerve cells activity but also epithelial components, modulating the intestinal barrier function. In IBS, the increased intestinal epithelial permeability could expose immune cells to an uncontrolled flux of luminal antigens, promoting the activity of nerve cells innervating the mucosa. Therefore, the remaining question is how immune, nervous and digestive systems interact with each other and the contribution these connections develop in the pathophysiology of IBS.

HYPOTHESIS AND OBJECTIVES

HYPOTHESIS

The increased activation of humoral responses in the intestinal mucosa of irritable bowel syndrome patients is promoted by enhanced neuro-immune interaction, leading to antibody production to minimize the consequences of the barrier dysfunction.

OBJECTIVES

MAIN OBJECTIVE

To characterize the activation of the humoral response in the intestinal mucosa of patients with diarrhea-predominant irritable bowel syndrome. For this purpose, we designed several a study in patients and healthy volunteers and assessed a B cell *in* vitro model.

SPECIFIC OBJECTIVES

1. To analyze the activation the antibody-mediated defense in the jejunum by:
    1. Establishing the local immunoglobulin production signature
    2. Identifying the target of the humoral response activation observed in the intestinal mucosa
    3. Studying the interaction between the nervous system and plasma cells
2. To determine the molecular pathways involved in humoral response activation which are up-regulated in comparison to health.
3. To obtain a B cell *in vitro* model which allows us to study the effect neuropeptides exert in B cell activation/differentiation and immunoglobulin production.

CHAPTER 1

6. **Mucosal IgG production and plasma cell-nerve fiber interaction: potential orchestrators of gut-brain axis dysfunction and clinical manifestations in Irritable Bowel Syndrome**

6.1. INTRODUCTION

Irritable Bowel Syndrome (IBS) is a chronic gastrointestinal disorder with a very broad reported prevalence: 1.1-45% of the adult population (1). Patients report abdominal pain and alterations in bowel frequency and stool consistency. The physiopathology of IBS has not been fully elucidated, despite the numerous efforts of the scientific community. However, based on the current knowledge, IBS is considered a disorder of gut-brain interaction with a biological basis, resembling more an organic disorder than a functional syndrome (2), as it has been long-time considered. In the last decade, structural abnormalities in the intestinal barrier affecting intercellular junctions have been described (3,4). These changes disable the integrity of the intestinal epithelium enhancing intestinal permeability and facilitating the onset of immune responses and mucosal inflammation.

In this sense, one of the leading hypothesis addressing the causes which may underlay IBS origin is that increased intestinal permeability promotes an uncontrolled access of luminal antigens to the *lamina propria*, evoking the activation of abnormal immune responses. This activation has been described by a number of studies, though discrepancies exist as to which immune cells are involved or whether the response keeps local or extends beyond the gut microenvironment (5). Some authors have reported elevated number of cytotoxic CD8+ T cells (6), expressing gut homing integrins, and a higher number of IgG+ B cells (7) in peripheral blood. A local immune response has been also described by several authors within the gut mucosa. In particular, mucosal infiltration driven mainly by T lymphocytes and mast cells has been observed in both large and small bowel (8), yet this is not a universal finding and seems to affect more to those suffering diarrhea and post-infectious IBS than to other subtypes of IBS (9). Mediators secreted by mast cells (such as histamine and proteases, particularly tryptase) are also found in higher amounts in jejunal (3) and colonic biopsies (10), as well as in supernatants and the intestinal lumen (11) from IBS patients, compared to healthy volunteers (HV).

B cells and plasma cells residing in the intestinal mucosa have also been described to be altered in number in IBS-D patients, with differences depending on the segment studied and the authors. Thus, in colonic biopsies, lower levels of IgA+ bearing-cells haven been found, with no difference in the counts of IgM+ and IgG+ lymphocytes, compared to controls (7). Regarding the jejunal mucosa, our group has shown increased numbers of B lymphocytes and plasma cells in IBS-D patients compared to HV. In this line, we have also observed an upregulated differential expression of germline transcripts and Ig genes at mRNA level, and at a protein level higher amounts of IgG in the intestinal content and an increased number of IgG+ positive cells in the

lamina propria of IBS-D patients (12). No changes were found in peripheral blood, suggesting this immune response is mostly mucosal.

All these studies reinforce the potential role of the immune system in the pathophysiology of IBS. Currently, antigens evoking this response are still unknown. Therefore, results related to the participation of B cells and antibody-producing cells (such as plasmablasts and plasma cells) may contribute to a deeper understanding of this syndrome. It has been stablished that infectious gastroenteritis is one the main risk-factors to IBS onset increasing the probability of developing IBS up to 6 times (13). Studies addressing this subject include infections caused by a broad range of virus, bacteria and parasites (14,15), highlighting how challenging it is to recognize the responsible initiating antigen. IgG against Candida albicans (16) and flagellin (17) are significantly increased in blood from IBS patients. Apart from flagellin, other elements of the bacterial cell walls, such as lipopolysaccharides (LPS), have also been linked to this disorder (17). Due to the improvement of the symptoms experienced by some IBS patients after excluding some aliments from the diet, another strategy adopted is to identify those alimentary-antigen specific antibodies involved. Zuo et al. showed several IgG anti-egg, shrimp, crab, soybean and wheat have been associated to IBS in blood samples (18). However, nothing has been already established and further research is needed to fully understand the target of this differential Ig production in IBS.

Interestingly, neuromediators may be also playing an effect in the activation or modulation of the immune system in this disorder. It is widely known that nervous system can modulate the immune response, inhibiting it through parasympathetic and sympathetic terminals, or promoting a local innate defense through peripheral nervous system (19). In fact, several neuromediators, such as norepinephrine, can modulate proliferation of B cells and Ig secretion (20) and peptidergic nerve fibers have been found to be in close proximity to B cells in ileal mucosal biopsies from Institute of Cancer Research (ICR) mice after receiving intraperitoneal LPS (21). There are evidences showing higher levels of certain neuromediators, including vasoactive intestinal peptide (VIP) in plasma and sigmoid tissue (22), substance P (SP) in rectum (23) and corticotropin realising factor (CRF) in jejunal biopsies in IBS patients (24). Besides these alterations in the levels of neuropeptides, we have previously described enhanced activity of humoral response in these patients, specifically transcription of heavy chains genes for IgG1, IgG2, and IgG4, that were positively correlated with psychological symptoms such as depression (12). The goal of this study is to identify the role of B cells and the potential modulation of the nervous system on antibody-mediated defensive responses associated with IBS pathophysiology.

6.2. EXPERIMENTAL DESIGN

A prospective observational study was designed in order to: (1) characterize the immunoglobulin production signature observed in IBS-D patients; (2) establish the target of the humoral response activation observed in the intestinal mucosa of IBS-D; and, (3) study the potential modulatory effect of the nervous system on plasma cell activation.

Clinical assessment and biological samples (feces, blood and jejunal mucosal biopsies) were obtained from all participants (Figure 1). Participants were classified into two groups: IBS-D patients or HV, according to their clinical features (see "Participant and clinical recruitment" section below). All samples used in this study were codified and blindly analyzed by the study researchers.

The experimental design was adapted to the main objectives of the study, as follows:

(1) Characterization of the immunoglobulin production signature observed in IBS-D patients

In order to obtain a deeper understanding of the humoral defense activation in the intestinal mucosa a characterization of the immunoglobulin production signature was carried out by quantification of the Ig subtypes present in the fecal supernatant of IBS-D and HV. The protocols available for Ig quantification by ELISA were mostly designed for blood samples. During this study, we adapted and optimized these protocols to make them suitable for fecal supernatants, taking into account the differences in stool consistency between IBS-D and HV groups.

(2) Identification of the antigens promoting the activation of the humoral response observed in the intestinal mucosa of IBS-D

The target/s of the increased antibody-production in IBS is still unknown, so identifying the antigens against which these antibodies are produced is one of the main objectives when studying IBS physiopathology. Previous results from our group and publications from other authors suggest a bacterial cell-wall element may be one of the triggers of humoral immune response. Therefore, to approach this objective the amount of LPS was determined in stool. Additionally, anti-LPS Igs, IgA, IgM and IgG were quantified in plasma and fecal supernatants from IBS-D and HV using an ELISA technique. Due to limitations in the number of samples, these analyses were performed in a randomly selected subgroup of patients from our groups of study. The methodology used in this section was also set up and adapted when using fecal samples.

(3) Study the potential modulatory effect of the nervous system on plasma cell activation

Characterization of the neuro-immune interaction was performed by immunofluorescence (IF). Proximity between plasma cells and nerve endings and their potential correlation to clinical variables was also assessed with another fragment of the biopsy during this study. Distance measurements were blindly carried out by transmission electron microscopy (TEM), obtaining always consistent results even when measured by several researchers.

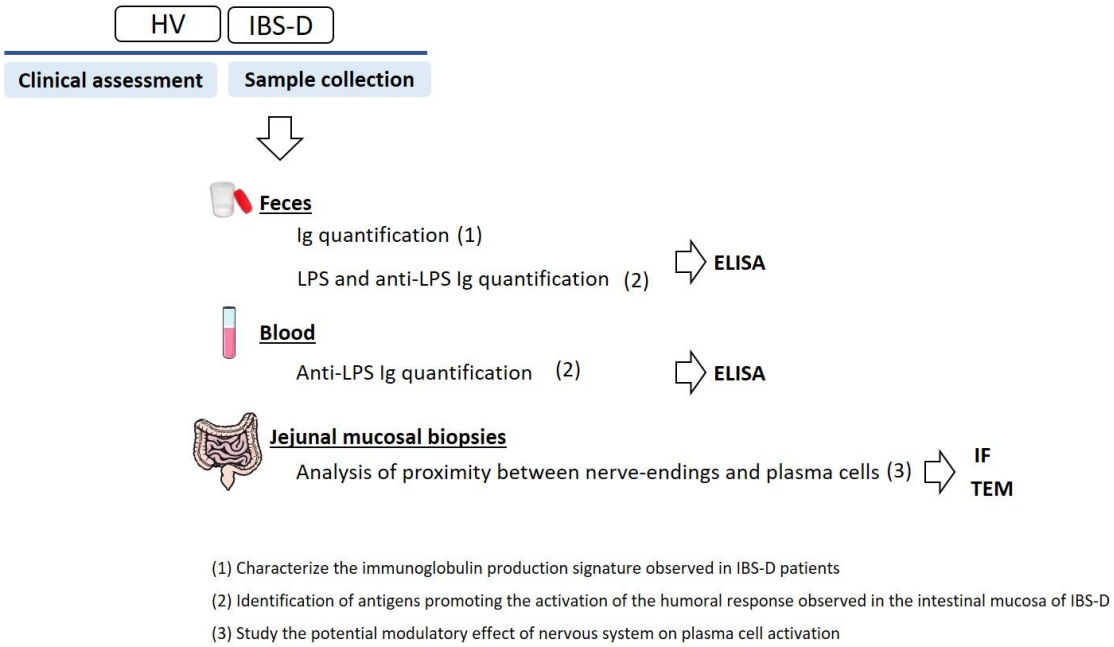

Figure 1: Experimental design of chapter 1. The numbers next to the techniques indicate the biological samples used for each part of the experimental design. ELISA: Enzyme-Linked ImmunoSorbent Assay; HV: Healthy volunteer; IBS-D: Diarrhea-prone Irritable Bowel Syndrome; IF: Immunofluorescence; Ig: Immunoglobulin; LPS: Lipopolysaccharide; TEM: Transmission Electron Microscopy.

6.3. METHODS

6.3.1. Participants and clinical assessment

All participants were evaluated by a gastroenterologist at the Gastroenterology department of the Vall d'Hebrón Barcelona Hospital Campus (Barcelona, Spain). Newly diagnosed diarrhea-predominant IBS (IBS-D) patients were retrospectively recruited from the outpatient gastroenterology clinic, all of them fulfilling Rome III criteria. Healthy volunteers (HV) were recruited from the general population by public advertising. A complete structured clinical questionnaire, evaluating digestive symptoms, was conducted in all candidates prior to entering

the study. Subjects with other gastrointestinal pathologies and volunteers with digestive symptoms were excluded from the study, as well as patients with dyspepsia, following the Rome III criteria. Past episodes of infectious gastroenteritis and other gastrointestinal comorbidities were assessed using biochemical and serological tests, including anti-transglutaminase antibodies and thyroid hormones. Inclusion and exclusion criteria are summarized in supplementary table S1.

All candidates underwent a battery of skin prick test (SPT) (Laboratorios Leti SA, Barcelona) for 22 common food allergens and 12 inhalants before the collection of jejunal mucosal biopsies. Histamine and saline were used as positive and negative controls, respectively. Results were evaluated by an allergist to discard food allergy. Participants showing positivity to food allergens by SPT or consistent with clinical history (digestive and/or extra-digestive symptoms associated with exposure to certain food components) were excluded. Digestive and psychological symptoms were assessed by clinical questionnaires completed daily during 10 days by all participants. The following symptoms were recorded: 1) pain severity (by a 100-point visual analogue scale, VAS) (25); 2) pain frequency (number of days with pain) (25); 3) stool frequency (maximum number of bowel movements); 4) stool form (by the Bristol Stool Chart score)(26); and 5) abdominal distention using a 6 point VAS (25). Background stress and depression levels were assessed using the validated Spanish versions of the Modified Social Readjustment Scale of Holmes-Rahe (27), the Perceived Stress Scale of Cohen (28), and the Beck's Depression Inventory (29), respectively. IBS severity was assessed by a severity scoring system (Francis Score) (25), a prompted visual analogue scale consisting in 5 questions, each one with a maximum score of 100, which evaluate bowel habits, distention, the intensity and frequency of pain and quality of life presented by the candidates. Written informed consent was obtained from each participant. The study protocol was approved by the Ethics Committee at Vall d'Hebrón Barcelona Hospital Campus (Barcelona, Spain) [(PR(AG)211/2018)] and informed consent was obtained in all participants. The study was carried out in accordance with the Declaration of Helsinki.

6.3.2. Collection of biological samples

6.3.2.1. Feces

Feces were collected in donor's residence 1 or 2 days prior to the obtaining of the jejunal mucosa biopsy. At the time of stool collection, urine contamination was avoided and sample was homogenized with a scraper, to ensure obtaining a representative stool fraction. Feces were

kept at -20ºC and transported to the laboratory at 4ºC, where they were stored at a -80ºC until further processing and analysis.

6.3.2.2. Peripheral blood

Blood from each participant was collected directly into BD Vacutainer EDTA tubes (BD Biosciences). Plasma was obtained by collecting supernatant after centrifugation at 1200xg during 10min at 4ºC, and stored at −40°C until analyzed.

6.3.2.3. Jejunal biopsies

Jejunal mucosal biopsies from IBS-D and HV were obtained using a Watson's capsule as previously described (11). Subjects were orally intubated, previous overnight fasted, and capsule was positioned 10 cm distal to the angle of Treitz's under fluoroscopic control. The resulting biopsy was divided in three pieces. One fragment was sent to the Pathology Department for histological analysis and exclusion of other pathologies. The two other fragments were used for further microscopic examinations: one was placed in formalin and embedded in paraffin for IF and the other one was fixed for ultrastructure evaluation by TEM.

6.3.3. Analytical procedures

6.3.3.1. Quantification of immunoglobulins

Feces from participants (200mg) were aliquoted in 2mL Lysing Matrix E Tubes (MP Biomedicals) and diluted in PBS (pH=7.4) supplemented with HaltTM protease Inhibitor (ThermoFisher) to the appropriate concentration, according to the isotype Ig: 0.08mg/mL for IgAs, 4mg/mL for IgM, 20mg/mL for IgG, 100mg/mL for IgG subtypes (IgG1, IgG2, IgG3, and IgG4) and 200mg/mL for IgE. Samples were homogenized 2 min by vortex for sIgA, IgM and IgG or using Mini-Beater-16 cell disrupter (Biospec products) in two rotations of 3min and 5min, respectively, separated by an incubation at 4ºC during 3 min for the others Ig. Homogenized feces were centrifuged at 4ºC 13,000xg during 5 min for Ig quantification. Afterwards, supernatants were transferred into a new tube and stored at -40ºC. Ig quantification was done in fecal supernatants using Ig-specific ELISA kits, according to the manufacturer's instructions: Human IgAs ELISA kit (Immunodiagnostik), Human IgM ELISA kit (Bethyl Laboratories), Human IgG ELISA kit (Bethyl Laboratories), Human IgG1 ELISA kit (Ray Biotech), Human IgG2 ELISA kit (Abcam), Human IgG3 ELISA kit (Abnova), Human IgG4 ELISA kit (Cloud-Immunoassay), Human IgE ELISA kit (Bethyl Laboratories). Due to the differences in stool consistency between groups, results were normalized by total quantity of protein. Protein quantification was performed by the Pierce BCA protein assay (ThermoFisher) method.

6.3.3.2. Quantification of LPS and anti-LPS immunoglobulins

Feces were aliquoted according to the previous protocol described for Ig quantification in fecal supernatants. For LPS quantification, stool was diluted at 100mg/mL PBS, homogenized using Mini-Beater-16 cell disrupter (Biospec products), as described previously, and centrifuged at 3,000xg 10 min. LPS was detected with LPS ELISA Kit (Elabscience). Low-binding and endotoxin-free material was used in LPS quantification to avoid unspecific signal. Luminal IgA anti-LPS was quantified in feces with the same protocol used for the quantification of the others Igs described above, at a concentration of 200 mg feces/mL PBS (EndoCab IgA ELISA kit (HycultBiotech)). Results obtained from LPS and luminal IgA anti-LPS quantification were also normalized by total quantity of protein.

Immunoglobulins anti-LPS were also quantified in plasma from all subjects by ELISA according to the manufacturer's instructions. The dilution conducted in plasma for each Ig quantification and the ELISA kit used were as follows: 1/50 IgA (EndoCab IgA ELISA kit (HycultBiotech)), 1/50 (IgM EndoCab IgM ELISA kit (HycultBiotech)) and 1/300 IgG (EndoCab IgG ELISA kit (HycultBiotech)).

6.3.3.3. Immunofluorescence staining

Jejunal mucosal biopsies were fixed in formalin, embedded in paraffin, cut at 4µm with a Microm HM325 microtome (ThermoFisher) and transferred to slides for further protein expression analysis of neuro-immune interaction by double-staining IF. Samples were heated at 65ºC overnight to remove paraffin excess and were deparaffined and dehydrated sequentially with xylene, ethanol and distilled water washes, following general procedures. Antigen retrieval was performed by heat induced epitope retrieval procedures. Tissue sections were submerged in a pH9 solution (Tris-EDTA buffer) and autoclaved for 10min at 120ºC. A permeabilization step for intracellular staining was included. Samples were incubated with the appropriate permeabilization solution (Table 1) for 5min at RT. Afterwards, samples were blocked with Dako Blocking Solution (Dako) for 3h at RT. Blocking Solution was supplemented with 15% normal goat serum (Sigma-Aldrich) and 0.5% of bovine serum albumin (Sigma-Aldrich) to reduce background signal (Table 1). Samples were stained with the pertinent primary antibody overnight at 4ºC except for anti-CD138, where the incubation step was reduced to 1h at RT due to the strong signal obtained with these antibodies. Tissues were then exposed to 1:500 appropriated secondary antibody Alexa Fluor 488/ Alexa Fluor 594 anti-rabbit/anti-mouse) (ThermoFisher) 30min in the dark at RT. Nucleus was stained with 10ng/mL 4',6- diamidino-2-phenylindole (DAPI) for 10min RT. Slides were mounted with Prolong antifade mountant media

(Invitrogen) and stored at 4ºC light protected. Slides exposed to these conditions, except for the primary antibody incubation step, were included as negative controls. During the procedure, tissue sections were washed (x3) in phosphate buffered-saline (PBS, pH=7.4) between steps. Results were visualized using a Zen 3.0 (Blue version) and built-in camera in a confocal fluorescence microscope LSM980 (Zeiss).

Ab	Marker	Ab Host and supplier	Permeabilization	Ab dilution
anti-CD38	Plasmablast/Plasma cell	Mouse; Nordic Biosite	0.075% Triton X-	1:100 O/N
anti-TACR1	Substance P receptor 1	Rabbit; ThermoFisher	100	1:50 O/N
anti-CD138	Plasma cell	Rabbit; Sigma-Aldrich	0.1% Triton X-100	1:100 1h
anti--PGP9.5	Neuron/Nerve ending	Mouse; Abcam		1:50 O/N

Table 1: Antibodies used for protein expression by immunofluorescence in jejunal biopsies from healthy volunteers and IBS-D patients. O/N: over-night incubation.

6.3.3.4. Transmission electron microscopy

Biopsies were cut in two or more different pieces, whenever possible, and tissue fragments were immersed in conventional fixative solution: 2.5% (v/v) glutaraldehyde (electron microscopy grade, Merck), 2% paraformaldehyde (PFA) (w/v) in 0.1M phosphate buffer during at least 48h at ºC RT. Biopsies were transferred into a maintenance solution (1% PFA in 0.1M phosphate buffer) and stored up to 1 month at 4ºC. Samples were post-fixed with 1% osmium tetroxide (Sigma) (w/v) and dehydrated sequentially. After dehydration, samples were embedded in Eponate 12 resin (Ted Pella Inc.), in different blocks when having two or more pieces, and polymerized at 60ºC. Biopsies were then cut in ultrathin (70-90nm) sections with a Leica Ultracut UCT microtome (Leica Micro-systems GmbH) and placed on gold grids (100mesh) with a film layer. The evaluation of the samples was performed using a TEM JEM-1400 (Jeol Ltd.) equipped with a CCD Gatan ES1000 W Erlangshen camera. Post-fixation procedures and sample observation were carried out at the Servei de Microscopia of Universitat Autònoma de Barcelona (Bellaterra). For the evaluation of ultrastructural features of plasma cell activation and its proximity to nerve endings, between 20-40 images were taken in non-overlapping fields from different blocks for each IBS-D (n=18) and control (n=15) samples at 12000-15000x magnification.

6.3.4. Statistical analysis

Normality of the data distribution was tested by the D'Agostino and Pearson omnibus normality test. Normally distributed parametric data are expressed as mean ± standard error of mean and compared by the compared by the unpaired Student's t test (two-tailed). For non-parametric distributed data, results are expressed as the median and the maximum-minimum range and analyzed by the Mann-Whitney U test was used. All the statistical analysis was performed using GraphPad Prism 6.0 software. Relationships between clinical variables and gene expression were assessed by Spearman's rho correlation. P-values ≤ 0.05 were considered significant.

6.4. RESULTS

6.4.1. Study population

A total of 44 HV and 45 IBS-D patients were included in the study (Table 2). There were no significant differences in the percentage of atopy (p=0.6104), and the IBS-D group showed higher ratio women: men (p=0.0481), and a higher median age than the HV group (p=0.0113). As widely described in the literature, patients had higher levels of acute stress (Cohen score, p<0.0001) and depression (Beck's Inventory, p<0.0001). Despite not reaching statistical differences, there was a trend in the IBS-D group to have increased levels of stress during last year (Holmes-Rahe score, p=0.1096). Approximately half of the IBS-D group declared symptoms of dyspepsia and distension, 56.25% and 46.15% respectively.

	HV (n=44)	IBS-D (n=45)	p-value
Gender, F:M	22:22	32:12	**0.0481***
Age, years	28 (18-54)	37 (21-64)	**0.0113***
Atopy, yes: no	9:16	18:23	0.6104
Intensity of the abdominal pain, score	0	50 (6-100)	-
Frequency of the abdominal pain, n days	0	5 (2-10)	-
Bowel movements (n)	1.25 (0.5-2)	3.5 (1-12)	**< 0.0001***
Stool form, Bristol scale	3.5 (2-4)	6 (4-7)	**< 0.0001***
Dyspepsia, yes:no	0	18:14	-
Abdominal distention	0	18:21	-
Holmes-Rahe Scale	75 (18-293)	123 (0-889)	0.1096
Cohen Scale	14 (4-53)	26(10-41)	**< 0.0001***
Beck's Depression Inventory	1 (0-22)	10.5 (1-31)	**< 0.0001***

Table 2: Clinical and demographic characteristics of participants. Results are expressed as median (minim –maximum values). * symbol highlights values with significant statistical differences between groups ($p<0.05$).

6.4.2. IBS-D patients present significantly higher concentration of luminal IgG

Quantification of Igs in fecal supernatant revealed a significantly increased amount of IgG in IBS-D, compared to HV. The concentration of sIgA showed also a trend to be higher in the IBS group. The subtypes of IgG were determined in stool from both groups: IgG2 and IgG3 were increased in IBS-D, the later not reaching statistically significance. No differences were found in IgM, IgE, IgG1 and IgG4 concentration between groups (Table 3).

Ig	HV (n=17)	IBS-D (n=19)	p-value
sIgA	11.55 (1.34-71.30)	26.78 (4.02-221.1)	0.0990
IgM	62.54 (3.44-1074)	109.1 (4.75-1854)	0.1282
IgG	3.68 (0.61-35.34)	11.38 (1.5-45.82)	**0.0412***
IgG1	0.15 (0.003-0.82)	0.07 (0.002-96)	0.2740
IgG2	0.40 ± 0.29	0.71 ± 0.53	**0.0395***
IgG3	0.91 (0.19-1.83)	1.05 (0.28-3.65)	0.0792
IgG4	0.028 ± 0.004	0.021 ± 0.004	0.1840
IgE	0.090 (0-0.472)	0.159 (0.012-2.590)	0.1040

Table 3: Values of Igs quantification in fecal supernatant from HV and IBS-D patients. Results are expressed as [ng Ig/mg protein], except for sIgA, which are [µg Ig/mg protein]. Ig: immunoglobulin. * indicates p-value <0.05.

6.4.3. Healthy volunteers and IBS-D patients show similar levels of LPS and anti-LPS immunoglobulins

IgG2 has been linked in the literature to the response against polysaccharides of the bacterial wall (30). We assessed the levels of luminal LPS, one of the main polysaccharides found in feces. We identified higher concentration of LPS in stool from IBS-D group (0.469 ± 0.086 ng LPS/mg protein) compared to HV (0.326 ± 0.041), despite not reaching statistical significance (p=0.411) (Figure 2). Igs against LPS were also determined in fecal supernatant. Due to technical limitations (Supplementary Table S2), only sIgA could be quantified, showing similar values for IBS-D patients (0.038 (0.019-0.140) UA IgA) and HV (0.027 (0.006-0.186) UA IgA, p=0.272) (Figure 2). No differences between groups were observed when evaluating the concentration of Igs against LPS in the systemic circulation for IgA anti-LPS (IBS-D: 58.3 ± 8.79 AU vs HV: 43.8 ± 8.38 AU,

p=0.253), IgM anti-LPS (IBS-D: 57.4 (14.2-138) AU vs HV: 59.8 (52.8-134), p= 0.573) and IgG anti-LPS (IBS-D: 96.2 (28.5-280) AU vs HV: 81.4 (42.3-195) AU, p=0.513) (Figure 3).

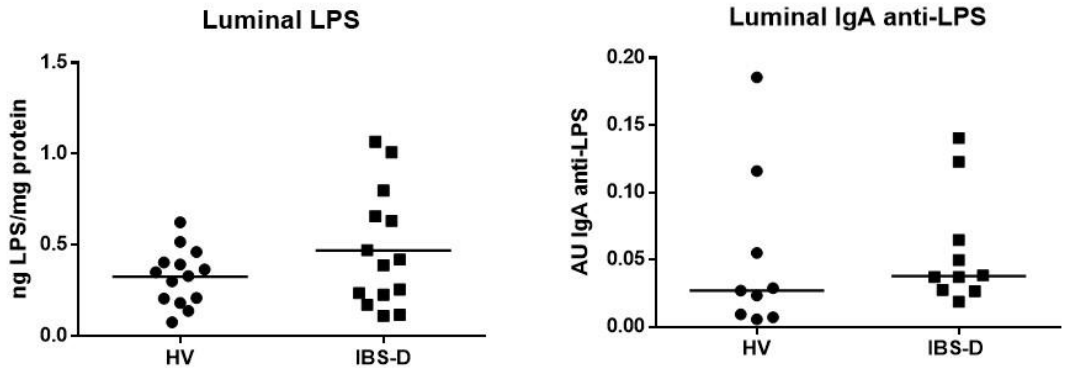

Figure 2: Representation of the concentration of LPS and IgA against LPS in fecal supernatant from HV and IBS-D patients. AU: Arbitrary units.

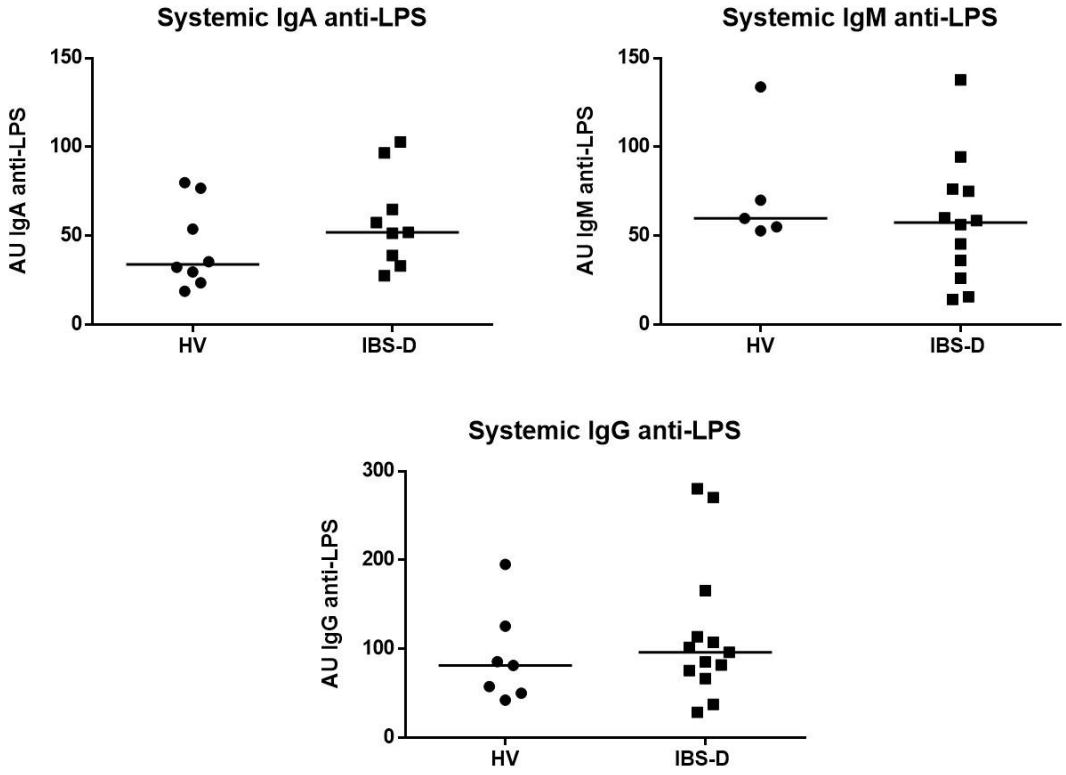

Figure 3: Representation of the amount of Igs against LPS in systemic circulation in HV and IBS-D patients. AU: Arbitrary units.

The analysis of correlation between the amount of luminal LPS detected in fecal supernatants and the expression of luminal or systemic IgA showed no association between any variable (Supplementary Table S5). Similarly, luminal and systemic expression of anti-LPS IgA did not show any correlation (r2= -0.022, p= 0.936).

6.4.4. Luminal IgG positively correlates with the intensity of abdominal pain in IBS-D

We determined the association of intestinal and psychological symptoms (abdominal pain, stool consistency (assessed by Bristol scale) with the concentration of IgG in the fecal content in the IBS-D group. The amount of IgG correlated with the intensity of the abdominal pain described by the patients (r2=0.498, p=0.0356) (Figure 4). IgG2, also significantly increased in fecal supernatant of IBS-D, did not show any correlation with the variables analysed.

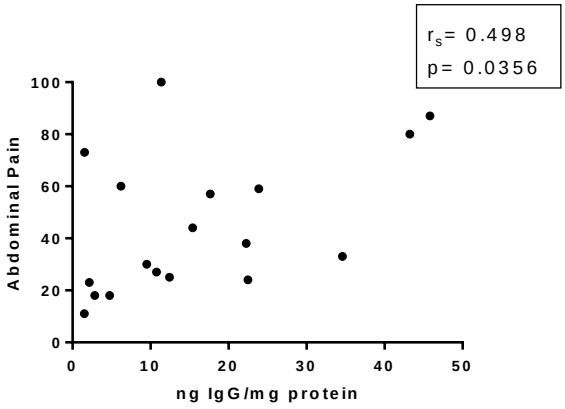

Figure 4: Representation of the correlation between IgG concentration in feces and the intensity of abdominal pain in the IBS-D group.

6.4.5. Phenotypical characterization of neuro-immune interactions in jejunal mucosa

In order to assess the role of neuro-immune interactions in the jejunal mucosa of participants, we performed double-staining IFs of antibody-producing cell markers (CD38 and CD138) and neuro-related proteins markers (TACR1 and PGP9.5). Figure 7 represents the double staining of CD138, terminally differentiated plasma cell marker, and PGP9.5, neuronal and axonal marker. As previously described by our group (12), plasma cells form clusters of cells in both HV and IBS-D. PGP9.5+ fibers innervates the whole *lamina propria*, especially immediately below the epithelium, and are also in close proximity to plasma cell clusters (Figure 5). No differences were observed between groups.

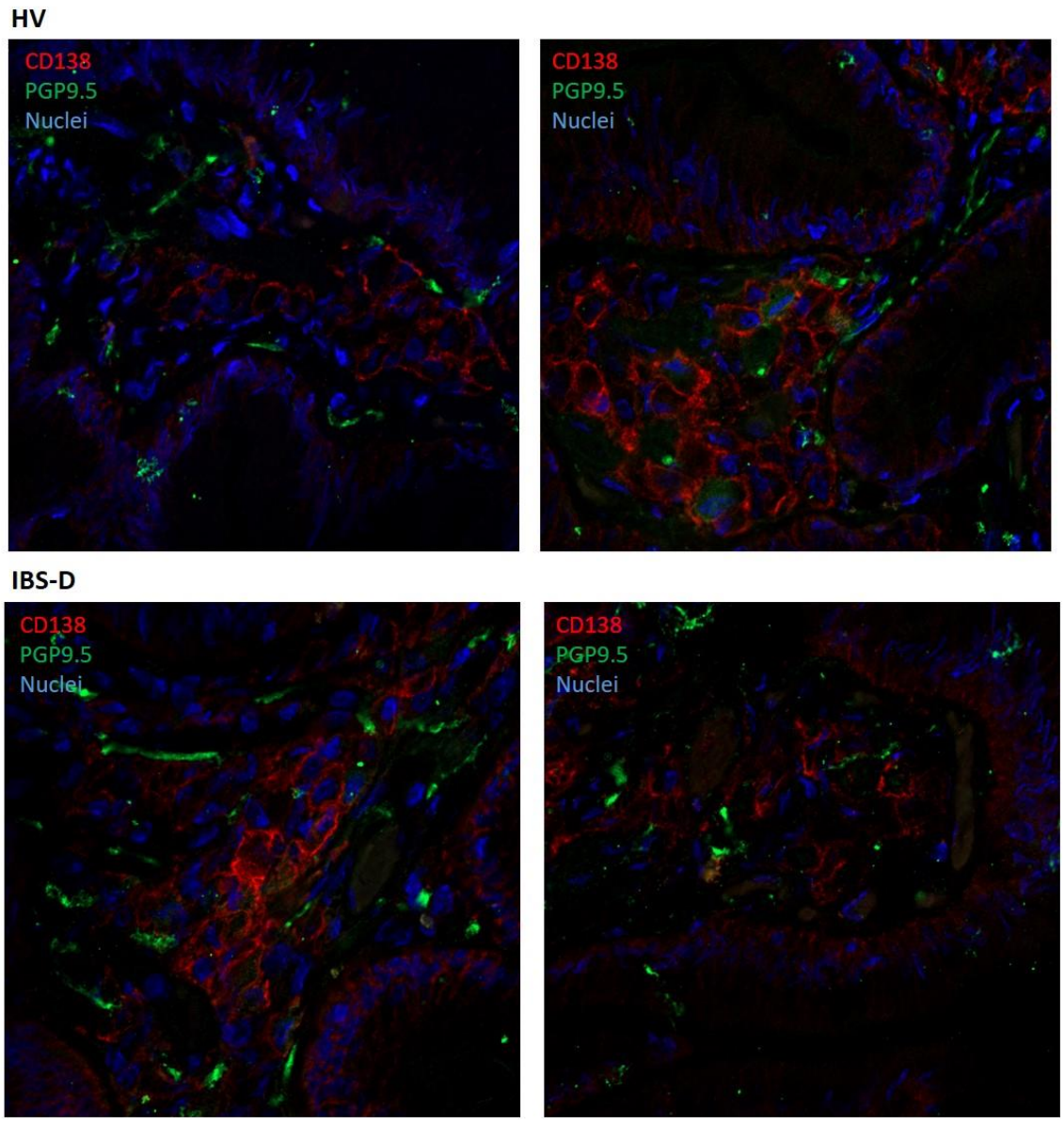

Figure 5: Representative images of CD138 (plasma cell marker) and PGP9.5 (neuronal marker) double-staining in jejunal mucosal biopsies of HV and IBS-D. Magnification: 600x

To assess whether those fibers nearby antibody-producing cells were positive for substance P receptor TACR1, involved in nociception, we performed TACR1+CD38 staining. CD38, expressed in both plasmablast and plasma cells, is detected in proximity to TACR1+ fibers in both controls and patients (Figure 6).

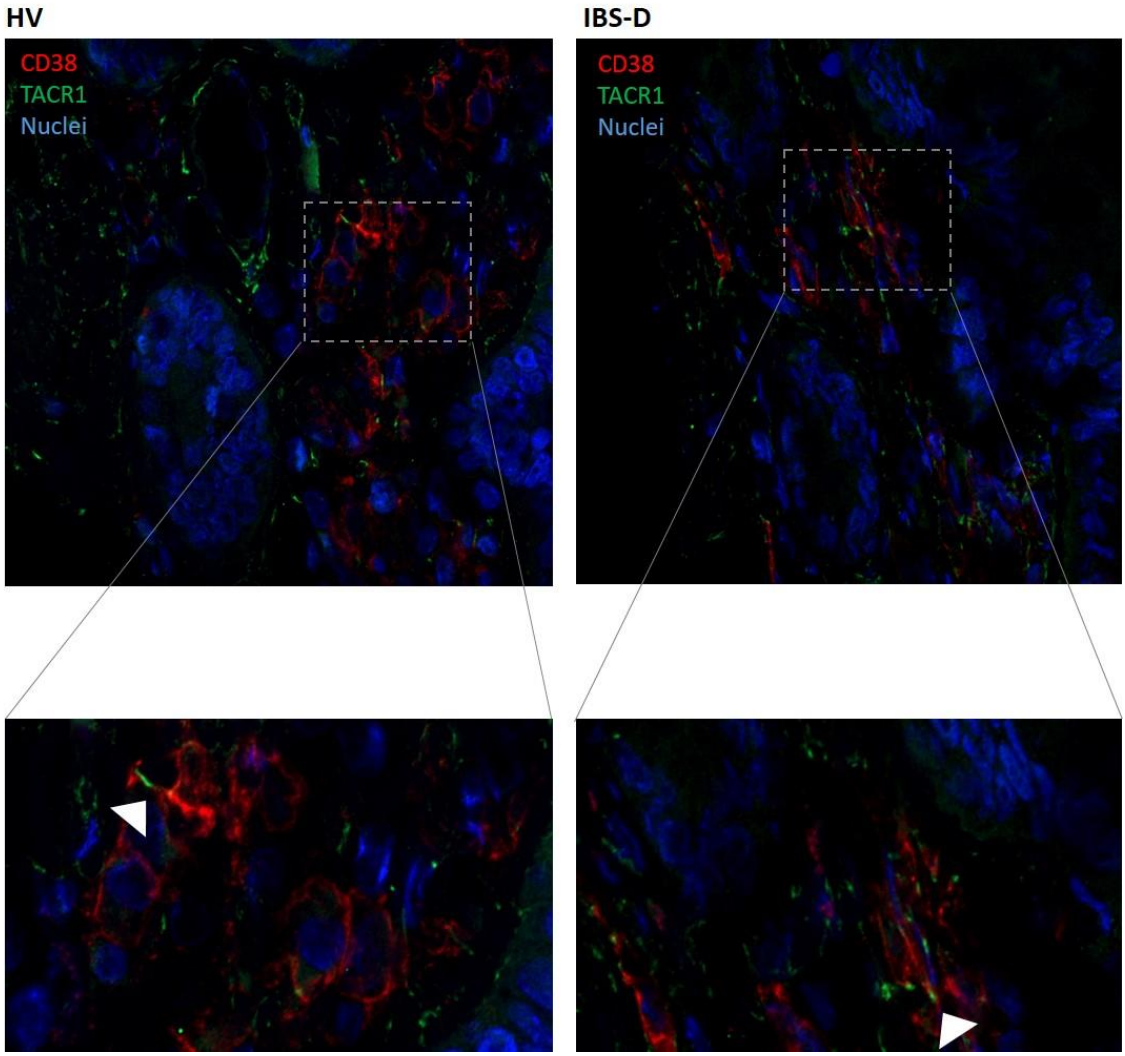

Figure 6: Representative images of CD38 (plasmablast and plasma cell marker) and TACR1 (Substance P receptor 1) double-staining in jejunal mucosal biopsies of HV and IBS-D. Magnification: 600x. White arrows indicate those zones where TACR1+ and CD38+ are in close proximity.

6.4.6. Association between plasma cells and nerve endings proximity and IBS-D psychological symptoms

In a previous work, we described clusters of long-lived plasma cells featured the jejunal mucosa of IBS-D, while individual plasma cells were observed scattered in the control group (12). To better characterize these cells, jejunal biopsies from both groups were analysed by TEM. Mucosal plasma cells are found in the subepithelial area and located in proximity to nerve endings. According to previous results published by our group, plasma cell present more signs of activation, including enlarged cytoplasm and expanded endoplasmic reticulum cisternae (Figure 7). Distance between plasma cell and nerve endings is significantly lower in samples from IBS-D (1.27 (0.21-4.25) µm) compared to HV (2.26 (0.26-7.06) µm); p=0.0066 (Figure 8 and 9).

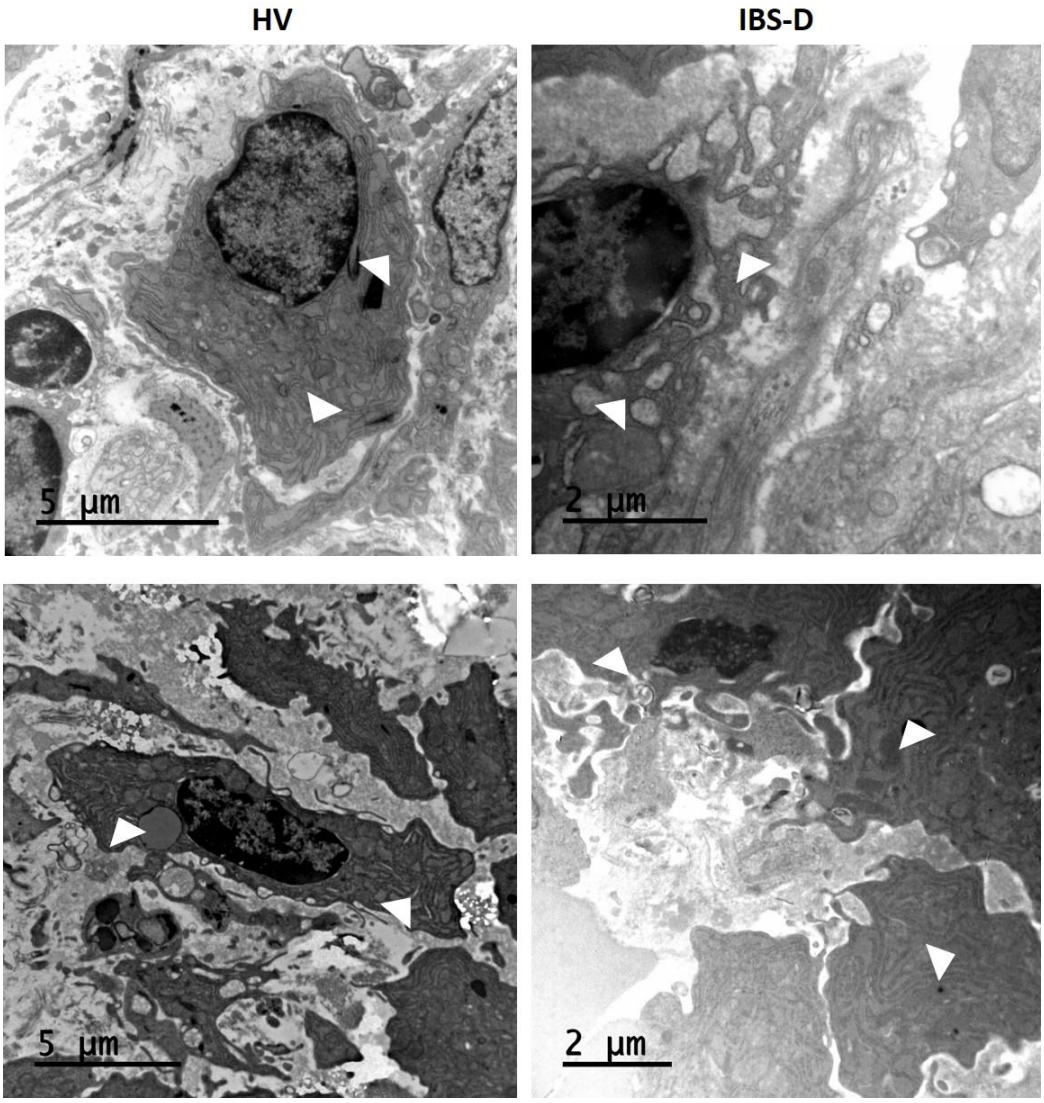

Figure 7: Representative images of the ultrastructure of mucosal plasma cells from HV and IBS-D. Signs of activation such as enlarged cisternae can be observed especially in the IBS-D group (white arrow heads)**.** Magnification 12,000x-20,000x

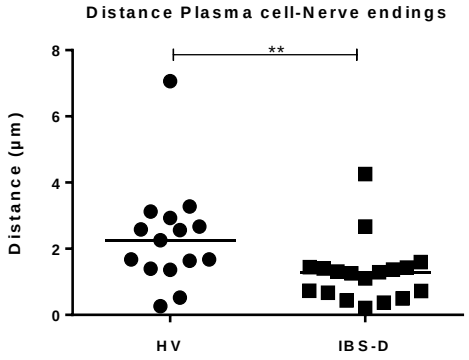

Figure 8: Distance between plasma cell and nerve endings in the *lamina propria* of jejunal biopsies from HV and IBS-D groups.

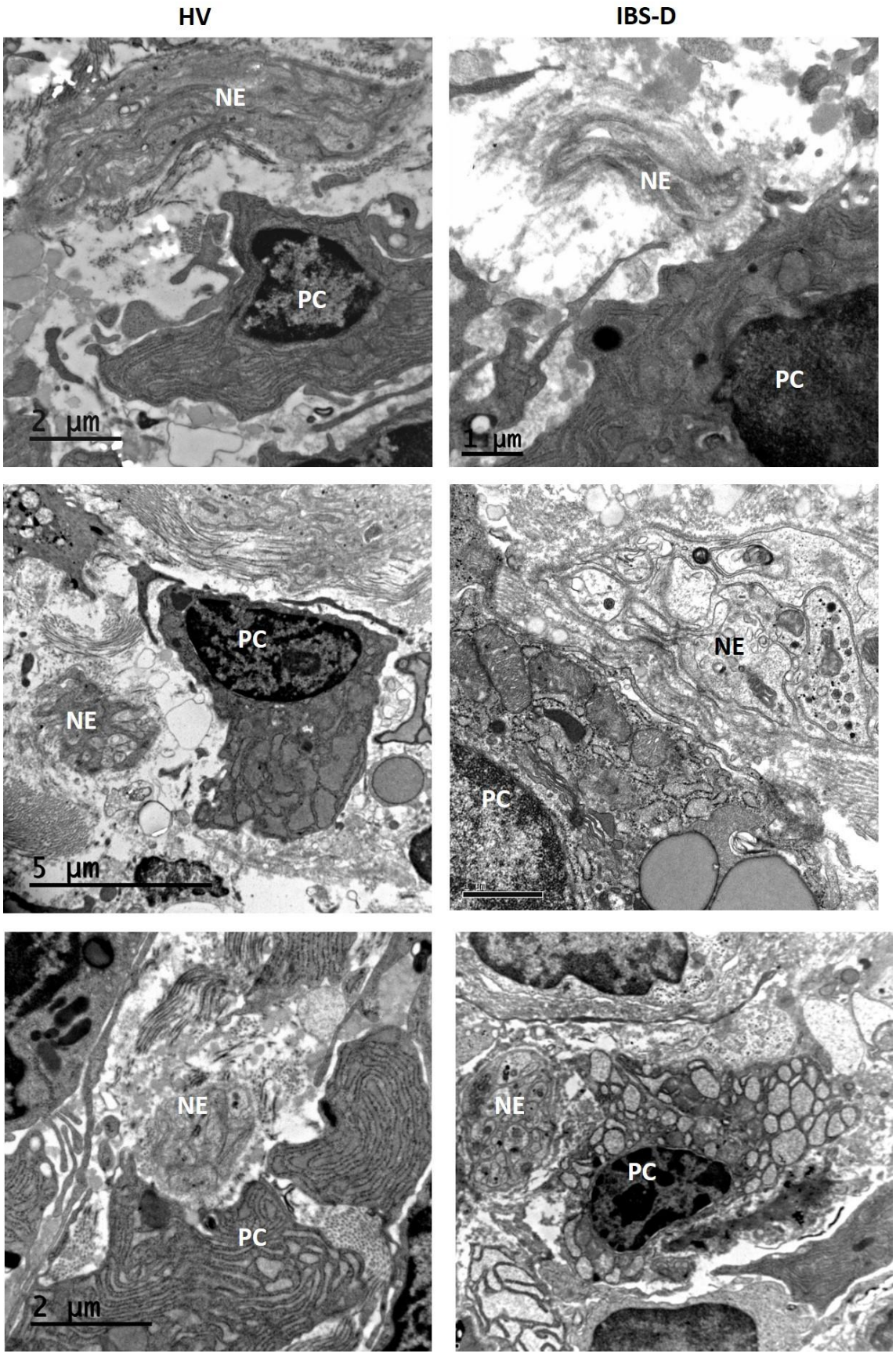

Figure 9: Representative images from plasma cell in close proximity with nerve endings in HV and IBS-D jejunal samples. Magnification 12,000x-20,000x. NE: Nerve ending; PC: Plasma cell.

Distance of plasma cells to nerves negatively correlated with psychological stress (r2=-0.0343; p=0.0394), measured with Cohen scale and symptoms of depression (r2=-0.354, p=0.0643), assessed by Beck's Inventory (Figure 10), the former not reaching statistical signification.

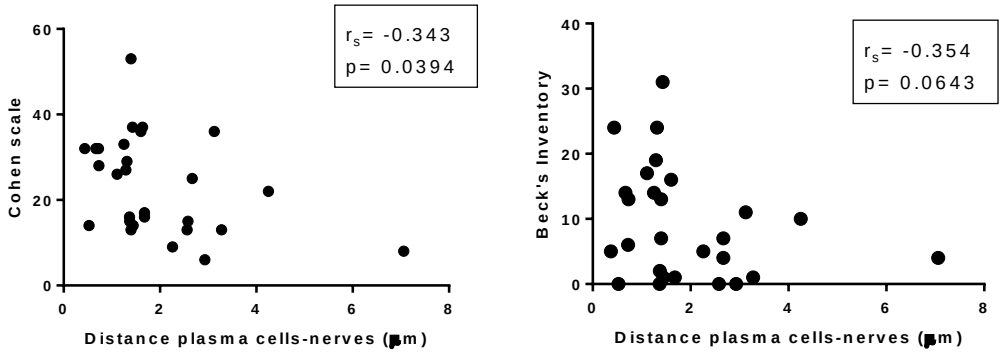

Figure 10: Representation of the correlation between plasma cell-nerve ending proximity with psychological stress and symptoms of depression, Cohen score and Beck's Inventory, respectively.

6.5. DISCUSSION

This is the first study identifying a clear interaction between plasma cells and nerve endings in the intestinal mucosa in IBS-D and its association with clinical severity, reinforcing the neuro-immune interaction contribution to IBS pathophysiology, specifically the antibody-mediated immune response.

Our aim was in this study was to deliver a useful non-invasive biomarker for IBS to help in diagnosis, as it is currently done by exclusion of other disorders and by symptomatic criteria following Rome criteria. Due to the disparities observed by some authors in blood samples (12), we quantified the amount of Ig in fecal supernatants. We observed that IBS-D patients showed significantly higher concentration of IgG than HV. These results are in line with our previous work (12), where we demonstrated higher levels of IgG in IBS-D patients, both in jejunal biopsies and the intestinal content. Simultaneously, we described higher humoral activity, observed as higher counts of B cells and plasma cells in the small bowel mucosa of patients, and higher gene expression of germline transcripts and IgG genes (12). All these results suggest IgG as the main participant in the IBS-driven mucosal humoral response. Despite some authors have described an increase in IgG also in peripheral blood (17,18), the leading theory is that the activation of the immune defence is a local response of the intestinal mucosa and not always visible in peripheral blood. We wanted to test if this rise in IgG levels could be also found in stool samples as it represents the passage through all the intestinal tract. Obtaining a sample which can

provide information of all the intestinal tract is very important because usually biological samples are limited to a very specific region of the bowel, most commonly to study gene and protein expression in mucosal or transmural biopsies (31–33). Therefore, stool appeared as a good candidate for approaching this issue. Since IgG is also increased in fecal supernatants, the hypothesis that those activated plasma cell found in the intestinal mucosa are producing IgG, which is released through the epithelium to the lumen, is reinforced. Despite the fact that we do not have a deep understanding of IBS physiopathology, and considering the increased epithelial permeability observed in some IBS patients (3,4), IgG may putatively reach the intestinal lumen due to the impaired barrier function and not only through the neonatal Fc receptor (FcRn) (34). Therefore, the target/s of this response should be present in the *lamina propria*, where B cells and plasma cells reside, and its presence in the feces or intestinal content in more proximal regions, would not have a biological reason other than a spillover or the presence of additional targets (e.g.: food or microbes) in these locations. This may be in fact the case of Inflammatory Bowel Disease (IBD), where higher levels of IgG and IgA as well as IgG- and IgA-coated bacteria have been described (35). Uncontrolled access to the lumen could explain why we observe higher levels of IgA in stool supernatant, despite not reaching statistical differences.

A better characterization of the IgG subtyped expression could give us information about the processes driving humoral response. When we quantified IgG subtypes, we observed higher levels of IgG2 and IgG3, the later not reaching statically significance, in stool supernatants from IBS-D patients. As noted in the introduction, several authors have attempted to identify the target antigen of these Ig (16–18), but with little success so far. Alimentary antigens have been raise as potential triggers of the humoral defense in IBS patients, due to the improvement of symptoms reported by some patients after following a food-exclusion diet (36–38). IgG4, together with IgE, is the main Ig subtype mediating response against food antigens (39), contrary to IgG2 and IgG3, which are produced against bacterial encapsulated polysaccharides and in pro-inflammatory mechanisms (30), respectively. Despite IgG3 results show a trend, we considered IgG2 was the Ig with a major contribution in IgG level variations between groups, hence it was the IgG subtype showing significant differences. Therefore, our results suggest that a polysaccharide could be involved in the activation of the antibody-mediated response; for that reason we proceeded to the quantification of LPS and anti-LPS Igs in HV and IBS-D biological samples.

Due to the link between LPS and intestinal inflammation (40,41) and visceral hypersensitivity (40) described in the literature, several assays in blood from IBS-D and HV have been published,

showing a higher LPS concentration in patients suffering from this syndrome (17). LPS is the major component of the gram negative bacterial outer membrane (42), therefore, it can be found at high concentrations in stool samples. When we compared the amount of LPS found in fecal supernatant, we observed that IBS-D group showed a slight rise of LPS concentration, not statistically different from HV. The quantification of anti-LPS IgA in stool did not offer differences between IBS-D and HV. Feces are not commonly used biological samples, which implies difficulties to find proper commercial kits for Ig detection (e.g: ELISA) together with the necessity to adapt the protocols. Despite the efforts, unfortunately, we were not able to detect anti-LPS IgM and anti-LPS IgG in stool; their concentration in fecal supernatant is very low and even using lyophilisation methods, we could not overcome the technical limitations (Supplementary Table SX). In IBS-D and HV blood, we quantified anti-LPS IgA, IgM and IgG, obtaining similar levels in both groups. The heterogeneity of IBS-D patients can be noted when observing the results; there is an extensive overlap between IBS-D and HV LPS and anti-LPS Ig levels. This overlap may indicate that not all patients are responding to the same antigen. The intestinal mucosa is challenged by a broad range of antigens, from different nature, including bacteria, virus and elements from the diet, which may penetrate the bowel barrier differently according to the subgroup of patients. Considering this heterogeneity, results from other authors where LPS levels are higher in blood from IBS-D patients (17) and our own results, where a trend to increased LPS in stool supernatant can be observed, we suggest LPS may be responsible for the priming of the humoral response in some of the patients or it is contributing synergistically together with other antigens to activate antibody-mediated processes. Further research is needed, to obtain a deeper characterization of this response as well as to identify the target antigen(s).

The role of gut-brain axis dysregulation is one of the remaining questions concerning IBS pathophysiology. The study performed in our participants revealed IBS-D patients showed higher scores of acute stress and depression, assessed with Cohen and Beck scales, respectively, as it has been previously described in the literature (43). Despite IBS has been widely associated to psychiatric comorbidities, the details of the interaction between nervous and digestive system have not been fully established yet. However, it is well-accepted the presence of a neuro-immune crosstalk in the intestinal mucosa (44,45), which is potentially involved in the onset and the outcome of this disorder. When we quantified IgG levels in fecal supernatants, we observed that IgG concentration positively correlated with the intensity of abdominal pain reported by the IBS-D group. Few studies address the communication between B cells/plasma cells and nerve-endings. It is known that neuropeptides can affect B cell proliferation, survival and activity

(46,47) and they can even secrete neuropeptides such as CRF in response to certain stimuli (e.g: stress) (48). In addition, it has been described that distribution of nerve-fibers can be affected by immune cell location (49). Therefore, this communication and neuro-modulation seems to be a bidirectional pathway, where both nervous and immune system are regulated by each other.

We decided to perform a phenotypical characterization of this neuro-immune interaction in jejunal mucosa considering that the intestinal tract is highly innervated, mainly by vagal fibers (44), and that a great number of B cells and plasma cells reside in the small bowel mucosa . We assessed by IF techniques the proximity between terminally differentiated plasma cells, CD138+, and nerve-endings, positive for the neuronal marker PGP9.5. We observed that plasma cells appeared in clusters in *lamina propria* of the jejunal mucosa, as we previously published (12). These cell clusters are in proximity to nerve endings, which were also mainly found beneath the epithelium. These results are in line with what has been described in the literature; the innervation of the intestinal epithelium plays a crucial role modulating its function, including intestinal permeability (50). One of the main symptoms reported by IBS-D patients is abdominal pain (51). Therefore, we wanted to characterize whether those nerve-endings nearby antibody-producing cells were positive for TACR1. TACR1 is the receptor with highest affinity to bind SP, which is involved in nociception (52), and CD38+ was used as an antibody-producing cell marker, as plasma cells and plasmablasts express high levels of this protein (53). We found TACR1 in proximity to cluster of CD38+ cells, suggesting these nerve-endings may be responding to SP secreted by antibody-producing cells. Despite this, conclusions from these results must be taken with caution. First of all, the complexity of the stainings did not allow us to perform a quantitative analysis; we conducted a qualitative assessment of PGP9.5+CD138+ and TACR1+CD38+ expression, obtaining no differences between HV and IBS-D groups. It has been published that the levels of SP are higher in IBS-D group (54) in rectal biopsies. We detected the neuropeptide receptor, TACR1, not SP, what may explain the controversy in the results. Second, it would be interesting to assess the levels of SP in this segment of the small bowel mucosa. SP can be produced by several neuroendocrine populations, even the same mucosal nerve fibers can express the receptor and release SP simultaneously (46,47,52); therefore, more experiments are needed to know the source of this neuropeptide and to confirm the effect of receptor activation/inhibition. However, this is the first study approaching this neuro-immune crosstalk in the jejunal mucosa in IBS-D, what can provide us with further insight into the role of gut-brain axis dysregulation in this disorder.

Mucosal plasma cells located in close proximity to nerves in the IBS-D group, with enlarged cytoplasm and expanded endoplasmic reticulum cisterna, as previously reported (12). Interestingly, this proximity inversely correlated with acute stress and depression levels described by patients. Hence, as proximity increases, the severity of symptoms rises. These results confirm that neuro-immune modulation is present in IBS. Other immune populations have been described to be in close proximity with nerve-endings in this syndrome, such as mast cells in the colonic mucosa (55).

A complete characterization of these neuro-immune interactions is needed to better define the underlying mechanisms associated with IBS pathophysiology. Identification of the neuropeptides mediating those interactions and the antigens eliciting such immune could help us to find therapeutic targets for this disorder**.**

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CHAPTER 2

7. The transcriptomic profile of the jejunal mucosa in diarrhea-predominant Irritable Bowel Syndrome reveals humoral response as a distinctive molecular feature.

7.1. INTRODUCTION

Irritable bowel syndrome (IBS) is a frequent chronic gastrointestinal disorder whose genetic pattern heritability has not been established yet. Its high prevalence among the population, together with the unclear mechanisms concerning the pathophysiology of this syndrome, entails the necessity of a deeper understanding regarding IBS specific gene-expression signature.

The study of the transcriptome is commonly used to address the differences in gene expression between health and pathology. This approach is notably useful considering the knowledge we can obtain referring to which proteins are affected in the disease, besides the molecular pathways involved, which modulate protein expression and activity. Therefore, transcriptomic analysis is a potent tool to contribute to the identification of the pathophysiological mechanisms of a certain disease. However, when talking about protein function, many mechanisms must be taken into account: not only post-transcriptional changes, which alter the final protein product, but also post-translational processes, such as modification of specific protein residues or protein diffusion and shuttling (1). Hence, transcriptomic studies are especially powerful when are integrated with other experimental techniques, leading us to a better comprehension of the regulatory and/or pathological mechanisms playing a role in the disease. Therefore, identifying a transcriptomic risk pattern for a disease is, although challenging, an optimal strategy to perform.

Some studies analyzing the transcriptome of the intestinal mucosa of IBS patients have been performed with different techniques, mostly using microarrays (2–6) but also RNA-seq (7,8). Both, the integrity of the intestinal epithelium and the differential immune activity pattern, potential cornerstones of IBS pathophysiology, have been found to be altered in transcriptomic analysis. Several authors reported a dysregulation of genes involved in the barrier function of the small bowel mucosa (5,8,9). These findings have been also confirmed at the protein level, resulting in increased paracellular permeability due to ultrastructural abnormalities in the apical junctional complex of the intestinal epithelium (5–7,9).

Changes in the transcriptome of IBS patients referring to host defense mechanisms include a hundred of genes associated to pro-inflammatory conditions, mucosal inflammation and immunity (3,9,10). Transcriptomic analysis describes an altered immune activation, antigen presentation pathways, humoral response and NF-kβ signaling (4,6,8,9). The differences in the intestinal mucosal transcriptome in IBS may also affect immune response indirectly, such as the reactive oxigen species (ROS) production regulated by an increased expression of dual oxidase 2 (DUOX2) (2,11), or directly, as is the case of upregulated germline transcripts and

immunoglobulin genes in IBS-D patients (4). Toll-like receptors (TLR) are located in the intestinal epithelium and constitute fundamental components of the mucosal innate response. Differences in mRNA TLR4, 5 and 9, receptors for lipopolysaccharide (LPS), flagellin and CpG oligonucleotides, respectively, have been also identified as differentially expressed in IBS (12).

Despite the number of studies addressing the role the small bowel in IBS pathophysiology has increased notably in the last decades, there is still a lack of research focusing on the contribution of the jejunum in this disorder, due to its very limited access through endoscopy. The jejunum presents a dynamic and highly developed *lamina propia* associated immune system, one of the most active of the gastrointestinal tract, being crucial in oral tolerance and intestinal homeostasis. These features, together with its morphological and ultrastructural composition, consisting of villi and microvilli, which determines anion and solute-absorption function and intestinal permeability characteristic of all the small bowel mucosa, highlights the jejunum as a key segment to contribute to . The objective of this study is to confirm and further analyze the transcriptome of the jejunal mucosa, specifically focusing on defensive genes associated with the immune activity, to further understand the contribution of the intestinal antibody-mediated response to IBS pathophysiology.

7.2. EXPERIMENTAL DESIGN

A prospective observational study was designed in order to: (1) determine those genes upregulated in the jejunal mucosa of IBS-D group (2) establish the immunological enriched pathways in the IBS-D patients compared to Healthy Volunteers (HV).

Jejunal mucosal biopsies and clinical assessment were obtained from all participants (Figure 1). Subjects were classified into IBS-D or HV group, according to their clinical features (see “Participants” section below) supervised by a team of gastroenterologist from the Digestive Unit (Vall d’Hebron Barcelona Hospital Campus, Barcelona, Spain). All samples were coded and blindly analyzed. The gene expression analysis was performed by 2 approaches to:

(1) Determine those genes upregulated in the jejunal mucosa of IBS-D group

A Differential expressed gene (DEG) analysis was performed after the normalization of the results obtained with RNA-seq. Once identified the upregulated genes in the intestinal mucosa of IBS-D patients, a gene enrichment analysis (GEA) from DEG results was carried out to determine whether any pathway was over/under-expressed compared to HV.

(2) Establish the immunological enriched pathways in the IBS-D patients compared to HV

A second approach was performed to study the immunological enriched pathways in the jejunal mucosa of the IBS-D group. Normalization of the raw data obtained with RNA-seq was followed by, a Gene set enrichment analysis (GSEA) considering all data, not only the differentially expressed genes, hence increasing the resolution of the method.

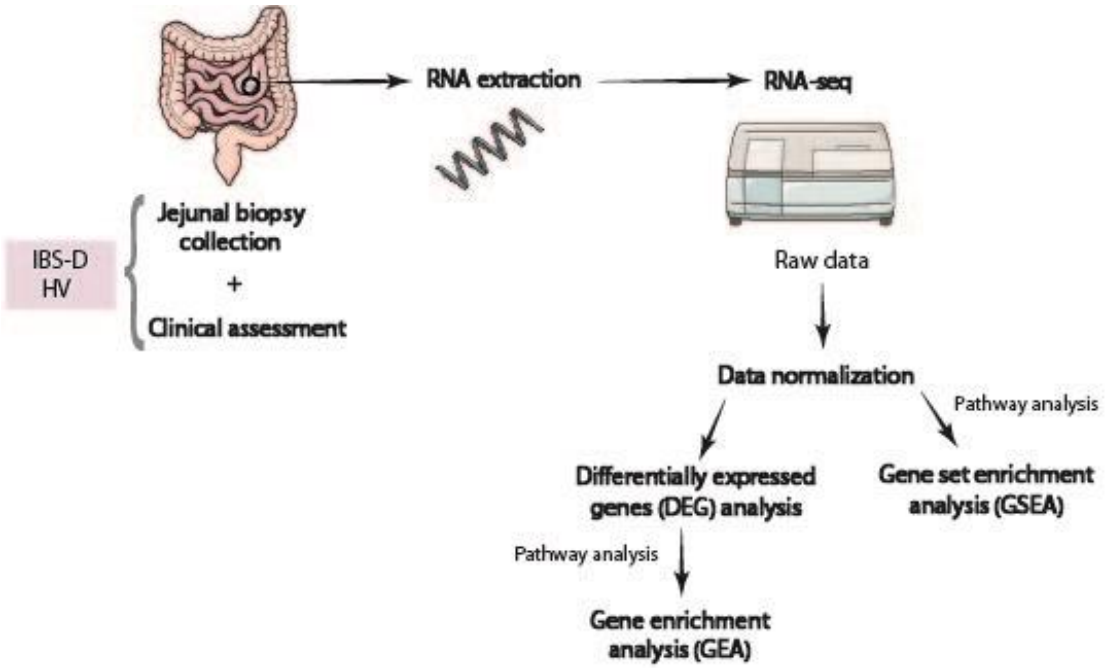

Figure 1: Experimental design. Jejunal biopsies from IBS-D and HV were collected and RNA was extracted. Raw data obtained from RNA-seq analysis was normalized using Variance Stabilizing Transformation method. Two different approaches were performed. First, the differentially expressed genes study that allowed to determine whether any pathway was upregulated by Gene enrichment analysis. The second approach was performed using a matrix with all the gene sets of interest and carrying out a pathway study by Gene set enrichment analysis. Clinical assessment from all participants was obtained for this study. IBS-D: Diarrhea-predominant Irritable Bowel Syndrome; HV: Healthy Volunteers.

7.3. METHODS

7.3.1. Participants

Newly diagnosed diarrhea-predominant Irritable Bowel Syndrome (IBS-D) patients and healthy volunteers (HV) were recruited from the outpatient gastroenterology clinic of Hospital Vall d'Hebrón (Barcelona, Spain) and the general population by public advertisement, respectively. The team of gastroenterologists evaluated all participants and ensured IBS-D patients fulfilled the Rome III criteria to be included in the study. A complete clinical assessment using a structured questionnaire for both IBS-D patients and HV was carried out, excluding all those subjects who presented other gastrointestinal pathologies or food allergies. Food allergy was evaluated by a battery of skin prick test (SPT) (Laboratorios Leti SA, Barcelona, Spain) for 22 common food allergens, using histamine as positive control and saline as a negative. Subjects underwent SPT previous obtaining of the jejunal mucosal biopsy and results were analyzed by an allergist. Positive reaction to SPT or clinical history associated to certain food exposure (including both digestive and extra-digestive symptoms) were considered exclusion criteria. All HV who entered this study did not present any digestive symptoms (including abdominal pain, distention or dyspepsia). Biochemical and serological tests were used to assess previous episodes of infectious gastroenteritis as well as other gastrointestinal pathologies. Table S1 in the supplementary data summarized inclusion and exclusion criteria; Table S2 details the clinical and demographical data of each participant.

Participants reported in clinical questionnaires digestive and psychological symptoms daily during 10 days previous to the collection of the jejunal mucosal biopsy. IBS-D participants completed a severity scoring system questionnaire (Francis Score), composed of 5 questions which evaluated severity and frequency of symptoms. A prompted visual analogue scale (VAS) was used to assess pain and distention severity, in order to avoid categorization of symptoms with continuum values. The following symptoms were reported: 1) pain severity (by a 100-point VAS) (13); 2) pain frequency (number of days with pain) (13); 3) stool frequency (maximum number of bowel movements); 4) stool form (by the Bristol Stool Chart score)(14); and 5) distention, using a 6-point VAS (13).

Depression and psychosocial stress, chronic and acute, were recorded using the validated Spanish versions of the Beck's Depression Inventory (15), the Modified Social Readjustment Scale of Holmes-Rahe (16) and the Perceived Stress Scale of Cohen (17), respectively. Written informed consent was obtained from each participant. All procedures were developed in

accordance with the ethical standards and with the Helsinki declaration and the study protocol was approved by the Ethical Committee of the Hospital Vall d´Hebron (PR(AG)211/2018).

7.3.2. Collection of jejunal biopsies

Jejunal mucosal biopsies obtained from IBS-D and HV were collected using a Watson's capsule as previously described (18). For sample obtaining, participants underwent overnight fasting and were orally intubated. Watson's Capsule was introduced and positioned to the proximal jejunum, 10 cm distal to the angle of Treitz's. Monitoring of the capsule position was performed under fluoroscopic control. Biopsy, obtained by suction with a 50-mL syringe, was divided in two pieces. One fragment was processed for histological analysis by a pathologist from the Pathology Department (Vall d'Hebron Barcelona Hospital Campus, Barcelona, Spain), in order to discard other pathologies. The remaining piece was immediately placed in RNAse-free tubes, embedded in 500µL of RNA Later Solution (Life Technologies) and stored at −80°C until processed for RNA extraction.

7.3.3. Analytical procedures

7.3.3.1. RNA extraction

RNA isolation from jejunal biopsies, frozen at -80ºC and immersed in RNA Later Solution, were lysed in 1mL of TRIzol Reagent (ThermoFisher Scientific) using FastPrep (MP Biomedicals). Sample processing was performed according to manufacturer's instructions. Quantity and quality of total RNA, obtained from the aqueous phase after being filtered and eluted through miRVANA columns, was analyzed by capillary electrophoresis (Agilent 2100 Bioanalyzer; Agilent Technologies). Samples showing a low RNA integrity number (RIN) were excluded from the study (Table S3 in Supplementary Data) and 20 subjects from each group, IBS-D and HV, were blindly selected for RNA-seq procedures.

7.3.3.2. RNA-seq analysis

mRNA expression from 40 selected samples were analysed by RNA-seq on an HiSeq 2500 at the CRG-Genomics Unit (Barcelona, Spain). RNA-seq raw data was sent to Bioinformatics Unit from Bellvitge Biomedical Research Institute (Bellvitge, Spain) for quality control, basic alignment statistics and further pathway and functional analysis. Quality control of the samples was performed using FASTQC tool (version 0.11.9) (19). All individual FASTQC reports were merged in a html file through MultiQC tool (20). RNA-seq raw reads presented good quality for downstream analysis. All samples had sequences of a single length (75bp) and the sequencing depth ranged between 5.8M and 23.10M (in average 15.7M).

STAR aligner tool (version 2.7.2b) (21) via the Galaxy Project (22) was used to align raw data to reference genome (hg38), downloaded from Ensembl: ftp://ftp.ebi.ac.uk/pub/databases/gencode/Gencode_human/release_33/.

A general exploration of the phenotypic data was conducted by using In-house R-scripts, to explore characteristic patterns and identify potential undesired effects. The exploratory analysis was divided into qualitative (sex, atopy and functional dyspepsia (FD)) and quantitative variables (age and Francis, Holmes-Rahe, abdominal pain, bowel movements and Bristol scores). The following analysis were inspected: a Principal Component Analysis (PCA), a hierarchical clustering based on Pearson correlation coefficient and read counts distribution visualized in violin plots. Stripcharts for every quantitative variable, contingency tables for qualitative variables and scatterplots of every combination of two quantitative variables were conducted. Data normalization was performed by applying the Variance Stabilizing Transformation (VST) and cluster robustness was assessed by means of a hierarchical clustering, implementing Pvclust CRAN package (v2.2.0) (23). A p-value per cluster was obtained indicating how strong the cluster was supported by the data.

7.3.4. Pathway and functional analysis

Two types of analysis were performed:

(1) Differentially expressed genes (DEG) and corresponding enriched biological processes analysis based on RNA-seq data associated with IBS-D.

(2) Gene set enrichment analysis (GSEA) based on the normalized expression data to uncover the enriched biological pathways in IBS-D patients when compared to HV.

7.3.4.1. DEGs

After the exploratory analysis, a generalized linear model (GLM), using DESeq2 Bioconductor package (v1.24.0) (24), was adjusted considering Sex and Group (IBS-D or HV) factors, to detect differential expression between phenotypes. Raw p-values were adjusted for multiple testing using the Benjamini and Hochberg False Discovery Rate (FDR) (25). We considered DEGs when FDR was lower than 0.05.

To put in biological context the obtained DEGs, a gene enrichment analysis (GEA) was conducted. GEA was computed over Kyoto Encyclopedia of Genes and Genomes (KEGG) PATHWAY, Reactome and GO databases (26–28). For each queried biological pathway or GO term, a p-value was calculated using a hypergeometric distribution test. Multiple testing

problem was controlled by FDR. FDR lower than 0.05 was considered as statistically significant. The background distribution was defined by all available annotations in the relevant database. GEA was conducted with clusterProfiler Bioconductor package (v3.12.0) (29).

7.3.4.2. GSEA

A functional analysis by Gene Set Enrichment Analysis (GSEA) was performed using the gene expression matrix approach. Raw counts from the RNA-seq data were normalized by applying the VST and genes were mapped using the Ensembl identifiers for running the GSEA. The matrix was constructed based on a Gene Cluster Text (GCT) file based on the normalized counts [gct] and a Categorical Class (CLS) file, using the metadata provided by the researcher [cls], which defines IBS-D and HV phenotypes. Gene sets were downloaded from the collections of the Molecular Signatures Database (v7.0 MSigDB), both the Gene Matrix Transposed (GMT) files, associated to the gene sets of interest, and The CHIP file, with the annotation matching the Ensemble IDs [chip]. 4872 gene sets were used in the analysis. Enrichment was considered significant when the FDR was < 25%.

7.4. RESULTS

7.4.1. Study population

The study included 20 HV participants and 20 IBS-D patients (Table 1 and Supplementary Table S2). There were no significant differences in the distribution of groups by sex neither in the percentage of atopy (p=0.281). The age of participants was different in each group (p< 0.0001), therefore, age was not further included as a variable in the functional and pathways analysis. As it has been described previously in the literature, depression levels, measured with Beck's Inventory, are higher in IBS-D group compared to HV (p< 0.0001). However, no differences were observed in the level of psychological stress, as assessed by Cohen's (p= 0.116) and Holmes-Rahe (p=0.203) questionnaires. There was a significant difference in the number of bowel movements (p< 0.0001) and the stool consistency (p< 0.0001) between groups.

	HV (n=20)	IBS-D (n=20)	p-value
Gender, F:M	12:8	12:8	> 0.9999
Age, years	22 (18-30)	34 (20-69)	**< 0.0001***
Atopy, yes: no	3:12	8:12	0.281
Intensity of the abdominal pain score	-	52.25 ±6.05	-
Bowel movements (n)	1.5 (0.5-2.5)	3 (1-6.5)	**< 0.0001***
Stool form, Bristol score	3.5 (2.5-5)	6 (4.5-6.5)	**< 0.0001***
Dyspepsia, yes:no	-	9:10	-
Francis	-	291 (0-438)	-
Holmes-Rahe Scale	77 (13-229)	143 (5-770)	0.203
Cohen Scale	16 (8-22)	22 (7-50)	0.116
Beck's Depression Inventory	0 (0-0)	6 (1-30)	**< 0.0001***

Table 1: Clinical and demographic characteristics of participants. Results with a normal distribution are expressed as the mean ± standard error of mean and non-parametric data as median (minim –maximum values). * symbol highlights values with significant statistical differences between groups ($p<0.05$). HV: Healthy Volunteer; IBS-D: diarrhea-predominant Irritable Bowel Syndrome; FDR: False-Discovery Rate; n: sample size.

7.4.2. Quality control of the samples

All samples were successfully aligned to the human reference genome (hg38) showing percentages of uniquely mapped reads between 84.3% and 94.1%. A complete gene annotation matrix was obtained from the .gtf annotation file which referred to 60,721 different genes by an Ensembl ID. Reads were mainly distributed in exons (coding sequence exons 61.1%-75.7%). Therefore, the percentage of assigned reads for gene quantification (counts matrix) was high (87.2% - 94%). Those genes with less than 10 reads among all samples were removed from the analysis. This led to a final raw counts matrix referring to 27,833 genes.

Three out of the 40 samples were discarded for DGE analysis based on the exploratory analysis result (Supplementary Figure S1). The final dataset included 18 samples from IBS-D and 19 samples from HV. A set of 27,669 genes were available for DGE analysis. Differences in age

between both groups impaired including this variable in the model for DGE analysis (Supplementary Figure S2).

7.4.3. Genes differentially expressed in the jejunal mucosa of IBS-D patients

DEGs was assessed in IBS-D mucosal jejunal samples compared to HV, considering FDR<0.05 as statistically significant. Fifty-seven 57 genes were found to be differentially expressed (Supplementary Table S4). An enrichment analysis (GEA) was performed to evaluate the presence of overrepresented biological pathways, but no significant results were obtained. Ninety-three per cent of the DEG are co-expressed or do not present any type of interaction (Figure 2), while a 7% showed a direct or physical interaction. Then, an enrichment analysis without considering on DEGs but all genes grouped in gene sets (GSEA) was performed to find potential up-regulated or down-regulated pathways in IBS-D compared to HV. GSEA allowed to detect smaller differences than GEA.

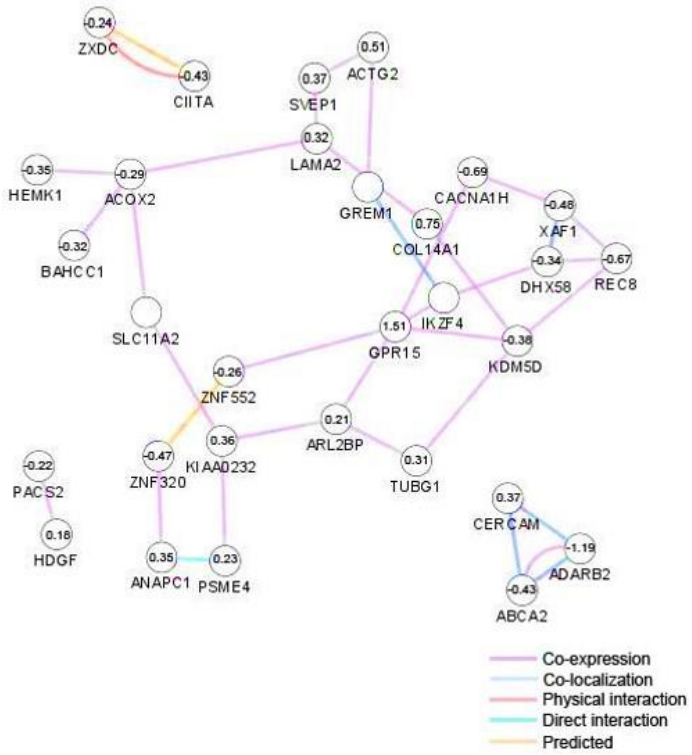

Figure 2: DEGs interactions when comparing IBS-D and HV groups. Color code indicate the type of interaction: co-expression, co-localization in the cell, physical interaction, direct interaction (identified to belong to the same pathway) or predicted interaction using Cytoscape v3.8.0. Log2 Fold Changes values are indicated inside the nodes. Those genes not presenting any interaction with other DEGs do not appear in the figure. A complete list of DEGs with log2 Fold Change and adjusted p-values are detailed in the Supplementary Data (Table S4).

7.4.4. B cell signaling is enhanced in IBS-D group compared to health

A GSEA analysis was conducted and gene sets representing cell types, states and alterations within the immune system (immunological signatures) were found to be overrepresented in the IBS-D group. Previous studies from our group demonstrated changes in antibody-mediated response of intestinal mucosa of IBS-D; a more detailed analysis enriched the immunological signature and allowed to identify that humoral response gene sets presented significantly higher gene expression levels in patients (Table 2). Immunological signatures involved in plasma cell activity were enriched, including upregulation of genes involved in antibody-producing cells than in naïve B cells from blood, memory B lymphocytes from spleen or memory IgM B cells isolated from blood and bone marrow.

Results also suggested an enhanced germinal center activity (Table 2): genes from pre-germinal B cells and germinal center B cells were upregulated compared to blood naïve B cells and dark zone germinal center cells, respectively. The analysis also showed an enrichment of lymphoid-primed multipotent progenitors, more specifically of B cells versus other immune cell populations (monocytes, macrophages and neutrophils). Dendritic cells, which originate also from lymphoid-primed multipotent progenitors, were promoted compared to B cells.

Other immunological signatures showed a predominance of antibody-producing cells from blood compared to memory B cells, regardless of being IgG+, IgA+ or IgM+ (Figure 3). Despite these gene sets are not statistically different (FDR= 0.4682 for IgM and FDR= 0.3133, for IgG and IgA), they belong to the 10% of immunological signatures with highest degree of overrepresentation in the IBS-D group, measured by the enrichment score (ES) (ES= 0.4287 and ES=0.4175, respectively). These results provide more evidences on the key role played by the antibody-mediated response in this syndrome.

Immunological Signature	Expression	FDR	ES
Plasma cell activity			
Naïve B cells versus blood antibody-producing cells	Down-regulated	0.2003	0.4939
Plasma cells versus memory B lymphocytes from spleen	Up-regulated	0.2274	0.5247
Memory IgM B cells versus antibody-producing cells from bone marrow and blood	Down-regulated	0.2264	0.4549
Germinal center activity			
Naïve B cell *versus* pre-germinal tonsil B cells	Down-regulated	0.2295	0.4634
Pre-germinal center B cells *versus* dark zone germinal center B cells	Down-regulated	0.2325	0.3345
Naïve B cell *versus* dark zone germinal center B cells	Down-regulated	0.2374	0.3478
Naïve follicular B cells *versus* early germinal center B cells	Down-regulated	0.2382	0.3494
Germinal center B cells *versus* naïve B cells	Up-regulated	0.2446	0.3938
Predominance of B cells			
Lymphoid-primed multipotent progenitors *versus* granulo-monocyte progenitors	Down-regulated	0.2464	0.3021
Naïve B cells *versus* dendritic cells	Down-regulated	0.2468	0.2942
B cells *versus* monocyte macrophages	Up-regulated	0.2481	0.2576
Neutrophils *versus* B cells	Down-regulated	0.2248	0.3753

Table 2: Representative selection of enriched immunological signatures in IBS-D by GSEA. Gene sets from the IBS-D phenotype were compared to immunological signatures published in the literature. Gene set expression from different immune population were confronted (e.g: population A vs B); when up-regulated expression is indicated, these gene set expression is higher in population A compared to B, and inversely when is down-regulated. Completed list with MiSigDB Standard Name is detailed in Supplementary Data (Supplementary Table S5). Significance of these enrichment and degree of overrepresentation are indicated with FDR and ES values, respectively. FDR: False-Discovery Rate; ES: Enrichment Score.

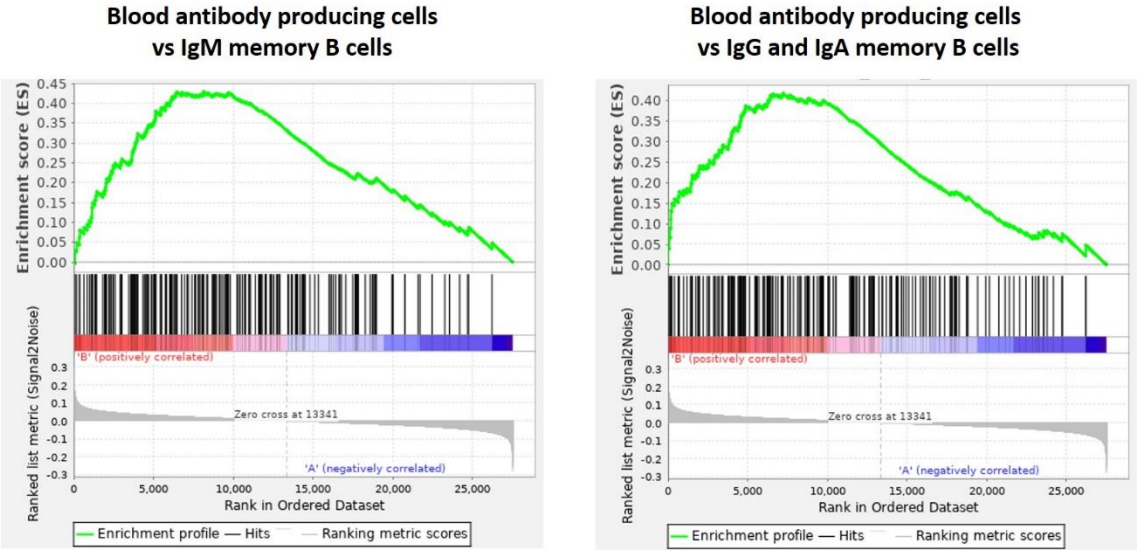

Figure 3. Representative enrichment plots of immunological signatures involved in humoral response activation with high Enrichment Score (ES) obtained by GSEA when comparing IBS-D versus HV. In the top portion of the plot, ES for those genes belonging to the gene set are represented. Special attention must be given to the distinct peak of the curve, which indicates the ES of the selected gene set and therefore, the overrepresentation degree of that immunological signature. In the middle portion, black lines represent genes from this gene set, distributed according to their position in the ranked list of genes. Genes contributing the most to the gene set ES were called leading edge subset; for positive ES were those genes ranked prior to the peak. In the bottom portion of the plot, correlation of genes with IBS-D and HV, measured by ranking metric scores, are represented. In this case, positive ranking metric score indicates there is a correlation with IBS-D profile (B) and negative values negatively correlates with HV group (A).

7.4.5. Expression of immunoglobulin structure genes are enriched in IBS-D patients

Confronting the top 50 genes with highest ES in IBS-D patients against HV (Figure 4 and Supplementary Table S6), the importance of humoral immune defense can be noted. When evaluating the differences between both profiles, we observed 58% of top 50 enriched genes in IBS-D were involved in the Ig structure (Figure 5). Despite these genes are not statistically significant (FDR<0.25), interestingly 93% of them encode for the antibody variable region (67% encodes for light chains and 33% for heavy chains). Of the two types of light chains (kappa and lambda), higher number of genes encoded for lambda were up-regulated compared to kappa light chains (67% versus 33%, respectively) and also presented higher ES. The Ig isotype is determined by the constant region of heavy chains; interestingly, genes determining IgG2 and IgG3 isotypes were also in the top 50 enriched genes in IBS-D.

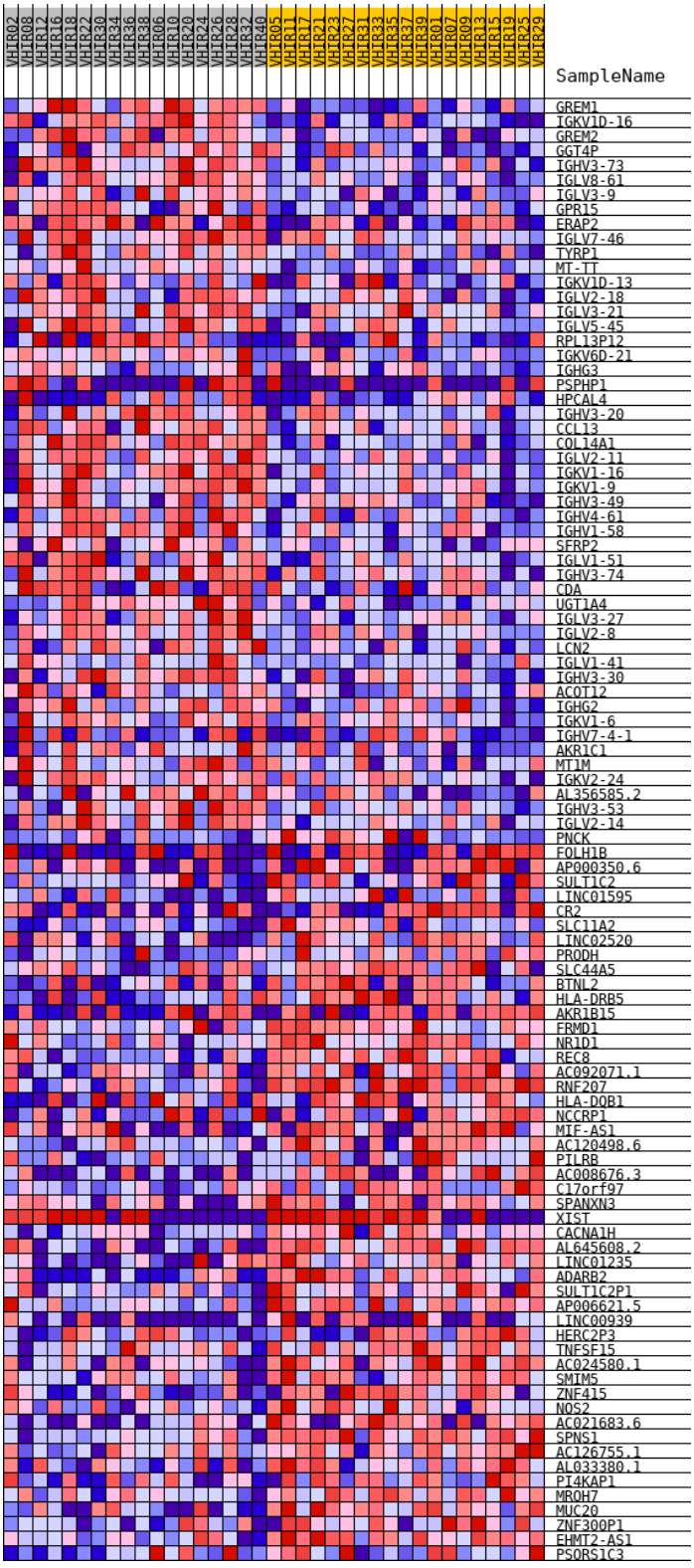

Figure 4. Heat map of the top 50 enriched genes for both phenotypes (IBS-D versus HV) obtained by GSEA. IBS-D subjects are highlighted in grey and HV in yellow and the name of the differentially expressed genes are indicated on the right hand-side of the heat map. Up-regulated and down-regulated genes are in red and blue colors, respectively. HV: Healthy Volunteer; IBS-D: diarrhea-predominant Irritable Bowel Syndrome.

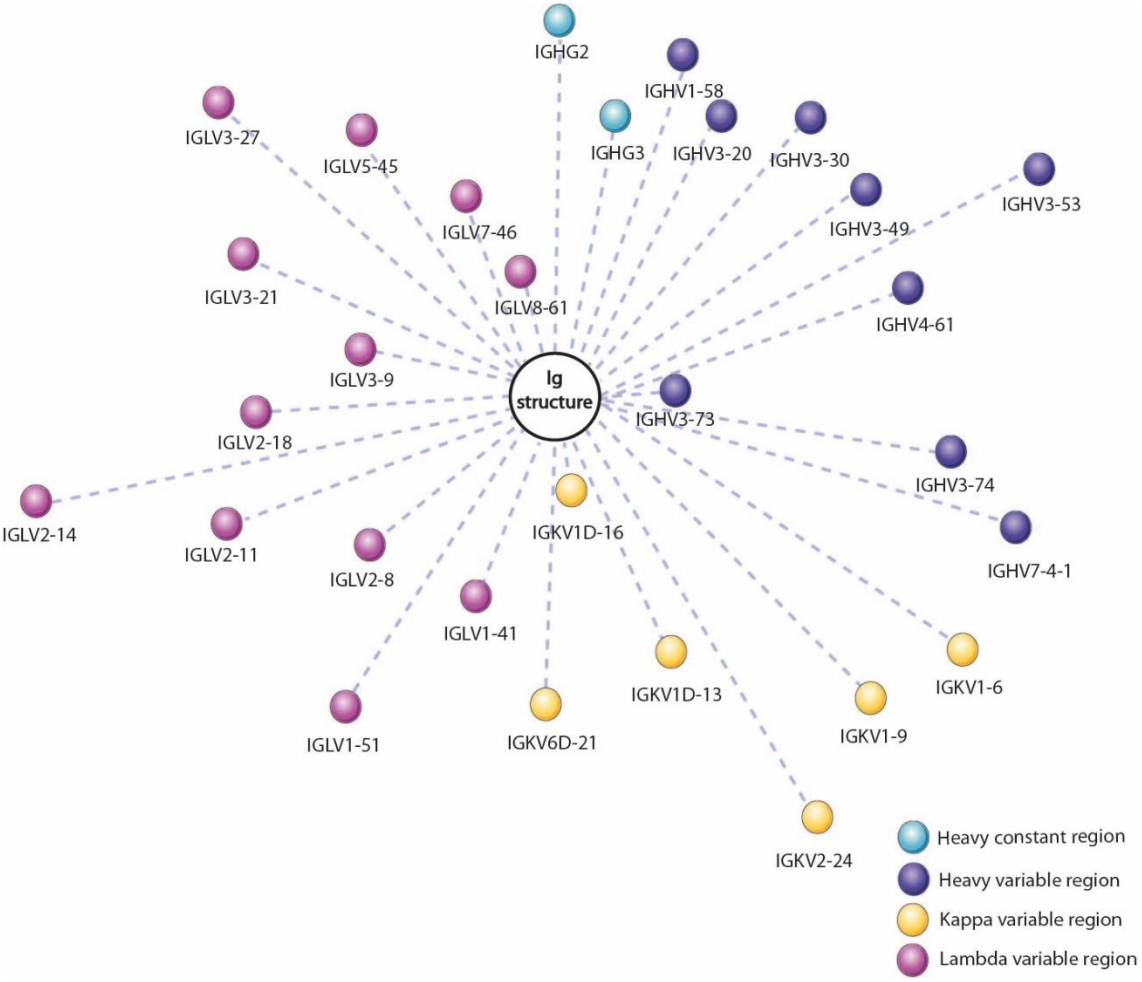

Figure 5. Representation of those genes associated with immunoglobulin (Ig) structure found in the top 50 enriched genes in IBS-D compared to HV by GSEA. Genes with higher Enrichment Score (ES) in patients are closer to the central node. HV: Healthy Volunteer; IBS-D: diarrhea-predominant Irritable Bowel Syndrome.

7.4.6. B cell receptor signaling and modulation of intestinal permeability pathways are overrepresented in IBS-D

Gene sets belonging to canonical pathways were identified by the Reactome pathway database. B cell receptor (BCR) signaling (FDR= 0.193, ES= 0.712) together with downstream signaling events triggered upon the receptor activation (FDR= 0.155, ES= 0.537) are up-regulated in the IBS-D group, including activation of NF-kβ in B cells (FDR= 0.148, ES=0.584). Antigen-mediated response was also enriched, including second messengers induced by antigen-triggered BCR activation (FDR= 0.212, ES= 0.865), Fc-gamma-receptor-dependent phagocytosis (FDR= 0.194, ES= 0.741) and antigen processing by ubiquitin-proteosome degradation (FDR= 0.214, ES= 0.236).

Uncontrolled access of antigens to the *lamina propria* of intestinal mucosa of IBS-D patients due to loss of barrier integrity is one of the leading hypothesis in the pathophysiology of this disorder. Pathways involved in intestinal barrier function, such as tight junction interactions (FDR= 0.2418, ES= 0.5168) and gap junction assembly (FDR= 0.2375, ES= 0.5535) and trafficking and regulation (FDR= 0.2402, ES= 0.4756) have been identified.

7.5. DISCUSSION

This transcriptomic analysis builds-up on our previous RNA-seq analysis conducted on jejunal biopsies from IBS patients. In the present study, we found a lower number of DEG compared to our previous study (57 DEG versus 3806 DEG (8). Several changes in the methodology conducted in this analysis may explain these differences: including a higher number of participants, longer RNA transcripts (75pb versus 50pb) and performing paired-end (two directions) RNA analysis, instead of a single-end study. These results must be validated by qPCR to exclude the possibility these results have been generated by chance. Due to time limitations, this validation could not be performed but it will be addressed in the near future. It is also must be noted, a single-jejunal biopsies was obtained from each participant due to the biopsy collection method used to reach jejunum (Watson's Capsule). Immune infiltration in the intestinal tract is discontinuous, therefore, obtaining a single biopsy may not be representative enough and may influence our results.

After identifying those DEG in the IBS-D group, we proceeded to evaluate whether these genes belong to any biological route but unfortunately, we could not find any overexpressed pathway by GEA. DEG are involved in multiple routes of cell signaling, without physical or direct interaction with the other DEG. Evaluating the potential over-enriched pathways only considering interaction between DEG has a notable limitation and slight differences between both groups cannot be detected. In order to overcome this problem, we conducted a more complete second analysis (GSEA), which comprises all genes organized as gene sets, increasing the resolution of the analysis, and studied the alterations in biological routes.

GSEA revealed those gene sets associated with immune pathways (immunological signatures) were enriched in IBS-D group as compared to HV. Those immunological signatures with FDR<0.25 were considered statistically different. This FDR cutoff was established considering GSEA and MSigDB Team recommendations (30); using a lower threshold may lead to overlook potentially significant results. Among the enriched immunological signatures, those involved in humoral defense are highlighted. Upregulated genes in antibody-producing cells are prominent

when we compared them to populations from B cell ontogeny, such as naïve or memory B cells. These results suggest not only B cell activation is promoted but the differentiation into plasmablasts and plasma cells. These findings go in line with previous results from our group, where DEG obtained from microarray belonged to humoral immune functions and an increased number of plasma cells in the jejunal mucosa of IBS-D patients was also reported (4). Interestingly, GSEA results show germinal center activity is increased, therefore it points out generation of antigen-specific-antibody-producing cells is promoted. Not only antibody-producing cells, but immunological signatures involved in B cell ontogeny are enriched when compared to granulocyte immune cells (neutrophils, monocytes and macrophages), pointing out adaptive immunity plays an important role in this syndrome. Notably, dendritic cells are overrepresented compared to naïve B cells; their function as antigen presenting-cells may be contributing to these differences.

When we compared the gene set expression of IBS-D *versus* HV, we obtained those genes with the highest ES in each group. ES gives us information about the overrepresentation degree of a gene in a gene set. Fifty-eight per cent of the top 50 genes with highest ES in IBS-D are involved in Ig structure, considering the background of this study, this result must receive special attention. The variable region is the most overrepresented among these genes. This part of the Ig is crucial in playing a role in the generation of antigen-specific antibodies, hence these results show again an upregulation of antigen recognition routes in IBS-D group. Genes associated with lambda light chain isotype, predominantly in mucosal antibodies (31), constitutes a higher percentage of enriched genes compared to those codifying for kappa light chain. These findings reinforce the hypothesis the activation of humoral defense in these patients is not a systemic but a local response.

The constant region of heavy chain genes of Ig, which determines the Ig isotype, also appeared in the upper portion of ES ranked list of genes, more specifically IGHG2 and IGHG3, codifying for IgG2 and IgG3, respectively. Increased levels of IgG at a protein level in IBS-D patients have been reported in the literature (4,32,33), but without specifying the IgG isotype. At gene expression level, Ig germline transcripts for IgG1 and IgG2 and heavy constant chain genes for IgG1 e IgG4 have been described (4). Interestingly, IgG2 and IgG3 were found at higher levels in stool supernatant from IBS-D group compared to HV (results detailed in 'Chapter 1'). Knowing the Ig isotype increased in this disorder is a way to approach the responsible antigen(s) of humoral defense activation in the intestinal mucosa of these patients. Increased LPS content and viral infection, associated with IgG2 and IgG3 responses, have been identified to be involved in IBS pathophysiology (12,34–36), but nothing has been established so far.

GSEA results show enriched gene sets in IBS-D group related to canonical pathways derived from the reactome database. Likewise, the results obtained by immunological signature analysis, routes involved in B cell immunity are enriched. Signaling by the BCR and all the cascade signaling triggered upon antigen recognition (e.g: Fc-gamma-receptor-dependent phagocytosis or antigen processing by ubiquitin-proteosome degradation) are up-regulated. Other authors' transcriptomic analysis revealed alterations in the immune system, including NF-kβ signaling (9), also enriched in our GSEA. NF-kβ signaling plays a role in immune and inflammatory processes in response to bacteria, viral infections and stress stimuli, among others (37). The intestinal mucosa of IBS-D patients does not present a proper inflammation, but a micro-inflammation characterized by mast cells and T and B lymphocytes mainly (38). All these results, taken as a whole, highlight the importance of adaptive immune activity in the intestinal mucosa of these patients, especially the antibody-mediated defense.

Transcriptomic results observed in IBS-D together with pathways analysis by GSEA revealed a differential expression of gene sets involved in the integrity of the intestinal epithelium (tight junction interactions, gap junction assembly, trafficking and regulation). It has been already been reported an alteration in the gene expression of proteins involved in small bowel barrier function, specifically those taking part in the apical junctional complex by our group (5,8). Interestingly, this differential expression in IBS patients correlated with bowel dysfunction. Our previous study showed how an alteration of apical junctions, and therefore, a loss of the barrier integrity due to an increased paracellular permeability in the small intestinal mucosa of IBS-D patients (5,8). Notably, genes and proteins playing a role in the formation of tight and adherens junctions, which belong to apical junctional complex structures, have been described to be dysregulated all along the intestinal mucosa (5–7,9). GSEA canonical pathways identified in the IBS-D group reinforces the leading hypothesis of antigens from the intestinal lumen crossing the epithelium through an uncontrolled way, due to a damaged barrier function, triggering immune populations residing in the *lamina propria*.

Results obtained from the bioinformatics analysis demonstrated the need for research in molecular mechanisms associated to IBS-D to better define the pathophysiology and identify biomarkers and potential therapeutic targets. The genes and pathways found to be overrepresented in this study must be validated to confirm their role in this disorder. Working with an antibody-producing cell in vitro model could be a good tool to achieve a deeper knowledge on how Ig production is affected in response to several antigens and stimuli, and therefore better understanding IBS-D pathophysiology.

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CHAPTER 3

8. B cells and plasma cells as a tool to study neuro-immune crosstalk: evaluation and selection of an *in vitro* model.

8.1. INTRODUCTION

Irritable Bowel Syndrome (IBS) is a disorder of the gut-brain axis in which recurrent abdominal pain is associated with defecation or a change in bowel habits (1). The pathophysiology of IBS is yet to be described, however, scientific evidence showing alterations in the intestinal mucosa (micro-inflammation, epithelial dysfunction) (2–5) associated with symptom severity suggest the existence of active peripheral responses that promote and maintain gut dysfunction. Additionally, the high prevalence of psychiatric comorbidities (4,6) together with higher mucosal innervation associated with abdominal pain scores, as compared to health (7), also supports that IBS can be explained by biological factors. Several neuropeptides have been described to play a role in this syndrome, including VIP (8), CRF (9) or SP (7,10,11). SP is one of the most studied neuropeptides due to its importance in the digestive system; it is released in high concentrations in the intestinal mucosa and its high-affinity receptor, tachykinin Receptor 1 (TACR1), also known as neurokinin 1 receptor (NK1R)), is present all along the gastrointestinal tract (12). Interestingly, in IBS, previous studies have identified higher density of nerve fibers positive for SP in the colon (10,11) and the rectum (7), the former found in close proximity to mast cells (13). IBS pathophysiology is considered a multifactorial gut-brain axis dysfunction that may also be influencing other systems, including the immune system. An increased activity of the humoral defense in IBS patients has been observed (4) and strong evidences suggest activation and differentiation of B cells residing in the intestinal mucosa may be promoted by the neuroendocrine system. It has been previously described that B cell and antibody-producing cells express TACR1 (12) and SP seems to be involved in enhancing the production of immunoglobulins (Ig) through direct and indirect pathways (14,15). This background highlight the need of studying the neuro-immune connection in IBS, more specifically the effect of SP on B cell activation and differentiation.

Selection of a suitable *in vitro* model is one of the main factors to consider when addressing an experimental design. Obtaining human antibody-producing cells has never been an easy task due to limitations such as the short life of terminally differentiated plasma cells in culture, the low yield of isolation techniques from biological samples, or the production of a specific subtype of Ig in response to in vitro-driven class-switching. Some protocols attempt to avoid the short frame time of primary cultures stimulating with mitogens (e.g. pokeweed mitogen (16)) or using immortalized B cells (17). The alternative, isolating antibody-producing cells, is also challenging. Most of the protocols described in the literature to produce them are based on a first isolation of B cells or plasmablast and evoke their differentiation in vitro, due to the low proliferation rate

of terminally differentiated plasma cells (18). Usually the source of lymphocytes are tonsil or blood, specially this last one as sample collection does not represent a limitation.

Approximately 65% of B lymphocytes circulating in peripheral blood are naïve B cells (19). Using the conditions under naïve B cells activate and differentiate in vivo is challenging. Most of the humoral activity in vivo derive from T-cell dependent responses, therefore, interleukins and other molecules provided by T helper cells (Th cells) are commonly used in experimental studies. Cytokines selected for driving the differentiation process must be chosen carefully. The pattern of cytokines secreted in a T-dependent response will determine the yield and the maturation state of the generated antibody-secreting cells, which will condition the studies resulting therefrom.

Elucidating the pathways involved in neuromodulation taking place at the intestinal mucosa might potentially benefit to those functional disorders associated to a dysfunction of the gut-brain axis. In this study, we explore different experimental conditions to better select a suitable in vitro model to evaluate the response of B cells to SP, in order to reach a deeper understanding of neuro-immune mechanisms that potentially contribute to IBS pathophysiology.

8.2. EXPERIMENTAL DESIGN

This study has approached several methodological techniques to establish a suitable and feasible protocol for the assessment of the effect of SP on activation of human B cells. Intestinal immune cells were isolated from ileocolectomy tissues from patients (ileal surgical specimens) and from HV (jejunal biopsies and blood) as primary B cell culture. B cells obtained from blood were also differentiated into plasma cells. An immortalized B cell line was also included a potential suitable in vitro model. All cells were evaluated phenotypically and functionally, in order to establish the best experimental approach. A characterization study was performed by gene expression using quantitative PCR (qPCR) and protein expression by immunofluorescence (IF) and flow cytometry. Besides the basal characterization, B cells isolated from blood (not differentiated into plasma cells) and 126BLCL cell line were also characterized after exposure to SP in a time course stimulation assay.

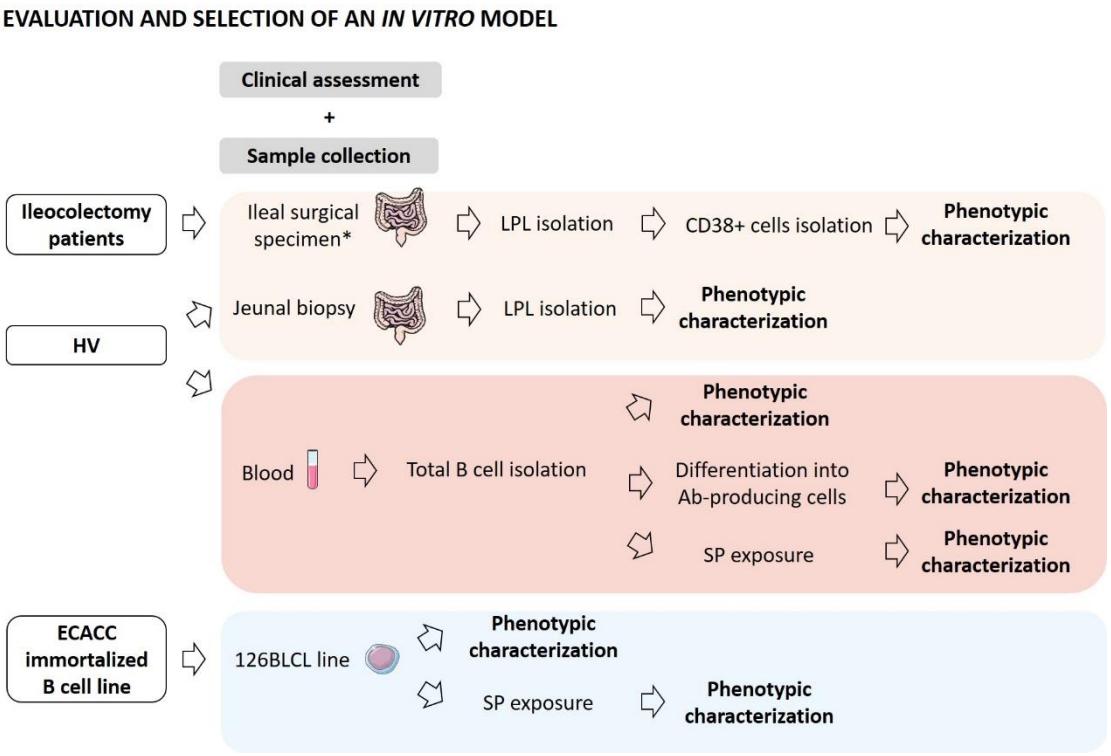

Figure 1: Experimental design conducted to evaluate and select different B cell sources to develop an *in vitro* model. Cells isolated from biological samples from participants and an immortalized B cell line were phenotypic assessed. Ab-producing cells: Antibody-producing cells; ECACC: European Collection of Authenticated Cell Cultures; HV: Healthy volunteers; LPL: *Lamina propria* lymphocytes; SP: Substance P. *Non-inflamed region.

8.3. MATERIAL AND METHODS

8.3.1. Participants and obtaining of biological samples

All participants were evaluated by a team of gastroenterologists from the Digestive Unit at Vall d'Hebrón Barcelona Hospital Campus, Barcelona, Spain. Jejunal mucosa biopsies and blood were collected from healthy volunteers (HV), recruited from the general population by public advertising. HV completed a clinical questionnaire reporting any digestive symptoms previous entering the study. All those candidates presenting gastrointestinal pathologies were excluded from the study (Supplementary Table S1 and S2). Pregnancy and food allergy were also a reason for study exclusion. The study protocol HV recruitment was approved by the Ethics Committee at Hospital Vall d'Hebron [(PR(AG)211/2018)]. Transmural ileal tissues from a non-inflamed region were obtained from patients undergoing ileocolectomy in General and Digestive Surgery Unit (Vall d'Hebron Barcelona Hospital Campus, Barcelona, Spain), informed consent was

obtained in all participants. The study was carried out in accordance with the Declaration of Helsinki.

8.3.2. B cell sources

Both primary B cell culture and immortalized B cell line were evaluated as in vitro models (Figure 2). For primary B cell culture, two different type of biological samples were compared: CD38+ cells obtained from intestinal tissue and B cells isolated from peripheral blood. The tested immortalized B cell line was a human Epstein-Barr virus (EBV) transformed lymphoblastoid cell line (126BLCL line). The purpose of 126BLCL line evaluation was to overcome the methodological limitations that working with a primary culture may involve.

8.3.3. Human intestinal B cells

Lamina propria lymphocytes (LPL) were obtained from the intestinal mucosa from two different intestinal regions, following different procedures:

1. Jejunal mucosa: A single biopsy from each participant was obtained using a Watson's capsule. Participants were orally intubated after over-night fasting, and biopsies were obtained when the capsule was located at 10cm distal to the angle of Treitz's, as previously described in the literature (19). The position of the capsule was monitored by fluoroscopic control. Biopsies were divided into two fragments: one for histological analysis, performed at the Pathology Department (Vall d'Hebron Barcelona Hospital Campus, Barcelona, Spain), and the other fragment for LPL isolation at the laboratory of Physiology and Physiopathology Digestive Unit (Vall d'Hebron Barcelona Hospital Campus, Barcelona, Spain).

2. Ileal transmural tissues were obtained from surgical specimens. Mucosa layer of non-inflamed zone of ileum was used for isolation of LPL at the laboratory of Physiology and Physiopathology Digestive Unit (Vall d'Hebron Barcelona Hospital Campus, Barcelona, Spain).

8.3.4. Immortalized B cell line

A human Epstein-Barr virus (EBV) transformed lymphoblastoid cell line, named 126BLCL line and supplied by the European Collection of Authenticated Cell Cultures (ECACC), was assayed as an in vitro model.

8.3.5. Experimental procedures

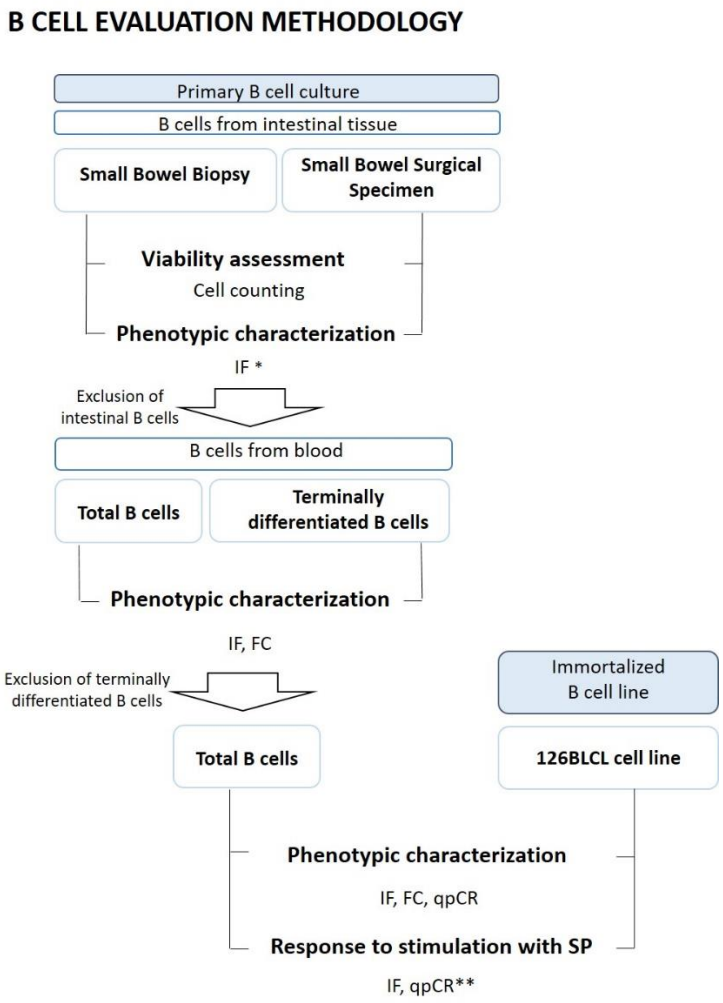

Figure 2: Methodology conducted to evaluate a suitable *in vitro* model to study the effect of SP on B cell activation. Substance P and its vehicle were used for stimulation experiments. IF: immunofluorescence; qPCR; quantitative PCR; FC: flow cytometry; SP: substance P. * Only in ileal specimens. ** Only in 126BLCL cell line.

8.3.5.1. Isolation of B cells from the intestinal mucosa

- Isolation of LPL from the jejunal mucosa: biopsies were cut in small pieces (≤1cm) and incubated 90min at RT under orbital rotation (112xg) with Hanks Balanced Salt Solution (HBSS) medium (Ca^{2+} Mg^{2+} free) supplemented with 1mM EDTA, 1mM dithiothreitol (DTT), 10% heat-inactivated Fetal Bovine Serum (FBS), 10% penicillin-streptomycin and 1% gentamicin to remove epithelium. After discarding intraepithelial lymphocytes found in the supernatant, the remaining tissue pieces were cut (≤2mm) and transferred to a digestion solution: RPMI1640-HEPES supplemented medium with 50mg/mL of collagenase, 9µL Dnase I, 10% heat-inactivated FBS, 10% penicillin-streptomycin, 1% gentamicin. Tissue was incubated with digestion solution at

37ºC during 2h under slow agitation. Cell clumps were disrupted using a syringe 21G mounted needle and LPL-containing cell suspension was filtered using a 100µm-nylon filter.

- Isolation of LPL from ileal mucosa: cell isolation was performed following the protocol described above, with the additional purification of CD38+ cells, isolated by positive magnetic selection, using CD38 MicroBead Kit human beads (Miltenyi Biotec), according to manufacturer instructions.

8.3.5.2. Isolation B cells from blood and differentiation into plasma cells

B cells from healthy donor's blood were isolated as described below. A second-step was added in the protocol to activate and differentiate these B cells into plasma cells. The yield of the procedure was evaluated.

- B cells isolation from peripheral blood: B cells were isolated from fresh peripheral blood by negative magnetic selection using MACSxpress B Cell Isolation Kit human (Miltenyi Biotec). For removal of erythrocytes, Red Blood Cell Lysis Solution (Miltenyi Biotec) was used. Isolation procedures were performed as stated in the manufacturer's instructions. Blood-derived B cells were seeded at a density of $5x10^5$ cells/mL or centrifuged at 300xg 10min RT for cell pellet collection and basal phenotypic evaluation.

- B cell differentiation into plasma cell: to generate antibody-producing cells, blood-derived B cells were treated with 10µg/mL of lectin from Phytolacca Americana (pokeweed, Sigma)) and 1µg/mL CD40Ligand (CD40L) (R&D systems) to activate mitogenic signaling. As antigenic factors, 100µg/mL of protein A from *Staphylococcus aureus* (Cowan Strain, Sigma) and 1ng/mL of oligodeoxyribonucleotides containing CpG motifs (CpG ODN2006, Miltenyi Biotec) were used. In order to activate and differentiate B lymphocytes, cells were stimulated with interleukines (IL) secreted by Th cells in the germinal centers: 25ng/mL IL-10 and 100ng/mL IL-21 (both from R&D systems). Cells were seeded at a density of $5x10^5$ cells/mL, without changing the culture media during the 7-days differentiation process (Figure 3). Non-stimulated cells were defined as control and used as reference group.

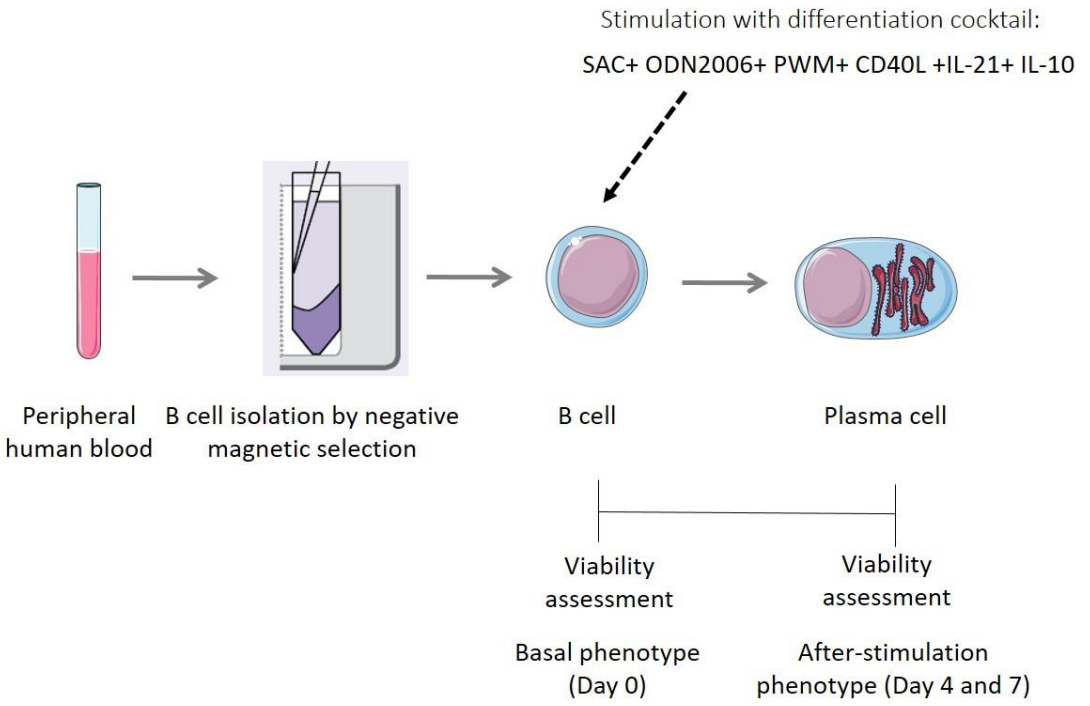

Figure 3: Experimental protocol performed for differentiation of isolated B cells from blood. Blood-derived B cells were stimulated with the differentiation cocktail, containing SAC, ODN2006, PWM; CD40L, IL-21 and IL-10, and kept in this medium during all the differentiation process. CD40L: CD40 ligand; IL-10: interleukin-10; IL-21: interleukin-21; ODN2006: oligodeoxyribonucleotides containing CpG motifs; PWM: lectin from Phytolacca Americana (pokeweed); SAC: protein A from *Staphylococcus aureus*.

8.3.5.3. Maintenance of the B cell line

The 126BLCL cell line was maintained in RPMI 1640 medium GLUTAMAX^{TM} supplemented with 10% heat-inactivated FBS, 1% penicillin-streptomycin and 1% gentamicin, seeded at a density of 5 x 10^5 cells/cm^2 and incubated at 37ºC in an environment of 95% of humidity and 5% CO^2.

8.3.5.4. Stimulation of B cells with Substance P

B cells isolated from blood (not differentiated into plasma cell) and 126BLCL cell line were seeded at 1x10^6cells/mL and were stimulated in a 96-well plate, with SP (Sigma), for 30 and 60 minutes, for gene expression studies and up to 4 days for further analysis of protein and gene expression. SP and SP vehicle (SP Vh, 0.05M acetic acid + 0.1%BSA + H_2O). Cells were exposed to a single exposure of 1nM and 10nM of SP, at time indicated, without changing cell medium until the end of the procedure. The basis for selecting the appropriate concentration of SP was defined according to the literature, when TACR1 presents a minimal and a high phosphorylation level, respectively (Supplementary Data).

8.3.6. Analytical procedures

8.3.6.1. RNA isolation

After incubation, cells were centrifuged at 300xg 10min RT pellet and supernatant was completely removed. Pelleted cells were diluted in RLT buffer and β-Mercaptoethanol and stored at -80ºC, until necessary. Total RNA was isolated using the RNAeasy Mini Kit (Qiagen), as indicated by the manufacturer. DNase treatment (Qiagen) was performed on columns at room temperature. RNA was eluted in 30µL of RNase-free water and stored at -80ºC until the next experimental step. Prior to gene analysis, RNA quantity and quality were confirmed by capillary electrophoresis (Agilent 2100 Bioanalyzer; Agilent Technologies). Only samples with values of RNA Integrity Number (RIN ≥ 7) were included in the analysis.

8.3.6.2. cDNA synthesis and quantitative PCR

cDNA was obtained by reverse transcription of 1µg total mRNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo-Fisher Scientific), following the instructions of the manufacturer's protocol. Gene expression analysis was performed for genes involved in B cell differentiation, Ig production and the SP receptor expression by qPCR on a ABI PRISM® 7500 FAST Sequence Detection System (applied Biosystems) using validated TaqMan® Gene Expression Assays (Applied Biosystems). Results are expressed as ΔCt, normalized by the house-keeping gene (PPIA) or as a fold-change (using Vh SP and non-treated cells as controls) when cells were exposed to SP. PPIA housekeeping gene was used to normalize the expression of all genes. Results were expressed as the fold change of the genes when stimulated with SP or the vehicle (vehicle vs non-stimulated as control), based upon changes in the delta cycle threshold values. TaqMan primer sets used are described in Table 1.

Gene assay	Gene name	Specie	TaqMan Assay
TACR1	Tachykinin receptor 1	*Homo sapiens*	Hs01025732_m1
BLIMP1	B lymphocyte-induced maturation protein-1	*Homo sapiens*	Hs00153357_m1
XBP1	X-box binding protein 1	*Homo sapiens*	Hs00231936_m1
PPIA	Peptidylprolyl isomerase A	*Homo sapiens*	H99999904_m1

Table 1: Gene expression assay probes used for qPCR analysis in B cells from blood and 126BLCL line.

8.3.6.3. Immunofluorescence staining

B lymphocytes isolated from the intestinal mucosa (jejunum or ileum) or blood and CD38+ cells from the ileum, were transferred into a Polystyrene Round-Bottom Tube (Corning Life Science) at a density of $8x10^4$ cells/100µL. When working with the 126BLCL line, cell suspension was set at $5x10^4$ cells, due to the greater size of these cells. Cells were charged in cytospin cassettes for a spin (6 min, 72xg) to immobilize cells on a slide (poly-L-lysine coated glass slides) and fixed 2min with PFA4% at room temperature. Samples were blocked with Dako Blocking Solution (Dako) for 30 minutes, except for IgG staining, where Blocking solution was supplemented with 10% human serum and 1% bovine serum albumin (BSA) to avoid unspecific binding of primary antibody to Fc receptors. Samples were incubated with primary antibody O/N at 4ºC: 1:200 monoclonal anti-CD20 (Dako), 1:100 mono-clonal anti-CD38 (Nordic BioSite), 1:200 polyclonal anti-CD138 (Sigma-Aldrich), 1:50 polyclonal anti-TACR1 (ThermoFisher), 1:100 polyclonal anti-IgM (Abcam) or 1:100 polyclonal anti-IgG H+L chain (Bethyl). Polyclonal anti-IgA (Bethyl) staining was incubated 1h RT at 1:300. Primary antibodies were diluted in the blocking solution, except for IgG, which was diluted in a lower concentration of human serum (blocking solution supplemented with 5% human serum and 1% BSA) to reduce background. After primary antibody incubation, cells were washed and exposed to the 1:500 appropriated secondary antibody Alexa Fluor 488/ Alexa Fluor 594 anti-rabbit/anti-mouse) (ThermoFisher) 30min in the dark at RT. Nuclei were counterstained with 10ng/mL 4',6- diamidino-2-phenylindole (DAPI) during 10min RT before mounting in Prolong antifade mountant media (Invitrogen). Slides were kept at 4ºC light protected until analysis. Fluorescence was visualized with a camera attached to the fluorescence microscope OLYMPUS BX61 (Olympus), using the CellSens Standard 1.7 software. Samples exposed to these conditions, excluding the primary antibody incubation, were used as negative controls of the technique.

8.3.6.4. Flow cytometry

B cells were pelleted ($2x10^5$ cells) in round-bottom polystyrene tubes and diluted in 500µL of cold phosphate buffered saline (PBS, pH=7.4) (Gibco) supplemented with 2% FBS and 0.1% of BSA. When permeabilization was required (for proteins with intracellular location), cells were treated with BD Cytofix/Cytoperm reagent (BD Biosciences) for 20 minutes. For surface staining, 5µL of primary antibody was added in a final volume of 100µL of PBS+2%FBS+0.1%BSA and incubated during 15min. For cytoplasmic staining, the procedure was the same, except the antibody diluent, which was Perm/Wash buffer (BD wash, BD Biosciences), to maintain the permeability of cell membrane. All antibodies were conjugated with fluorochromes, except for

TACR1 (ThermoFisher), for which an additional step was needed: staining with the appropriate secondary antibody, 1:500 Alexa 488 anti-rabbit (ThermoFisher) during 15min in the dark. Details of antibodies used in this procedure are summarized in Table 2. The whole procedure was perfomed at 4ºC. Cells were washed twice in PBS+2% FBS+0.1% BSA or Perm/Wash Buffer, according to permeabilization, between steps. Cells were collected and kept at 4ºC and light-protected until analyzed (maximum two days). Cell suspensions were processed using a LRS Fortessa flow cytometer (BD Biosciences) and samples analyzed with the FCS Express version 3 research edition.

Antibody	Supplier	Conjugated fluorochrome	Marker location
Anti-CD20	BD Bioscience	PE	Membrane
Anti-CD38	BD Bioscience	BV605	Membrane
Anti-IgM	BD Bioscience	BV421	Cytoplasmic
Anti-IgG	BD Bioscience	BV786	Cytoplasmic

Table 2: Summary of antibodies used for protein expression analysis in B lymphocytes from blood by flow cytometry.

8.3.7. Statistical analysis

This work consists of different methodological approaches to establish and select a suitable *in* vitro model for the study of B cell activation by neuropeptides. Normality of the data distribution was tested by the D'Agostino and Pearson omnibus normality test. Normally distributed parametric data are expressed as mean ± standard error of mean and compared by the compared by the unpaired Student's *t* test (two-tailed). For non-parametric distributed data, results are expressed as the median and the maximum-minimum range and analyzed by the Mann-Whitney *U* test was used. All the statistical analysis were performed using GraphPad Prism 6.0 software. Due to the low sample size, it was only possible to conduct a proper statistical analysis for LPL isolation. For the other assays, coefficient variation of the results has been considered, in order to validate the reproducibility of the experiments.

8.4. RESULTS

Several sources of B cells were evaluated to establish the most suitable *in vitro* model for assessing B cell response to SP. This study tested B cells isolated from biological samples, intestinal tissue and blood, from healthy donors and a lymphoblastoid cell line.

8.4.1. LPL isolated from jejunal biopsies

Small bowel mucosal samples from jejunal biopsies had a weigh of 70-80mg, and ileal specimens allowed the collection of 600-1000mg of mucosa. The number of LPL isolated were $5.5x10^5 \pm 6.1x10^4$ (n=16) and $1.9x10^7$ ($1.10x10^7$– $2.23x10^7$, n=5), respectively (Supplementary Table S3). From ileal tissue, 10.2% (9.24 – 12.5, n=3) of isolated cells were CD38+. Considering these results, we expected approximately $6.84x10^4$ CD38+ cells from biopsies, a suboptimal number of cells to conduct our experiments.

8.4.2. Expression of surface receptors of isolated cells

8.4.2.1. Expression of CD38 marker

The expression of CD38 in Isolated LPL was confirmed by immunofluorescence in cell suspensions obtained after LPL isolation from ileal specimens. Figure 4 shows that isolated positive cells clustered together. Although not calculated, most cells were positive for the CD38 marker**.** Most of the isolated cells where positive for CD38 and for TACR1.

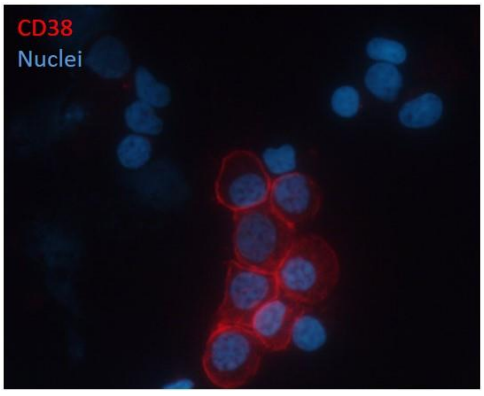

Figure 4: Identification of CD38+ cells in intestinal LPL cells. Cells were isolated from the mucosa obtained from ileal specimens. Magnification x600.

8.4.2.2. Expression of SP receptors

The expression of SP receptor in Isolated LPL was confirmed by immunofluorescence in cell suspensions obtained after LPL isolation from ileal specimens. Figure 5 shows not all LPL express TACR1.

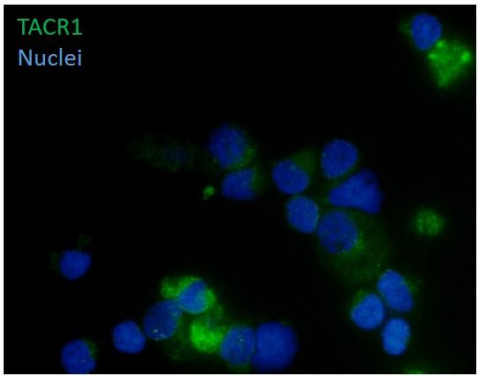

Figure 5: Identification of TACR1+ cells isolated from the ileal mucosa. Clusters of cells can be observed. Magnification x600.

8.4.3. Differentiation of blood-derived B cells into antibody-producing cells

8.4.3.1. Population purity assessment

Before proceeding to differentiation into antibody-producing cells, the purity of blood isolated B cells was assessed by flow cytometry using the pan-B cell marker CD20 (Figure 6). In all the isolation procedures, a pure population, with more than 90% of cells expressing the CD20 marker was consistently obtained.

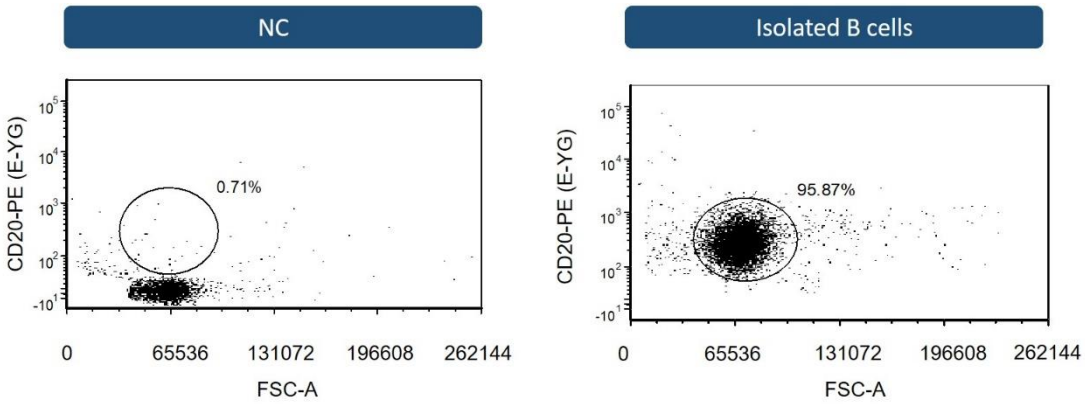

Figure 6: Representative dot plot of flow cytometry analysis for CD20+ cells from B cells isolated from blood. The percentage of gated cells is established with a negative control (cells not incubated with the anti-CD20 antibody). NC: Negative Control; FSC-A: Forward Scatter Area.

8.4.3.2. Viability and morphological assessment

B cells in culture showed a reduction in viability from day 1 (45-50%) until day 7 (10-20%) of the experimental procedure. Reduction in viability was observed in both conditions tested: stimulated and non-stimulated for differentiation into plasma cells (Figure 7). Viability strongly decreased the first 24h for both conditions and cells progressively died along the days of culture. Those cells stimulated to differentiate into plasma cells increase their size (identified by higher side scatter by flow cytometry) and present a higher complexity of the cytoplasm, compared to basal phenotype and non-stimulated cells (Figure 8).

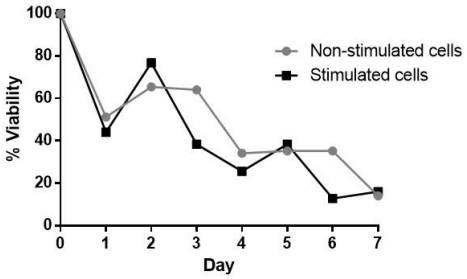

Figure 7: Time course of the number of viable cells in culture. At day 0, cells were exposed to a differentiation cocktail (stimulated-cells) or not exposed (non-stimulated cells). Each point represents the median of the replicates (n=5).

8.4.4. Expression of plasma cells markers

The terminally differentiated antibody-producing cell marker CD138 was identified already at day 4 after stimulation, as some cells were positive for CD138 and maintained the expression until day 7 (Figure 9a). No differences were observed before and after the differentiation process when assessing the levels of CD38+ by flow cytometry (Figure 9b). Despite cells differentiated into plasma cells, the low viability and number of CD138+ cells did not allow to conduct the subsequent proposed experiments of stimulation with SP.

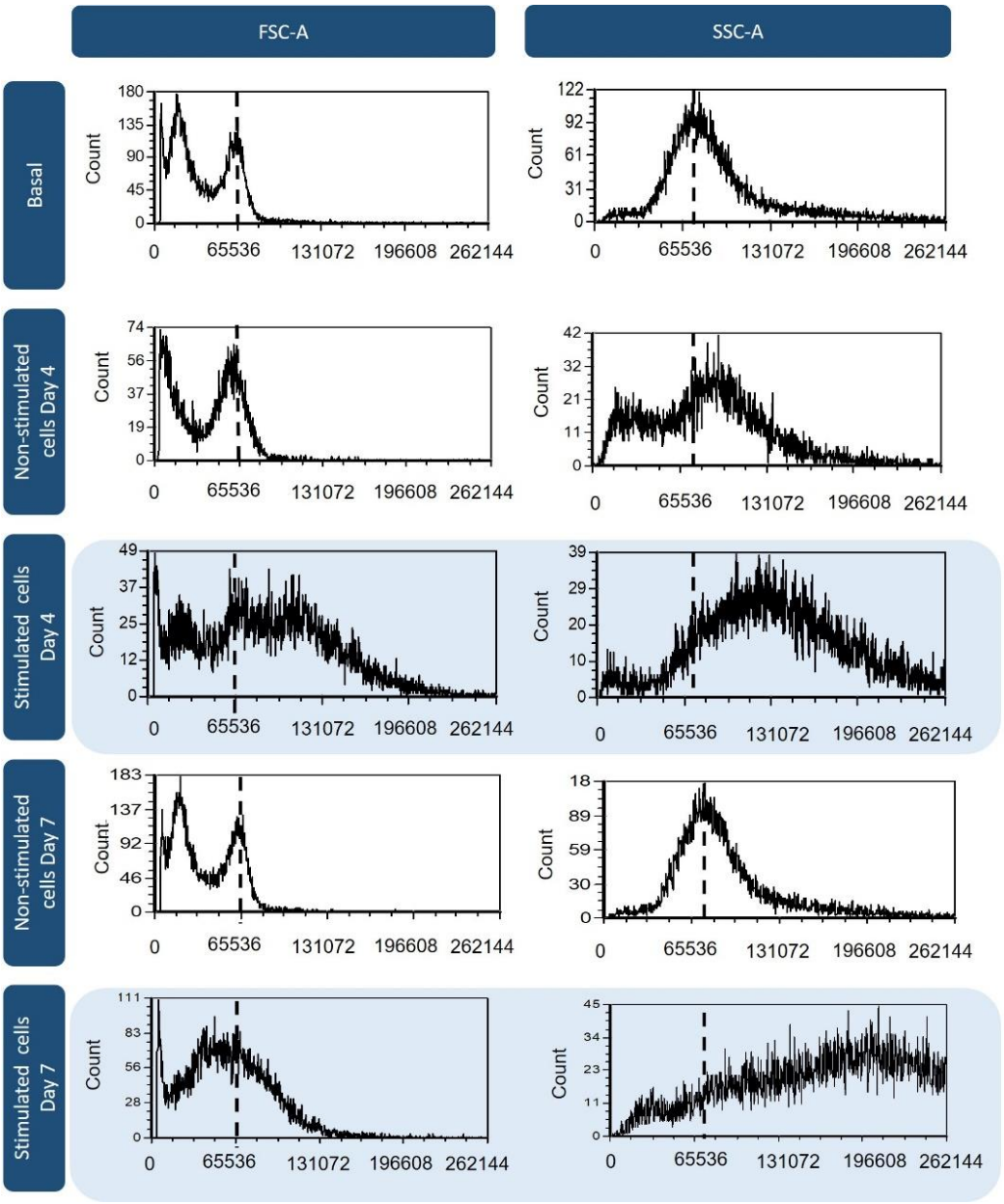

Figure 8: Representative of histograms of B cells cultured for 7 days. The size of the cells and the complexity of the cytoplasm can be observed in the histograms (FSC-A and SSC-A, respectively). Those cells highlighted in blue are B cells stimulated to differentiate into plasma cells. FSC-A: Forward Scatter Area; SSC-A: Side Scatter Area.

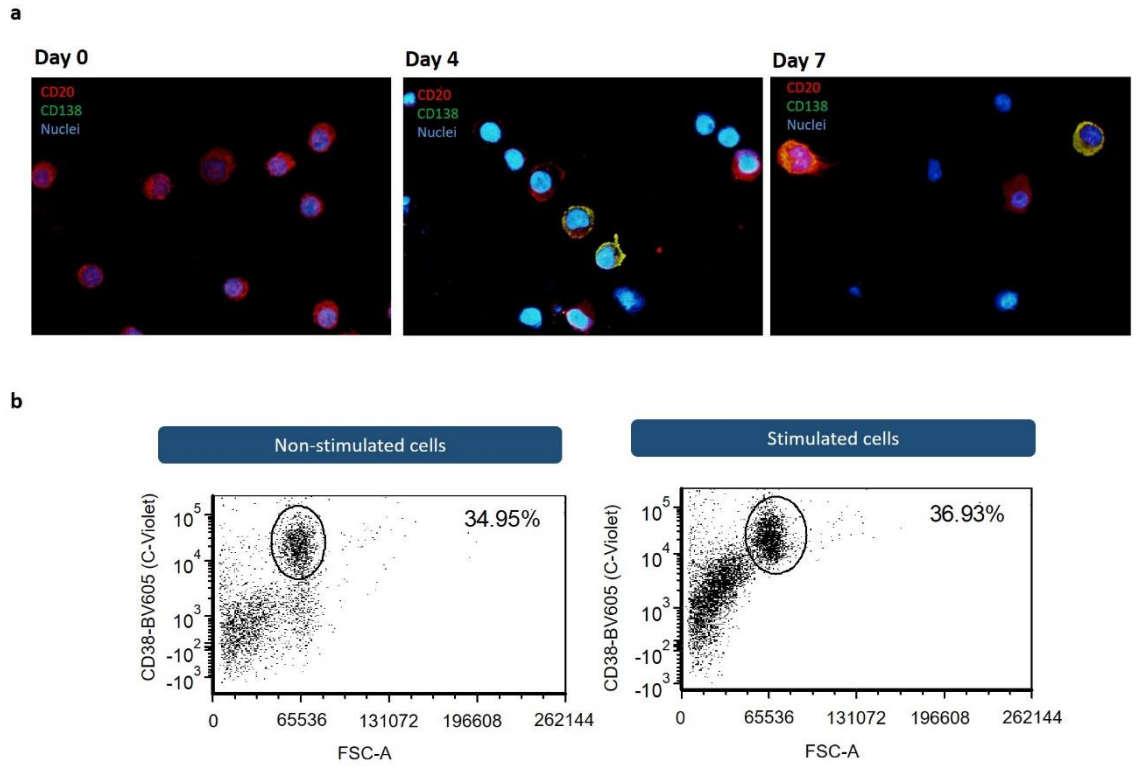

Figure 9: Expression of B cell and plasma cell markers after exposure of blood-derived B cells to the differentiation cocktail. a) Representative images of CD138 and CD20 staining in Isolated B cells from blood before and after exposure to differentiation factors by means of immunofluorescence. Magnification x400. b) Representative dot plot of flow cytometry analysis for CD38+ cells non-stimulated and stimulated with the differentiation cocktail at day 4. The percentage of gated cells was established with a negative control (only cells). FSC-A: Forward Scatter Area.

8.4.5. Characterization of the lymphoblastoid cell line 126BLCL

The principal Ig isotype expressed by the 126BLCL cells was IgM (98.5% of cells), followed by a low expression of IgG (1.45%). According to the literature, most of the B cells isolated from blood, approximately 65%, present a naïve phenotype (19). As a reference, B cells isolated from human blood showed that 81.6% expressed IgM+ and 12.2% expressed IgG+ (Figure 10).

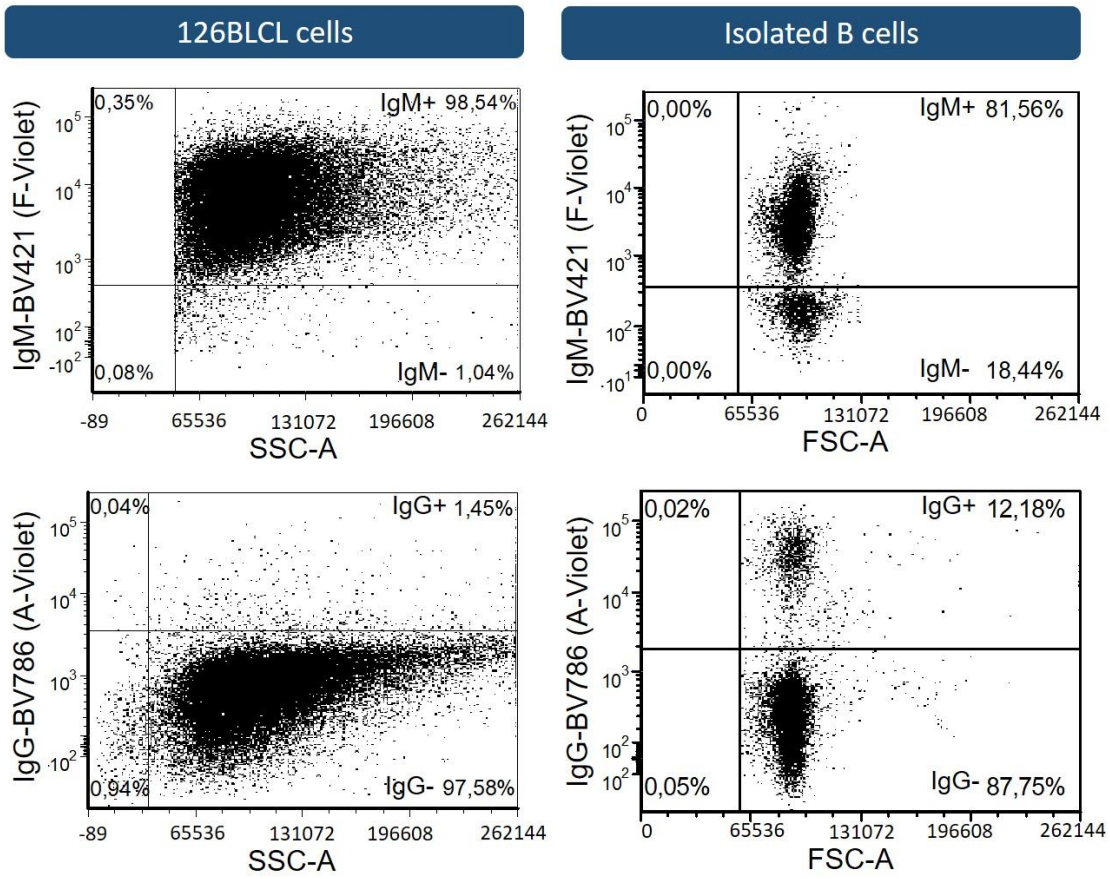

Figure 10: Representative dot plot of flow cytometry analysis for IgM and IgG expression in blood-derived B cells and the 126BLCL line. The percentage of cells in different quadrants were established with a negative control (only cells, without IgM-BV421 and IgG-BV786 antibodies, respectively). Blood was obtained from healthy volunteer donors. FSC-A: Forward Scatter Area; SSC-A: Side Scatter Area.

Regarding the expression of specific genes, 126BLCL expressed genes associated to B cell differentiation, *XBP1* (1.49±0.013, Cv=1,.8%), *BLIMP1* (5.06±0.46, Cv=12.8%), as well as *TACR1* (11.75±2.06, Cv=24.8%) at basal conditions (Figure 11a). Blood-derived B cells also expressed *XBP1* (5.32±0.12, Cv=3.23%) and *BLIMP1* (9.01±0.15, Cv=2.4%) at a higher ΔCt, therefore lower levels than 126BLCL but similar the expression of *TACR1* was similar in B cells from both sources. The protein expression of TACR1 was not detected at basal conditions in 126BLCL cells, contrary to blood-derived B cells, which were positive for the receptor, as detected by by IF (Figure 11b). Both, blood and immortalized B cells expressed CD20 and CD38 but no expression of CD138 was detected.

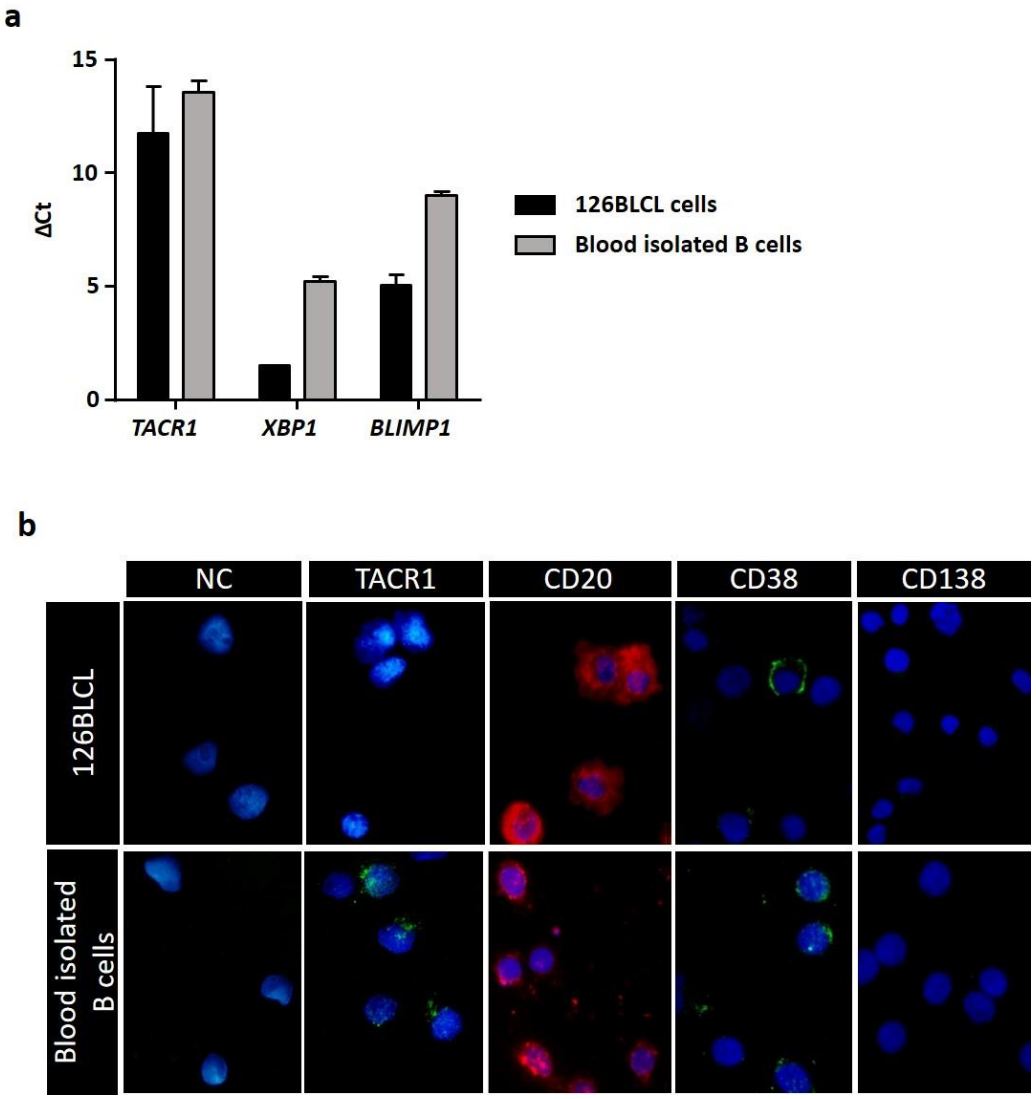

Figure 11: Expression of SP receptor and B cell differentiation genes in B cells. a) Gene expression of TACR1, XBP1 and BLIMP1 in 126BLCL cells and blood isolated B cells expressed in ΔCt normalized by the house-keeping gene (PPIA). **b)** Protein expression of TACR1, CD20, CD38 and CD138 assessed by immunofluorescence in 126BLCL cells and blood-derived B cells (isolated from blood). Magnification 600x.

The stimulation of 126BLCL cell line with SP at 1nM and 10nM did not modify cell viability, as it remained constant throughout the exposure time, independently of the stimulus (Figure 12). As observed in the figure, the number of cells increased probably due to the proliferation rate of these cells (cell cycle estimated to be approximately of 48h).

TACR1 protein expression in response to SP was increased at 10nM SP stimulation during 24h in inmortalized B cells, compared with both control cells and the rest of stimuli (SP or SP vehicle at 1nM) (Figure 13).

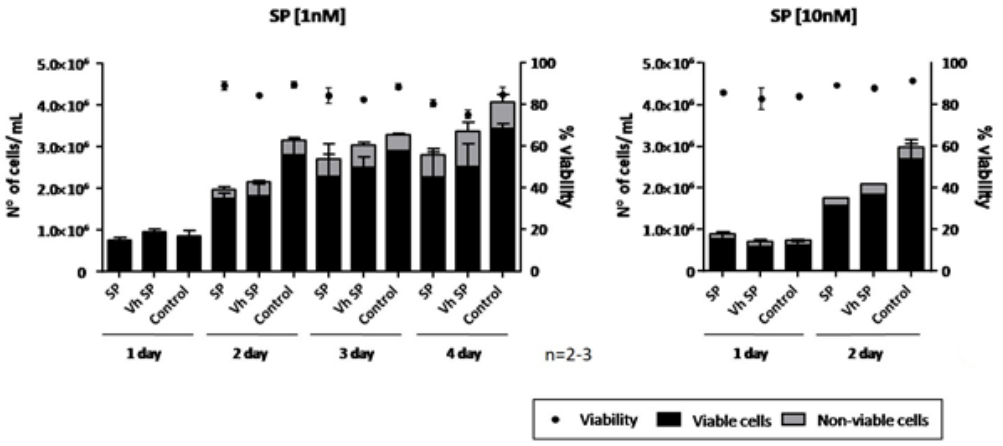

Figure 12: Cell growth and viability of 126BLCL cell line in response to different stimuli. Representation of cell viability and viable and non-viable cells through 4 days of exposure to 1nM of SP and through 2 days of exposure to 10nM of SP. Non-exposed cells were used as control (Control). Data shown are the mean of the fold-change according to cells treated with Vh for cells exposed to SP or to non-stimulated cells (control) for cells exposed to Vh. Bars represent standard deviation of two independent experiments**.** SP: Substance P; Vh SP: Vehicle Substance P.

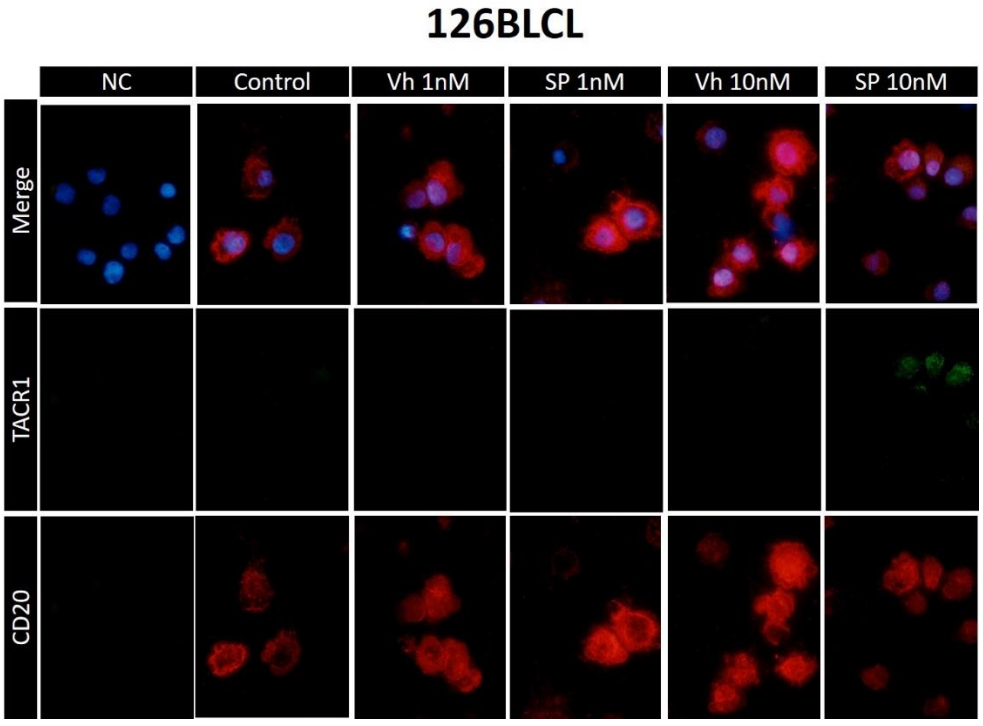

Figure 13: Representative images of TACR1 and CD20 staining in 126BLCL cell line after exposure to SP and SP vehicle at 1 and 10nM during 24h by means of immunofluorescence. Blue signal: DAPI. Negative Control (NC). Magnification 600x.

TACR1 expression in B cells isolated from blood was not altered when stimulating with SP or SP vehicle at 1nM, but similarly to 126BLCL cell line, a higher fluorescence signal was observed with SP at 10nM. A slight increase in the signal was also observed when cells were treated with vehicle, however, despite no quantification was performed, visual analysis differentiated an upregulation of TACR1 at the highest concentration tested (Figure 14). This slight increase in signal was also be observed in CD20 staining, which presented a light and non-homogenous signal in the membrane in control and in Vh 1nM exposed cells but it is distributed along the membrane surface in the other conditions.

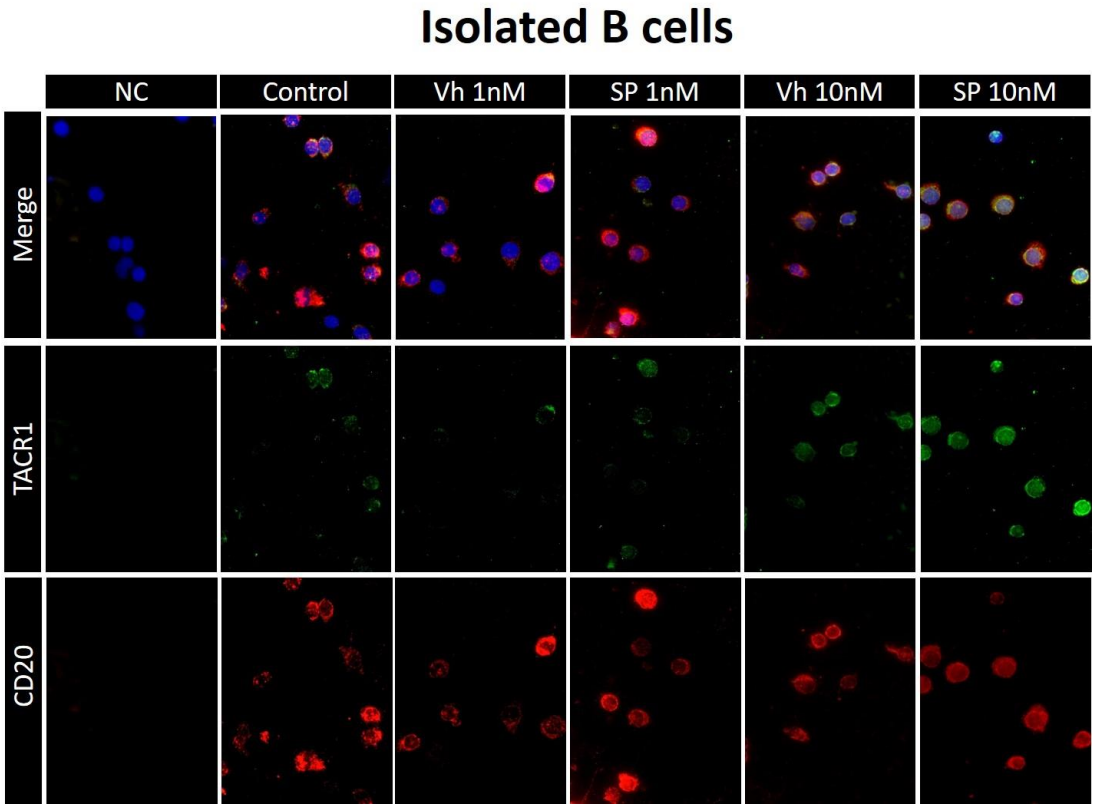

Figure 14: Representative images of TACR1 and CD20 staining in isolated B cells from blood after exposure to SP and SP vehicle at 1 and 10nM during 24h by means of immunofluorescence. Blue signal: DAPI. Negative Control (NC). Magnification 600x.

The effect of SP and/or vehicle on 126BCLC cells was assessed by quantifying the gene expression of TACR1, BLIMP1 and XBP1 under different incubation conditions. The results showed no differences at the time points and concentrations of SP tested (Figure 15). A tendency of a higher TACR1 expression after 24h when stimulating with 10nM was identified (fold change = 2), despite it would be necessary to increase the sample size (n=2) to run a proper statistical analysis.

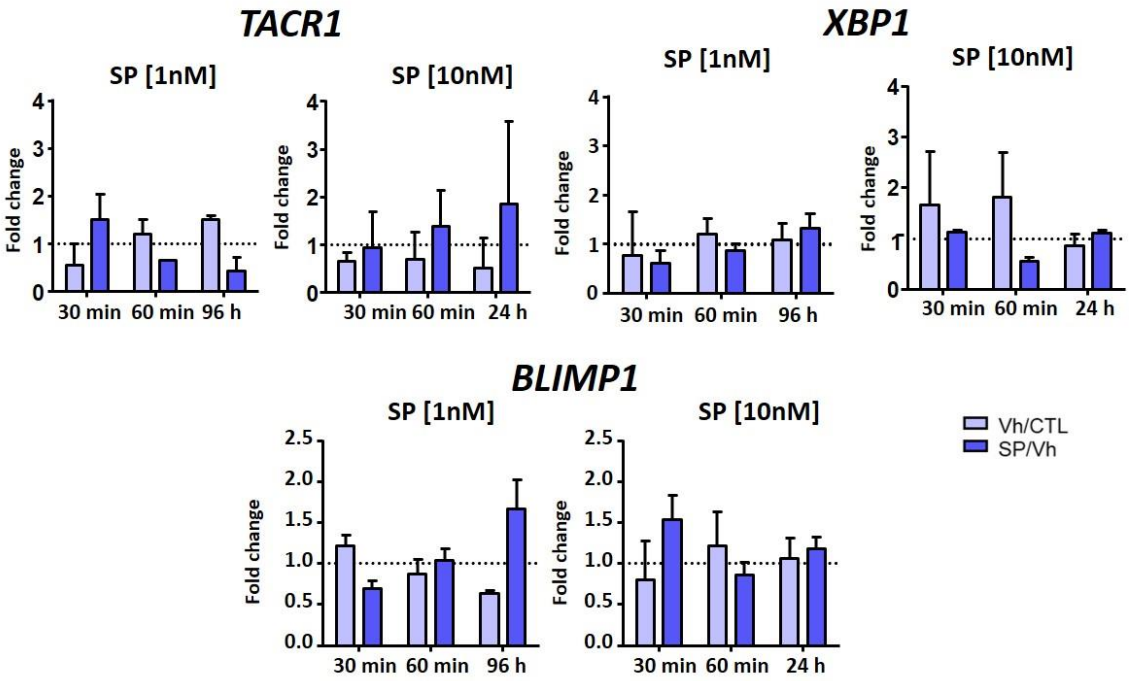

Figure 15: Gene expression time course of TACR1, XBP1 and BLIMP1 in 126BLCL cells with SP at 1nM and 10nM. Results are expressed as fold-change according to cells treated with Vh for cells exposed to SP or to non-stimulated cells (control) for cells exposed to Vh. Data shown represent the mean and the standard deviation of two independent experiments**.** Vh: Vehicle Substance P; CTL: Control; SP: Substance P.

8.4.6. Summary of the B cells *in vitro* models

Incubation of cells from intestinal tissue was discarded as model due to the difficulties in obtaining a viable single-cell suspension, together with other features such as the high invasive procedure needed for ileal specimen collection or the low number of cells isolated from jejunal biopsies. Viability and low yield of the differentiation protocol were the crucial factors for excluding terminally differentiated B cells for this study. The 126BLCL cell line was not suitable for these protocol due to the changes experimented during the immortalization process, therefore, isolated B cells from blood, without being differentiated into plasma cells, were chosen as the most feasible option. A summary of the results obtained for the selection of a B cell *in vitro* model are represented in Figure 16.

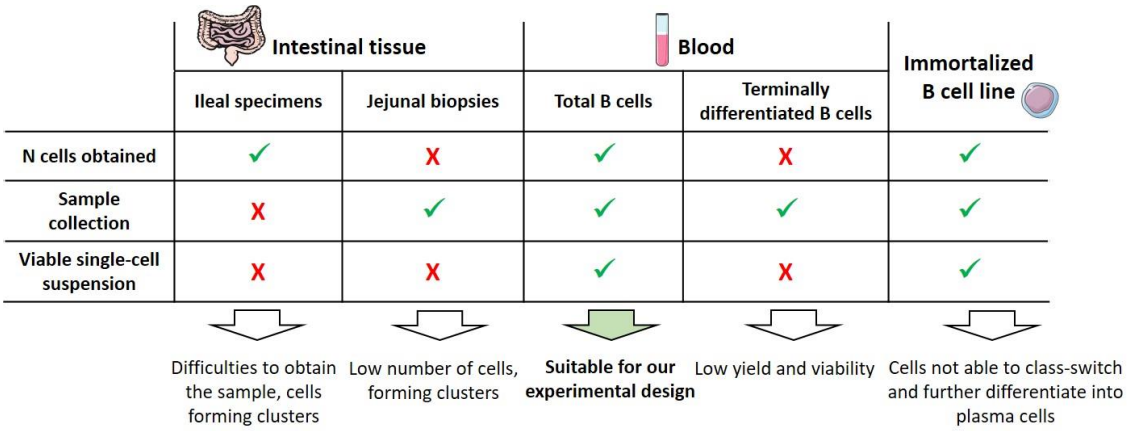

Figure 16: Summary of the different sources for obtaining human B cells and their characteristics.

8.5. DISCUSSION

The objective of this study was to establish a feasible and suitable in vitro model to study the potential role of SP in humoral response activation. Neuropeptides secreted by nerve endings and neuroendocrine cells exert an effect on immune cell populations, modulating their activity and proliferation (20,21). In IBS, an over activated antibody-mediated response has been postulated to play a role in IBS pathophysiology (1). Several neuropeptides have been described to be associated with this syndrome, but SP seems to be a focus of attention due to its involvement in nociceptive sensitization and the higher number of nerve fibers that stain for SP in the intestinal mucosa of IBS patients (7,10,11).

Considering previous evidences, plasma cell obtaining from intestinal tissue seemed the most suitable option to study neuro-immune mechanisms in this disorder. However, in this study, the type of intestinal sample collected yield low number of cells, therefore, limiting the type of experiments to perform. The study design included two ways for intestinal tissue collection: ileal specimen obtained by surgical procedures and jejunal biopsies by Watson's capsule. Ileal specimens were collected from donors, from which the intestinal non-inflamed region was used for cell isolation. However, the number of intestinal interventions were limited and not always is possible to collect the sample, what makes difficult the development of the experimental procedures. With Watson's capsule, the sample collection procedure is less complex and performed at our unit, the jejunal region is easy reachable and healthy volunteers can participate in the study. Despite this, the size of the collected biopsy is significantly smaller than the ileal surgical specimen, which can be reflected in the lower number of LPL obtained in jejunum. Another option would have been the collection of endoscopic biopsies, but they are

much smaller (around 10mg/each biopsy) than the ones obtained with the capsule. We did not include this method in the B cell isolation protocol since we would have needed a large number of endoscopic biopsies to conduct the assays.

LPL isolation is a necessary step for obtaining plasma cell from the intestinal tissue. Carrasco et al. optimized a protocol for LPL isolation from human colonic endoscopic biopsies, obtaining a range of 6.75-7.38 x104 cells/biopsy (8.44-9.22 x103 LPL/mg approximately) (22). We obtained similar results for isolation from jejunal biopsies (8.37-9.58 x103 LPL/mg) and a higher yield when obtained from ileal specimens (1.94-3.23 x104 LPL/mg), both from small bowel mucosa. Therefore, these results suggest that our protocol to isolate LPL from intestinal mucosa was working properly.

Setting up a protocol for plasma cell isolation may involve several drawbacks, including the few available commercial options to isolate CD138+ cells. Since CD38 is also an antibody-producing marker and other immune cell populations are also positive for this receptor, such as memory T cells or dendritic cells, even though representing a significantly lower percentage of cells residing in the *lamina propria* (23), we decided to use CD38 for plasmablasts and plasma cells isolation. CD38 isolation was only conducted with LPL isolated from ileal specimens, and not in the biopsies due to the low number of cells obtained. Around 10% of isolated LPL were CD38+. This percentage was lower than expected, considering that only IgA-producing cells represent 30-40% of LPL (23). Other authors had obtained similar results working with colonic biopsies (6.6-12.3% CD19+ cells, a pan-B cell marker) (22). These results may be explained by several factors. Firstly, during LPL isolation, biopsies are subjected to an aggressive enzymatic digestion with collagenase, which may dissect the membrane receptors, among them CD38. Secondly, clusters of cells were observed in IF images, which may also interfere in the antibody recognition and therefore, in the isolation process.

Enzymatic digestion is a yield-determinant step due to the activity of collagenase and DNase I differ from commercial brands and even between batches from the same supplier; when the enzymatic digestion is too aggressive, it disrupts the tissue and, on the contrary, clusters of cells can be observed when is insufficient. The complexity of this protocol has been noted before in the literature (22,24). Technical hitches in obtaining a single-cell suspension together with difficulties to sample collection led us to use other B cell sources.

Peripheral blood was used as an alternative biological sample to isolate B cells. B cells differentiate into plasma cells in secondary lymphoid tissues, therefore they cannot be found circulating. We wanted to generate antibody-producing cells and evaluate them as a potential

in vitro model. To achieve this purpose, B cells isolated from blood were exposed to ILs, antigens and mitogens, in order to reproduce as much as possible the physiological conditions of the intestinal mucosa, as described in the literature (25,26).

Considering we were working with a primary cell culture, one of the determinant steps was the viability assessment. In the differentiation protocol, lectin from Phytolacca Americana and CD40L were used as mitogens to increase the number of viable cells. It was observed, in isolated cells, that both non-stimulated and stimulated with the differentiation cocktail, present a short-life span. During the first day of culture, the number of viable cells drops drastically, it recovers 24h later and viability progressively decreases the next days. Primary culture is usually sensitive to alterations in cell media, what may explain the accentuated decrease in viability observed during day 1, when B cells are adapting to the new environment. Mimicking the physiological conditions B cells are exposed in vivo is challenging, so not reaching sufficient optimal conditions may explain the low viability observed during the culture procedure for primary cells. However, as it has been previously noted, this study consists of the evaluation of several techniques to obtaining a suitable in vitro model. Setting up a protocol is time-consuming, therefore, some of the assays have only been replicated 2-3 times and conclusions must be extracted with caution. Experimental protocol to differentiate B cells is a long procedure; it requires B cells to be exposed to the differentiation cocktail in culture at least 4 days. Hence, the reduction in viability during cell culture is a notable inconvenient. When evaluating the morphological features, it can be observed that those cells stimulated to differentiate into plasma cells present a higher size and a more complex cytoplasm than non-stimulated B cells or the basal phenotype, indicating the differentiation protocol is working, as plasma cells are larger in size and more granular than B cells.

We assessed CD20, pan B-cell marker, and CD138 expression to those cells treated with the differentiation cocktail at Day 4 and Day of 7 of stimulation. In the IF images, it can be observed not all the cells are CD20+, despite we obtained a pure population after the isolation. Since B cells decrease CD20 expression at late phases of the ontogeny (27,28), in vitro highly stimulation used to trigger activation and differentiation into plasma cells may be playing a role in losing CD20 expression. CD138 expression can be detected in a few number of cells, suggesting this protocol is working but with a low yield. Optimization of the yield is challenging due to the generation of antibody-producing cell protocol differs according to the maturation state and the origin of B cells used (26,29–33). Our results refer to isolated total B cells from blood. According to the literature, 65% of these cells are naïve B cells, only around 30% are CD38+ cells (circulating memory B cells) (19). Triggering differentiation into plasma cells is more challenging in naïve B

cells compared to other populations such as plasmablasts or memory B cells, which are already activated and the process has been initiated, often with bacterial antigens (16) or oligonucleotides not found in mammal cells (34). Taking into account these results, obtaining enough differentiated cells to generate a suitable in vitro model would imply extracting a great amount of blood from donors, despite the high yield of B cell isolation procedure. We concluded that the differentiation protocol was not a feasible option for our purpose.

Using an immortalized B cell line was an alternative to the short life-span problem observed when working with a primary culture. A basal phenotype characterization to establish in which phase of the ontogeny are 126BLCL cells is necessary to evaluate whether they are antibody-producing cells or whether they can respond to the further stimulus (SP) to they will be subjected. We compared the phenotype to isolated B cells from blood, as these cells are the more feasible alternative to our model.

We observed most of cells from both, 126BLCL and isolated B cells from blood, express IgM, what means they have not undergone class-switching, most likely due to their naïve phenotype. (19). IgM+ cells are potentially a good in vitro model for our objective as our hypothesis proposed that SP can induce B cell activation and, probably, isotype class-switching. Regarding genes associated to B cell differentiation, *XBP1* and *BLIMP1*, higher gene expression levels can be detected in 126BLCL, which suggests these cells are more advanced in the maturation and differentiation process than isolated blood B cells. However, one crucial feature of our cells must be the capacity to respond to SP. We observed TACR1 protein expression can be detected at a basal level in blood B cells but not in 126BLCL. TACR1 can be internalized and stored intracellularly and its location and transport to membrane is stimulus-dependent (35). For this reason, we did not discard these cells as a potential model. Following our observations, we proceeded to SP exposure to evaluate the response of both B cell types.

It has been previously published that TACR1 activates at 1nM of SP and the cascade signaling triggered by its ligand remains for hours >10nM (12). Therefore, these two concentrations were used to stimulate B cells. The 126BLCL was also monitored along the time course assay. We did not observe differences in the viability with any of the concentrations used, only at Day 2. Proliferation rate of 126BLCL is approximately 48h, therefore it cannot be concluded this increment is due to SP effect. No differences were observed when cells were exposed 4 days to 1nM SP, considering the possibility a lower concentration needs more time to induce activation, despite a decrease in the proliferation rate was noted, likely because of the maintenance of the cell culture media during the assay.

Interestingly, B cells isolated from blood responded at lower concentrations of SP compared to 126BLCL cells, based on changes in TACR1 protein expression. Of note, Vh SP (0.05M acetic) increases TACR1 expression when assessed by IF. Diluting SP in another vehicle would avoid this effect. CD20 also modifies its expression pattern at higher concentration of both SP and Vh SP. The function of this receptor remains still unclear, but it has been described it can be modulated by some stimulus, such as TNFα or IFNα (36,37); exposure to Vh SP and SP maybe activating CD20. However, more research must be conducted to understand these changes in staining pattern.

The immortalization process of 126BLCL with EBV implies some irreversible epigenetic modifications which impairs the activation of genes involved in differentiation process to plasma cells (38). Hence expression of genes involved in this pathway, such as XBP1 and BLIMP1, may be blocked, gene levels detected may correspond to the maturation state previous to the transformation. The lack of response to 1nM SP, when TACR1 initiates activation (12), could be related to these irreversible modifications or to the absence of protein expression of the receptor observed by IF. These results revealed that 126BLCL are not a good B cell in vitro model to study the potential effect of SP on class-switch recombination and plasma cell differentiation.

Following our observations, we concluded that B cells isolated from blood is the most suitable option for developing an in vitro model for the study of B cell activation by neuropeptides. B cells were not differentiated into plasma cells, therefore could be stimulated to class-switch and keep stimulating these cells to further differentiate into plasma cells. Moreover, as the experimental procedure is not long, and viability is not compromised, SP stimulation could be used for other B cell-associated neuro-immune mechanisms, not addressed in the present study.

Selecting an in vitro model is crucial to conduct a proper experimental design. Despite the use of different sources of B cells were compared and confronted to each other, we were not able to perform as many assays as desired, and we could not further study the mechanisms by which SP activates B cells. Nevertheless, the importance of this project relies on obtaining a tool for the study of B cell stimulation by neuromediators. Therefore, this project will potentially contribute to achieve a deeper understanding on the role of neuropeptides, especially SP, in the neuro-immune interactions that take place in the intestinal mucosa.

8.6. BIBLIOGRAPHY

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DISCUSSION

9. DISCUSSION

Irritable Bowel Syndrome (IBS) is a prevalent and chronic gastrointestinal disorder (150) with a high impact in quality of life; patients suffering from this disorder may be out of work several days due to its incapacitating symptoms or even suffer from presenteeism (151). The incomplete understanding of IBS pathophysiology, the lack of sensitive and specific biomarkers for its diagnosis together with unsatisfactory treatments, it all makes economic-social and working impact enormous and growing. Despite the causes leading to the onset and the outcome of this syndrome are still partially unknown, an increased immune response (125,134,152–157) together with altered epithelial integrity (119,137) have been postulated as major factors involved in IBS pathophysiology. Moreover, an alteration of gut-brain signaling has been also associated with IBS (158). Patients often report psychiatric symptoms, including stress, anxiety and depression (159–163) and some disclose a distinct brain signature (93) higher innervation of the intestinal mucosa and higher levels of neuromediators (VIP, SP, CRF, etc) (132,145,164) compared to healthy subjects, particularly at the level of the intestinal mucosa. Stressful and early-life traumatic events are also involved in the severity of symptoms in IBS (158,159,165–167).

The intestinal tract is a highly innervated organ with a notably immune activity, due to the constant challenge by luminal factors in the mucosa. It has been described the existence of a neuro-immune crosstalk (148,168–170), which modulates the proliferation and the activity of immune populations including resident cells in the *lamina propria* of the intestinal mucosa (90,169). Considering the potential role of the immune response in this syndrome, it is not unexpected that the small bowel, the region with a highest immune defense activity of the gastrointestinal tract, has been described to be affected in this syndrome, in contrast with the traditional vision focused only on the role of the colonic layers (171,172). Hence, further research is needed to improve current understating of the underlying causes of this disorder and how a dysfunction in neuro-immune communication could potentially be involved in the modulation of intestinal motility, immune activity and visceral hypersensitivity. For this reason, this thesis was designed to characterize the local humoral response in IBS intestinal mucosa and the potential interaction with the nervous system.

Studying the antibody-mediated response in feces from IBS-D patients not only provide us information about the defence response taking place in both small and large intestinal mucosa, but also it also represents a non-invasive procedure, which could be helpful in the future to find disease biomarkers. Quantification of immunoglobulins (Ig) in stool supernatant shows a

significant increased concentration of IgG in IBS-D group compared to healthy volunteers (HV). These results are in line with what was previously reported by our group, where we described an increased humoral response in the jejunal mucosa (125). Considering the decreased epithelial integrity observed in IBS-D patients (119,137), IgG production/secretion could be promoted by the uncontrolled access of luminal antigens to the *lamina propia* immune cells. Increased permeability may also affect IgG transport, increasing or facilitating it through non-regulated pathways in addition of being transported by neonatal Fc receptor (FcRn) (32). Further research is needed to establish the reason of this high levels of Ig in intestinal content (125) and stools.

Knowing which IgG isotype is increased may provide us useful information about the responsible antigen implicated in the activation of humoral response in IBS. Lipopolysaccharide (LPS) and flagellin bacterial lipopolysaccharides have been associated with IBS as higher levels have been observed in peripheral blood compared to HV (157). More specifically, LPS has been linked with intestinal inflammation (173,174), visceral hypersensitivity (173), and increased gene expression of its receptor, TLR4, have been found in jejunal biopsies of IBS patients (175). All together with findings showing significant increased levels of IgG2 in stool, led us to conduct a quantification of LPS and anti-LPS Ig in HV and IBS-D samples. However, we did not observe differences between groups, neither in feces nor in peripheral blood. Of note, an overlap between IBS-D and HV could be observed in all the quantifications, what may suggest there are different subgroups of IBS-D patients. Considering this heterogeneity in patients, we cannot fully discard that LPS is triggering immune activation in a subgroup of patients. Obviously, other patients may respond against other antigen(s) including other bacteria, virus and elements from the diet.

One of the key systems modulating humoral activity is the gut-brain axis (176,177). Increased IgG levels found in stool supernatant of IBS-D group positively correlated with the intensity of abdominal pain in the IBS-D group. It has been previously described by other authors the association between the higher innervation in IBS-D group and pain (158,178), but few studies address the communication between B cells/plasma cells and nerve-endings. We studied this interaction by immunofluorescence and observed clusters of plasma cells in close proximity to nerve-endings and TACR1 positive fibers. B cells can secrete neuropeptides (168,170), suggesting that SP secretion by antibody-producing cells could modulate the activity of nerve-endings. However, a quantitative analysis of immunofluorescence stainings, assessing SP expression and its receptor, and more experiments to confirm the source of SP secretion (produced by nerve-fibers and several neuroendocrine cells (90,147,170) are needed to test if SP is the main mediator in this immune-nerve crosstalk.

When we assessed the proximity between nerve-fibers and antibody-producing cells in jejunal mucosal biopsies with a higher resolution technique, transmission electron microscopy (TEM), we observed that this distance was significantly reduced in the IBS-D group and correlated with acute stress and depression levels, the later not reaching significance: the closer they are, the higher the severity of the symptoms. Plasma cells from jejunum have been also found to be in close association to mast cells (125), another immune cell population described to be in proximity to nerve-endings in IBS colonic mucosa (179). It seems clear that this could represent an interaction between immune cells residing the *lamina propria* but we still cannot elucidate how they modulate each other. In addition, though less likely, this proximity could also represent an epiphenomenon related to mucosal inflammation as have been described also in IBD (180).

In order to obtain a deeper characterization of the distinctive local humoral response observed in IBS-D patients, we conducted a transcriptomic study of jejunal mucosal biopsies with RNA-seq. The transcriptomic analysis showed a low number of differential expressed genes (DEG) in IBS-D group, not constituting any overrepresented biological route. DEG must be validated by qPCR, in the future to increase its potential significance.

We also conducted a more complete transcriptomic analysis by Gene Set Enrichment Analysis (GSEA), which is not limited to DEG but comprises all the genes, organized in gene sets. This different approach allowed us to detect some changes between both groups. Gene sets associated to immune pathways and immunological signatures were found to be enriched in IBS-D compared to HV. Interestingly, genes associated with antibody-producing cells were up-regulated when confronted to genes linked to naïve or memory B cells as well as an increased germinal center activity, suggesting that activation and differentiation into plasma cells is enhanced. B cell immunological signatures were overrepresented when compared to innate immune cell populations (monocytes and macrophages), reinforcing the importance of humoral defense in this syndrome. These results are in line with the increased levels of IgG, IgG2 and IgG3 observed in stool supernatant from patients and the increased antibody-mediated response in jejunal biopsies and intestinal content previously reported by our group (125). Genes involved in dendritic cell activity were also overrepresented compared to naïve B cells; their function as antigen presenting-cells may explain this enrichment over other immune cells.

Genes involved in Ig structure are the ones with highest enrichment score (ES) in IBS-D, especially the ones encoding for lambda variable chain isotype, predominant in mucosal antibodies (181), pointing out antigen-recognition processes as potential factors in IBS-D pathophysiology. Two genes of the heavy constant part of Ig were found to be overrepresented,

the ones defining IgG2 and IgG3 isotype, which were also increased in IBS-D stool. Interestingly, pathways involved in BCR and antigen-recognition signaling and barrier function were also overrepresented in patients, in line with what is published in the literature (119,137,182,183). These results are in agreement with the activation of humoral response observed at the protein level and confirm that the role of humoral defense in this syndrome should not be ignored. These results add more evidence to one of the leading the hypothesis about IBS pathophysiology: increased intestinal permeability evokes an uncontrolled access of luminal antigens and the consequent activation of plasma cell residing in *lamina propria*.

We did not observe any overrepresentation of genes related to the nervous system, despite its connection to B cells and plasma cells demonstrated at a protein level is clear. Transcriptomic analysis provides a lot of information but we may have missed post-transcriptional changes that affect differently patients and HV. For this reason, working with an antibody-producing cell *in vitro* model to study how neuropeptides affect Ig production (168,169) could be a good approach to obtain a better understanding of IBS-D pathophysiology, specifically SP, involved in nociception and described as increased in IBS (178,184,185).

In order to develop a tool to study the neuro-immune cross talk between B cells and SP in IBS, we evaluated different *in vitro* models to select the one most suitable to address our objective. Considering the evidence supporting the local activation of humoral response, first we approached our aim isolating plasma cells from the intestinal mucosa by two different procedures: ileal specimens from surgery and jejunal biopsies collected in vivo with the Watson's capsule. The sample weight from surgical specimens were notably higher than those obtained with the capsule in jejunum, as were the number of LPLs. When comparing the yield of our protocol to other studies, normalizing the number of isolated cells per biopsy weight, we observed similar results obtained from jejunal biopsies by other authors (186), confirming the procedure was working properly.

We used CD38 as antibody-producing cell marker for further plasma cell isolation from ileal specimens; jejunal biopsies were excluded due to the insufficient number of isolated LPL to conduct the next steps of the protocol. Around 10% of isolated LPL were CD38+, a percentage lower than expected considering only IgA-producing cells represent 30-40% of LPLs (187), but in line to other author's work, which obtained 7-12% CD19+ cells, a pan-B cell marker (186). These results may be explained by the aggressive enzymatic digestion with collagenase, which can dissect CD38. In fact, enzymatic digestion is the main drawback when performing this protocol. Collagenase activity differs among commercial brands and even between batches from the same

supplier and establishing the right concentration can be quite challenging (too aggressive damage the tissue and too light is no sufficient). These difficulties, noted before in the literature (186,188), lead us to consider blood B cells as an alternative for the *in vitro* model.

We induced the differentiation process into plasma cells from blood isolated B cells with interleukins, antigens and mitogens, mimicking the physiological conditions found in secondary lymph nodes and previously used in the literature (83,189–193). However, the short-life span characteristic of primary culture, and also observed in these cells, represents a handicap to perform further stimulation assays with this model. When we evaluated differentiated cells, we observed they increase their size and showed a more granular cytoplasm, according to flow cytometry results, similar to plasma cells when they differentiate and initiate Ig production. Not all these cells are positive for the pan-B cell marker CD20 at day 4 and 7 of the differentiation, despite its expression can be detected upon isolation, may be because they are highly stimulated as they decrease CD20 expression in late phases of the ontogeny (57,59). CD138 terminally-differentiated plasma cell marker can be detected at day 4 and 7, demonstrating the success of the protocol despite a low yield. However, optimizing this procedure is again challenging and time-consuming. For this study, we used total B cells isolated from blood, which according to the literature contains 65% of naïve B cells (194), and triggering differentiation is more complex as it requires activating first these cells and then inducing the differentiation. Hence, we decided to stop the characterization of the differentiated B cells and to evaluate total B cells isolated from blood and an immortalized B cell line (126BLCL cells) as potential *in vitro* models.

We characterized both B cell lines at baseline and how they responded to SP stimulus to decide which cells are the most feasible model. Despite 126BLCL are in a more differentiated state of the B cell ontogeny compared to B cells isolated from blood, as shown by the higher levels of XBP1 and BLIMP1, genes associated with B cell differentiation, we could not detect TACR1 protein expression at a basal level, contrary to blood cells. The ability to respond to SP was key for our experimental design, but this receptor can also be found intracellularly and its transport is stimulus-depend (195); consequently, we stimulated both cell lines to choose the best option. B cells isolated from blood responded at lower concentrations of SP, based on changes in TACR1 protein expression. No changes were observed in 126BLCL when exposed to SP at a gene expression level.

In the literature, it has been described immortalization with EBV can cause irreversible epigenetic alterations in genes involved in activation and differentiation process into plasma cell, including *XBP1* and *BLIMP1* (196). Hence, the difficulties observed when inducing changes

in TACR1 expression in 126BLCL may be explained by the EVB transformation process. The inability to trigger the differentiation in these cells excludes them as a good B cell *in vitro* model to study the effect of SP in humoral response activation. Therefore, B cells from blood, without undergoing plasma cell differentiation, remained the most suitable option.

This study confronts several B cell *in* vitro models from different sources, both primary culture and an established cell line. This project allowed us to obtain a useful tool to analyze in the future how SP affects humoral response. The proper B cell response to SP assays will be performed, including the agonist and antagonists in the experimental design, and a full phenotypical characterization of the cells before and after the exposure will be carried out. Despite we have focused our research on SP, this *in* vitro model may be suitable for other neuropeptides, including VIP or CRF.

In summary, our results highlight the relevance of antibody-mediated responses in IBS. More research testing the antibody-recognition to different epitopes by mucosal Ig content from IBS patients could provide evidence of which antigens are triggering this response. Identifying the antigen responsible of this activation is as interesting as challenging; it may be different for the subgroups of patients and acting synergistically with other antigens. The link of the immune defense with the nervous systems, confirmed by the proximity found between plasma cell and nerve endings, together with the correlation between the amount of IgG and the intensity of abdominal pain in patients, is also crucial to understand the origin and evolution of symptoms in IBS and to design new strategies for management. B cell *in vitro* models, such as the one evaluated in this thesis could help us to achieve a deeper understanding of the neuro-immune crosstalk and give us more information about the underlying causes of IBS and other functional disorders of the gut-brain axis.

CONCLUSIONS

10. CONCLUSIONS

The results of this thesis have generated the following conclusions:

1. The intestinal concentration of IgG and of the subtype IgG2 are higher in IBS-D than in controls and positively correlates with the intensity of abdominal pain.

2. The amount of intestinal and systemic LPS and immunoglobulins anti-LPS are similar in IBS-D and controls.

3. The higher proximity between plasma cells and nerve endings in the jejunal mucosa of IBS-D respect to controls, and its association with stress and depression levels reported by patients highlights neuro-immune interactions as key factors in IBS pathophysiology.

4. Mucosal immune signatures associated with the humoral response are overrepresented at a gene expression level in the jejunal mucosa of IBS-D patients.

5. Gene expression analysis identifies the immunoglobulin structure as highly enriched in the jejunal mucosa of IBS-D as compared with controls.

6. Blood isolated B cells are the most suitable in vitro model to study the effect of neuropeptides on activation and differentiation of B cells.

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ANNEX 1

11. ANNEX 1

11.1. SUPPLEMENTARY DATA CHAPER 1

Clinical and demographic characteristics of participants and experimental procedures

	HV	IBS-D
Inclusion criteria		
Age: 18-60 years	yes	yes
Rome III criteria for IBS-D	no	yes
Naïve (newly-diagnosed)	no	yes
Acceptance of the study protocol	yes	yes
Exclusion criteria		
Clinical history of food allergy	yes	yes
Positivity to SPT to foodstuff	yes	yes
Infectious gastroenteritis	yes	yes
Gastrointestinal comorbidities	yes	yes
Pregnancy	yes	yes
Major psychiatric disorders	yes	yes
Use of medication (steroids, immunosuppressive drugs, anti-histaminic and mast cell stabilizers)	yes	yes

Table S1: Inclusion and exclusion criteria for the study participants. HV: Healthy Volunteer**;** IBS-D: Diarrhea-predominant Irritable Bowel Syndrome; SPT: skin prick test.

	Gender	Atopy	Age (years)	Bowel movements	Bristol score (1-7)	Holmes score	Cohen score	Beck's Index	Frecuency abdominal pain	Intensity abdominal pain (0-100)	Distention	Dyspepsia
HV1	M	-	26	1.5	3.5	45	7	0	0	0	0	0
HV2	F	-	26	1	3	53	12	3	0	0	0	0
HV3	F	-	26	1	3.5	25	11	0	0	0	0	0
HV4	M	-	30	2	3	50	14	0	0	0	0	0
HV5	M	-	**35**	**-**	**-**	**-**	**-**	**-**	0	0	0	0
HV6	F	-	37	-	-	-	-	-	0	0	0	0
HV7	F	-	41	-	-	-	-	-	0	0	0	0
HV8	M	1	22	-	-	-	-	-	0	0	0	0
HV9	F	1	24	-	-	-	-	-	0	0	0	0
HV10	M	-	22	-	-	-	-	-	0	0	0	0
HV11	M	-	-	-	-	-	-	-	0	0	0	0
HV12	F	-	-	-	-	-	-	-	0	0	0	0
HV13	M	-	40	-	-	-	-	-	0	0	0	0
HV14	M	-	22	-	-	-	-	-	0	0	0	0
HV15	M	-	36	-	-	-	-	-	0	0	0	0
HV16	M	1	29	-	3	151	15	0	0	0	0	0
HV17	F	1	27	-	-	64	14	1	0	0	0	0
HV18	M	0	35	-	4	64	13	1	0	0	0	0
HV19	M	1	31	-	-	179	17	1	0	0	0	0
HV20	M	-	32	-	-	90	17	2	0	0	0	0
HV21	F	0	41	-	-	75	37	22	0	0	0	0
HV22	F	-	28	-	-	95	25	4	0	0	0	0
HV23	F	0	34	-	-	18	13	1	0	0	0	0
HV24	M	1	42	-	-	75	25	1	0	0	0	0
HV25	M	0	23	-	-	69	22	1	0	0	0	0
HV26	M	0	21	-	-	112	6	0	0	0	0	0
HV27	F	0	48	-	-	63	12	0	0	0	0	0
HV28	F	1	45	-	-	127	14	5	0	0	0	0
HV29	F	0	28	1	4	189	16	2	0	0	0	0
HV30	F	0	53	1	2	121	8	4	0	0	0	0
HV31	F	1	23	-	-	261	16	5	0	0	0	0
HV32	F	0	18	1.5	3.5	64	19	0	0	0	0	0
HV33	M	0	19	2	4	61	9	0	0	0	0	0
HV34	M	0	25	0.5	3.5	136	13	1	0	0	0	0

	Gender	Atopy	Age (years)	Bowel movements	Bristol score (1-7)	Holmes s ore	Cohen score	Beck's Inventoy	Frecuency abdominal pain	Intensity abdominal pain (0-100)	Distention	Dyspepsia
HV35	F	0	19	1	2.5	25	13	0	0	0	0	0
HV36	F	0	54	2	4	88	4	0	0	0	0	0
HV37	F	1	25	2	4	25	11	0	0	0	0	0
HV38	M	-	36	1	3.5	73	9	5	0	0	0	0
HV39	F	-	24	-	-	114	16	0	0	0	0	0
HV40	M	0	26	1.5	3	293	36	11	0	0	0	0
HV41	M	0	25	2.5	3.5	133	14	0	0	0	0	0
HV42	F	-	32	1.5	3.5	179	53	7	0	0	0	0
HV43	M	0	36	-	-	228	13	0	0	0	0	0
HV44	F	-	26	1	3	53	6	0	0	0	0	0
IBS-D1	F	1	37	6	6.5	397	37	31	10	81	-	-
IBS-D2	F	0	38	4	6.1	142	27	19	5	45	-	-
IBS-D3	F	1	36	4	5.4	71	32	6	3	15	-	-
IBS-D4	F	0	29	6	6.5	186	26	17	5	15	-	-
IBS-D5	M	0	37	8	5.8	25	13	2	6	53	-	-
IBS-D6	F	0	59	3	6	246	32	24	10	67	-	-
IBS-D7	M	0	39	5	6.6	20	13	13	2	50	-	-
IBS-D8	F	0	34	-	-	123	28	8	-	-	-	1
IBS-D9	F	1	25	-	-	151	32	22	-	-	-	1
IBS-D10	M	1	37	-	-	25	14	11	-	-	-	0
IBS-D11	F	0	41	-	-	197	17	4	-	-	-	-
IBS-D12	F	0	34	3	6	166	41	30	-	100	-	1
IBS-D13	F	0	51	3.5	6	49	15	7	-	59	-	0
IBS-D14	M	1	37	1	5.5	226	17	1	-	33	-	0
IBS-D15	F	-	-	-	-	-	11	3	-	-	-	-
IBS-D16	F	0	37	-	-	103	15	14	-	-	-	1
IBS-D17	F	1	38	-	-	306	30	13	-	-	-	1
IBS-D18	F	-	21	-	-	88	16	4	-	-	-	1
IBS-D19	F	0	49	-	-	238	24	11	-	-	-	1
IBS-D20	F	0	44	-	-	152	37	21	-	-	-	0
IBS-D21	F	1	27	-	-	133	13	1	-	-	-	0
IBS-D22	F	1	32	2.5	4.5	261	28	6	-	18	-	1

	Gender	Atopy	Age (years)	Bowel movements	Bristol score (1-7)	Holmes score	Cohen score	Beck's Inventory	Frecuency abdominal pain	Intensity abdominal pain (0-100)	Distention	Dyspepsia
IBS-D23	M	1	34	2.5	6	167	36	10	-	60	-	0
IBS-D24	M	0	31	3.5	5.5	138	10	2	-	58	-	1
IBS-D25	M	1	36	-	-	58	18	10	-	-	-	0
IBS-D26	F	1	25	-	-	0	27	2	-	-	-	0
IBS-D27	F	1	39	-		123	34	13	-	-	-	0
IBS-D28	F	1	56	1.5	6	110	16	12	-	-	-	-
IBS-D29	F	0	64	2	6.5	0	29	8	10	62.5	56	0
IBS-D30	F	-	24	-	4	25	14	1	-	-	-	-
IBS-D31	-	-	-	-	-	-	-	-	-	-	-	-
IBS-D32	F	0	43	5	7	69	29	24	-	-	-	-
IBS-D33	F	0	39	10	5.5	119	36	16	8	30	70	1
IBS-D34	M	0	41	12	7	99	15	2	5	30	0	0
IBS-D35	M	0	34	2	5.5	54	32	14	4	25	15	1
IBS-D36	F	0	50	3	6	227	25	7	5	65	67	1
IBS-D37	F	1	36	3	6	73	33	14	5	39	0	0
IBS-D38	M	1	27	1	5	87	24	2	10	6	9	1
IBS-D39	F	0	35	5	6	350	29	23	5	60	78	1
IBS-D40	M	0	28	3.5	6.5	308	20	8	4	43	22	0
IBS-D41	F	1	21	7	6.5	274	22	10	10	80	61	1
IBS-D42	F	0	39	10	6.5	64	18	13	3	100	100	1
IBS-D43	F	1	24	7	6.5	25	28	13	8	17	19	1
IBS-D44	F	1	62	4	6	889	37	24	10	76	47	1
IBS-D45	M	0	43	3	6.5	113	26	5	2	31	47	0

Table S2. Clinical and demographical characteristics of participants. F: Female; M: Male; 0: Negative; 1: Positive. Non-available data is represented with a hyphen.

Participant	Experimental procedure
HV1	13
HV2	5,6,8,9,12,13
HV3	5,6,8,9,12,13
HV4	5,6,8,9,12,13
HV5	5,6,8,12,13
HV6	1,2,3,4,5,6,7,8,12,13
HV7	1,2,3,4,7,8,12,13
HV8	1,2,3,4,6,7,8,12
HV9	1,2,3,4,5,6,7,8
HV10	1,2,3,4,5,6,7,8,12
HV11	1,2,3,4,5,6,7,8
HV12	1,2,3,4,5,6,7,8,13
HV13	1,2,3,4,5,6,8,12
HV14	1,2,3,4,7,8,13
HV15	1,2,3,4,5,6,8,12
HV16	14
HV17	14
HV18	14
HV19	14
HV20	14
HV21	14
HV22	14
HV23	14
HV24	14
HV25	14
HV26	14
HV27	14
HV28	14
HV29	14
HV30	14
HV31	1,2,3,4,5,6,7,8,9,10,11,12,13,16
HV32	1,2,3,4,5,6,7,8,9,10,11,12,13,15
HV33	1,2,3,4,5,6,7,8,9,10,11,12,13,15
HV34	1,2,3,4,5,6,7,8,11,12
HV35	1,2,3,4,5,6,7,8,9,10,11,12,13
HV36	1,2,3,4,5,6,7,9,10,11,12,13,15,16

Participant	Experimental procedure
HV37	1,2,3,4,5,6,7,8,9,11,12,13
HV38	14
HV39	14
HV40	14
HV41	14
HV42	14
HV43	14
HV44	14
IBS-D1	14
IBS-D2	14
IBS-D3	14
IBS-D4	14
IBS-D5	14
IBS-D6	14
IBS-D7	14
IBS-D8	1,2,3,4,6,7,8,9,10,11,12
IBS-D9	1,2,3,4,5,6,7,8,9,10,11,12
IBS-D10	1,2,3,4,5,6,7,8,9,10,11,12,13
IBS-D11	1,2,3,4,5,6,7,8,10,11
IBS-D12	1,2,3,4,5,6,7,8,11,12,13
IBS-D13	1,2,3,4,5,6,7,8,9,10,11,12,13
IBS-D14	1,2,3,4,5,6,7,8,9,10,11,12,13
IBS-D15	1,2,3,4,5,6,7,8,9,10,11,12,13
IBS-D16	1,2,3,4,5,6,7,8,9,10,11,12,13
IBS-17	1,2,3,4,5,6,7,8,10,11,13
IBS-D18	1,2,3,4,5,6,7,8,9,10,11,12,13
IBS-D19	1,2,3,5,6,7,8,10,11,13
IBS-D20	1,2,3,4,5,6,7,8,10,11
IBS-D21	1,2,3,4,5,6,7,8
IBS-D22	1,2,3,4,5,6,8,13
IBS-D23	1,2,3,4,5,6,7,8,13
IBS-D24	15
IBS-D25	1,2,3,4,5,6,8,13
IBS-D26	1,2,3,4,5,6,8,9,12,13
IBS-D27	1,2,3,4,5,6,8,13
IBS-D28	15,16

Participant	Experimental procedure
IBS-D29	15,16
IBS-D30	14
IBS-D31	14
IBS-D32	14
IBS-D33	14
IBS-D34	14
IBS-D35	14
IBS-D36	14
IBS-D37	14

Participant	Experimental procedure
IBS-D38	14
IBS-D39	14
IBS-D40	14
IBS-D41	14
IBS-D42	14
IBS-D43	14
IBS-D44	14
IBS-D45	14

Table S3. Experimental procedures performed with participant biological samples. 1-8: Immunoglobulin quantification in fecal supernatant by ELISA (sIgA, IgM, IgG, IgG1, IgG2, IgG3, IgG4, IgE, respectively); 9-11: anti-lipopolysaccharide immunoglobulin quantification in plasma by ELISA (IgA, IgM, IgG, respectively); 12: anti-lipopolysaccharide immunoglobulin A quantification in fecal supernatant by ELISA; 13: lipopolysaccharide quantification in fecal supernatant by ELISA; 14: Transmission Electron Microscopy; 15-16: D138+PGP9.5+ and D38+TA R1+ immunofluorescence stainings, respectively.

	Stool weight/aliquot	Concentration	Homogenization method	Centrifugation conditions	Conclusions
sIgA, IgM, IgG					
Condition 1	100mg	≥20mg/mL	Vortex	13000g 5min 4º	Detectable
IgG1, IgG2, IgG3, IgG4					
Condition 1	100mg	10-20mg/mL	Vortex	13000g 5min 4º	Low signal
Condition 2	100mg	40-100mg/mL	Vortex	13000g 5min 4º	Low signal, deficient homogenization
Condition 3	120-160mg	100mg/mL	Bead Beater	13000g 5min 4º	Low signal
Condition 4	200mg	100mg/mL	Bead Beater	13000g 5min 4º	Detectable
IgE					
Condition 1	200mg	100mg/mL	Bead Beater	13000g 5min 4º	Low signal
Condition 2	200mg	200mg/mL	Bead Beater	13000g 5min 4º	Detectable
anti-LPS IgA					
Condition 1	200mg	200mg/mL	Bead Beater	13000g 5min 4º	Detectable
Anti-LPS IgM, anti-LPS IgG					
Condition 1	200mg	200mg/mL	Bead Beater	13000g 5min 4º	Non-detectable
Condition 2	900mg	360mg/mL	Bead Beater	13000g 5min 4º	Non-detectable
Condition 3	900mg	1500-2000mg/mL	Bead Beater	13000g 5min 4º	Deficient homogenization, low signal
Condition 4	900mg	1500-2000mg/mL	Liofilization 24h/48h	-	Deficient homogenization
LPS					
Condition 1	200mg	20-100mg/mL	Bead Beater	4000g 10min 4º	Better signal at 100mg/mL, large pellet
Condition 2	200mg	100mg/mL	Bead Beater	1000g 20-10min 4º	Deficient centrifugation
Condition 3	200mg	100mg/mL	Bead Beater	3000g 10min 4º	Detectable

Table S4: Summary of the conditions tested for Ig and LPS determination in stool supernatant. Vortex protocol consist of sample homogenization in a vortex during 2min at room temperature. For Bead Beater method, samples are homogenized in a Mini-Beater-16 ell disrupter (Biospec products) in two rotations of 3min and 5min at room temperature, respectively, separated by an incubation at 4º during 3min. Ig: Immunoglobulin; LPS: Lipopolysaccharide.

	r_s	p-value	HV	IBS-D
Luminal IgA and anti-LPS	-0.0351	0.887	11	8
Systemic IgA and anti-LPS	0.0179	0.954	8	7
Systemic IgM and anti-LPS	-0.242	0.426	5	8
Systemic IgG and anti-LPS	-0.0107	0.974	6	9

Table S5: Analysis of the correlation between luminal LPS and anti-LPS luminal and systemic Igs. HV: Healthy Volunteer; IBS-D: Diarrhea-predominant Irritable Bowel Syndrome; LPS: Lipopolysaccharide.

11.2. SUPPLEMENTARY DATA CHAPER 2

Clinical and demographic characteristics of participants

	HV	IBS-D
Inclusion criteria		
Age: 18-60 years	yes	yes
Rome III criteria for IBS-D	no	yes
Naïve (newly-diagnosed)	no	yes
Acceptance of the study protocol	yes	yes
Exclusion criteria		
Clinical history of food allergy	yes	yes
Positivity to SPT to foodstuff	yes	yes
Infectious gastroenteritis	yes	yes
Gastrointestinal comorbidities	yes	yes
Pregnancy	yes	yes
Major psychiatric disorders	yes	yes
Use of medication (steroids, immunosuppressive drugs, anti-histaminic and mast cell stabilizers)	yes	yes

Table S1: Inclusion and exclusion criteria for the study participants. HV: Healthy Volunteer**;** IBS-D: Diarrhea-predominant Irritable Bowel Syndrome; SPT: skin prick test.

	Group	Gender	Atopy	Age (years)	Bowel movements	Bristol score (1-7)	Holmes score	Cohen score	Beck's Inventory	Intensity abdominal pain (0-100)	Francis	Dyspepsia
VHIR1	HV	M	1	23	2	3.5	51	14	0	0	24	0
VHIR2	IBS-D	F	0	25	2	5	318	24	13	57	288	1
VHIR3	HV	F	0	22	1	3.5	229	8	0	0	30	0
VHIR4	IBS-D	F	1	69	3	6	81	22	1	16	129	0
VHIR5	HV	F	0	28	2	3	33	-	-	0	2	0
VHIR6	IBS-D	M	0	48	3	5	103	7	3	18	240	0
VHIR7	HV	M	1	22	1.5	3	37	-	-	0	10	0
VHIR8	IBS-D	F	0	20	3.5	5	49	26	6	63	330	-
VHIR9	HV	M	0	23	1.5	3.5	222	-	-	0	0	0
VHIR10	IBS-D	M	1	20	3	6	148	29	9	76	286	1
VHIR11	HV	F	0	22	1	4	88	-	-	0	15	0
VHIR12	IBS-D	F	1	31	5	6.5	50	50	15	75	438	1
VHIR13	HV	M	0	22	2	3.5	69	-	-	0	280	0
VHIR14	IBS-D	F	0	31	2.5	4.8	79	17	6	69	289	1
VHIR15	HV	M	0	22	1	4	133	-	-	0	13	0
VHIR16	IBS-D	F	0	34	3	6	166	41	30	100	426	1
VHIR17	HV	F	0	19	1.5	3.5	95	-	-	0	18	0
VHIR18	IBS-D	F	0	51	3.5	6	49	15	7	59	352	0
VHIR19	HV	M	1	22	2.5	3	209	-	-	0	39	0
VHIR20	IBS-D	M	1	37	1	5.5	226	17	1	33	302	0
VHIR21	HV	F	0	30	1	2.5	170	-	-	0	236	0
VHIR22	IBS-D	F	1	32	2.5	4.5	261	28	6	18	258	1
VHIR23	HV	F	-	-	-	-	-	-	-	-	-	-
VHIR24	IBS-D	M	1	34	2.5	6	167	36	10	60	372	0
VHIR25	HV	M	-	-	-	-	-	-	-	-	-	-
VHIR26	IBS-D	M	0	41	2	6	109	19	4	47	221	0
VHIR27	HV	F	-	-	-	-	-	-	-	-	-	-
VHIR28	IBS-D	M	0	31	3.5	5.5	138	10	2	58	374	1

	Group	Gender	Atopy	Age (years)	Bowel movements	Bristol score (1-7)	Holmes score	Cohen score	Beck's Inventory	Intensity abdominal pain (0-100)	Francis	Dyspepsia
VHIR29	HV	M	-	-	-	-	-	-	-	-	-	-
VHIR30	IBS-D	F	0	42	6.5	6	155	21	7	95	371	0
VHIR31	HV	F	-	18	1.5	3.5	64	19	0	0	8	0
VHIR32	IBS-D	M	1	43	1	5	168	10	4	0	0	0
VHIR33	HV	F	0	19	1	2.5	25	13	0	0	30	0
VHIR34	IBS-D	F	0	29	3	6.5	770	22	11	75	236	0
VHIR35	HV	F	0	20	1	5	25	18	0	0	42	0
VHIR36	IBS-D	F	0	48	3.5	6.5	5	29	-	40	293	1
VHIR37	HV	F	0	19	0.5	3.5	85	16	0	0	15	0
VHIR38	IBS-D	F	0	40	2	6	13	8	3	59	364	1
VHIR39	HV	F	0	22	1.5	3.5	13	22	0	0	18	0
VHIR40	IBS-D	M	1	33	2.5	5.5	160	-	-	27	159	0

Table S2. Clinical and demographical characteristics of participants. Non-available data is represented with a hyphen. F: Female; M: Male.

Code	RIN	Code	RIN	Code	RIN
VHIR 1	8.2	**VHIR 15**	8.1	**VHIR 29**	9.3
VHIR 2	7.5	**VHIR 16**	8.6	**VHIR 30**	7.3
VHIR 3	9.1	**VHIR 17**	9.1	**VHIR 31**	7
VHIR 4	8.9	**VHIR 18**	7.7	**VHIR 32**	8.5
VHIR 5	7.7	**VHIR 19**	7.4	**VHIR 33**	8.9
VHIR 6	7.3	**VHIR 20**	8.8	**VHIR 34**	9.2
VHIR 7	8.6	**VHIR 21**	9.2	**VHIR 35**	7.9
VHIR 8	7.2	**VHIR 22**	8.2	**VHIR 36**	8.8
VHIR 9	8.6	**VHIR 23**	9	**VHIR 37**	7.2
VHIR 10	7.8	**VHIR 24**	8.4	**VHIR 38**	7.5
VHIR 11	7.7	**VHIR 25**	8.7	**VHIR 39**	8.3
VHIR 12	7.3	**VHIR 26**	7.6	**VHIR 40**	7.9
VHIR 13	9.2	**VHIR 27**	9.1		
VHIR 14	8.7	**VHIR 28**	8		

Table S3. RNA Integrity Number (RIN) of samples included in this study to evaluate the RNA quality.

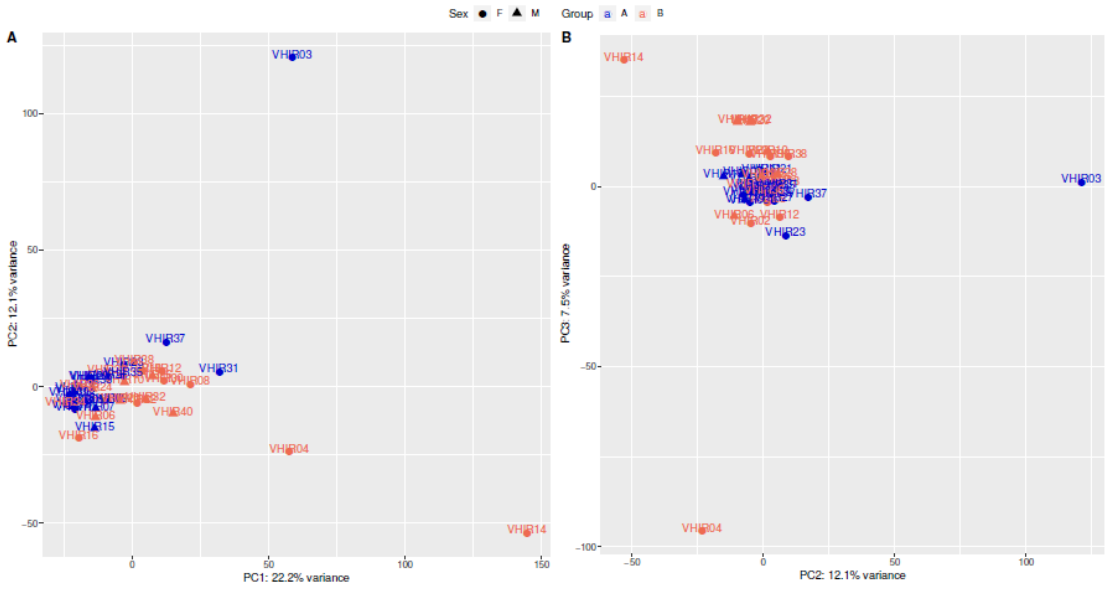

Figure S1: Exploratory analysis of the transformed read counts of 27,669 genes included in this study. The first three principal components are shown obtained from the PCA computed over all samples. Blue refers to HV (Healthy Volunteers) and orange to IBS-D (Diarrhea-predominant Irritable Bowel Syndrome). Left figure corresponds to PC1-PC2 and right figure corresponds to PC2-PC3. As a result of the exploratory analysis, two samples from the IBS-D group (VHIR4 and VHIR 14) and one from the HV group (VHIR3) were excluded from the study due to their identified behavior.

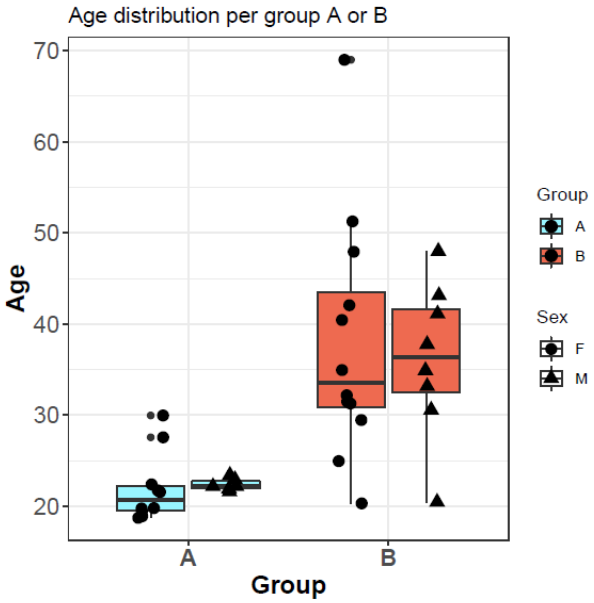

Figure S2: Boxplot and stripchart of quantitative variable Age divided per HV and IBS-D. Dotted black line refers to mean +/- one standard deviation. Variable Age was not included in DEG analysis due to the differences presented between groups.

Gene Name	Description	log2FC	p adj
COL14A1	Collagen Type XIV Alpha 1 Chain	0.7529	0.0027
HEMK1	HemK Methyltransferase Family Member 1	-0.3459	0.0027
AC026803.3	Uncharacterized	**-0.5517**	**0.0027**
GREM1	Gremlin 1, DAN Family BMP Antagonist		0.0027
ZNF320	Zinc finger protein 320	-0.4721	0.0027
DHX58	DExH-Box Helicase 58	-0.3363	0.0083
IGKV1D-16	Immunoglobulin Kappa Variable 1D-16	12.030	0.0093
TP73-AS1	TP73 Antisense RNA 1	-0.3871	0.0099
KIAA0232	Uncharacterized	0.3638	0.0145
RNF207	Ring Finger Protein 207	-0.6362	0.0145
CERCAM	Cerebral Endothelial Cell Adhesion Molecule	0.3660	0.0242
ZNF552	Zinc Finger Protein 552	-0.2643	0.0254
REC8	REC8 Meiotic Recombination Protein	-0.6737	0.0259
CCDC24	Coiled-Coil Domain Containing 24	-0.3422	0.0263
AC008760.1	Uncharacterized	-0.7764	0.0263
XAF1	XIAP Associated Factor 1	-0.4827	0.0263
LAMA2	Laminin Subunit Alpha 2	0.3176	0.0273
SPNS1	Sphingolipid Transporter 1 (Putative)	-0.7370	0.0273
GADD45A	Growth Arrest And DNA Damage Inducible Alpha	0.4376	0.0282
GPR15	G Protein-Coupled Receptor 15	15.059	0.0282
HDGF	Heparin Binding Growth Factor	0.1810	0.0282
CACNA1H	Calcium Voltage-Gated Channel Subunit Alpha1 A	-0.6903	0.0282
ACOX2	Acyl-CoA Oxidase 2	-0.2922	0.0302
TXLNGY	Taxilin Gamma Pseudogene, Y-Linked		0.0302
BAHCC1	BAH Domain And Coiled-Coil Containing 1	-0.3226	0.0303
CLSPN	Claspin	0.4455	0.0336
NXPE2	Neurexophilin And PC-Esterase Domain Family Member 2	13.603	0.0336
AC008763.1	Uncharacterized	-0.9207	0.0336
ARL2BP	ADP Ribosylation Factor Like GTPase 2 Binding Protein	0.2084	0.0336
C1GALT1C1	C1GALT1 Specific Chaperone 1	0.2157	0.0336
KDM5D	Lysine Demethylase 5D		0.0336
ANAPC1	Anaphase Promoting Complex Subunit 1	0.3509	0.0343
OBSCN	Obscurin	-0.4762	0.0343
ZXDC	ZXD Family Zinc Finger	-0.2387	0.0343
IGLV8-61	Immunoglobulin Lambda Variable 8-61		0.0343

Gene Name	Description	log2FC	p adj
IKZF4	IKAROS Family Zinc Finger 4	-0.3822	0.0343
TUBG1	Tubulin Gamma 1	0.3113	0.0343
ACTG2	Actin Gamma 2	0.5121	0.0365
ADARB2	Adenosine Deaminase RNA Specific B2	-11.921	0.0365
AC087222.1	Uncharacterized		0.0365
PSME4	Proteasome Activator Subunit 4	0.2316	0.0366
CLUHP3	Clustered Mitochondria Homolog Pseudogene 3	-0.3308	0.0419
IGLV1-51	Immunoglobulin Lambda Variable 1-51	0.7883	0.0419
PACS2	Phosphofurin Acidic Cluster Sorting Protein 2	-0.2195	0.0428
AC000123.2	Uncharacterized	-0.5422	0.0446
AC093827.4	Uncharacterized	-0.5520	0.0446
LINC00526	Long Intergenic Non-Protein Coding RNA 526	0.3027	0.0446
NPTN-IT1	NPTN Intronic Transcript 1	0.7511	0.0446
SHANK3	SH3 And Multiple Ankyrin Repeat Domains 3	-0.4561	0.0446
TIAF1	TGFB1-Induced Anti-Apoptotic Factor 1	-0.4808	0.0446
ABCA2	ATP Binding Cassette Subfamily A Member 2	-0.4321	0.0473
SVEP1	Sushi, Von Willebrand Factor Type A, EGF And Pentraxin Domain Containing 1	0.3671	0.0473
MLLT6	Myeloid/Lymphoid Or Mixed-Lineage Leukemia, Translocated To, 6	-0.2802	0.0473
NEURL2	Neuralized E3 Ubiquitin Protein Ligase 2	-0.4696	0.0473
SLC11A2	Solute Carrier Family 11 Member 2		0.0473
CIITA	Class II Major Histocompatibility Complex Transactivator	-0.4303	0.0482
ZNF740	Zinc Finger Protein 740	-0.1960	0.0503

Table S4. DEGs when comparing IBS-D *versus* HV. log2FC: log2 Fold Change; p adj: adjusted p-value.

MSigDB ID	Immunological signatures associated to humoral defense Standard name	FDR q-value
GSE22886	**NAIVE_BCELL_VS_BLOOD_PLASMA_CELL_DN**	0.2003
GSE37301	**LYMPHOID_PRIMED_MPP_VS_PRO_BCELL_UP**	0.2189
GSE29618	**BCELL_VS_PDC_DN**	0.2224
GSE3982	**NEUTROPHIL_VS_BCELL_DN**	0.2248
GSE12845	**IGD_NEG_BLOOD_VS_NAIVE_TONSIL_BCELL_UP**	0.2249
GSE22886	**IGM_MEMORY_BCELL_VS_BM_PLASMA_CELL_DN**	0.2264
GSE13411	**PLASMA_CELL_VS_MEMORY_BCELL_UP**	0.2273
GSE13547	**CTRL_VS_ANTI_IGM_STIM_ZFX_KO_BCELL_2H_UP**	0.2274
GSE13547	**WT_VS_ZFX_KO_BCELL_ANTI_IGM_STIM_2H_UP**	0.2285
GSE21063	**CTRL_VS_ANTI_IGM_STIM_BCELL_NFATC1_KO_8H_DN**	0.2265
GSE12845	**NAIVE_VS_PRE_GC_TONSIL_BCELL_DN**	0.2295
GSE12366	**PLASMA_CELL_VS_MEMORY_BCELL_UP**	0.2307
GSE12845	**IGD_POS_BLOOD_VS_DARKZONE_GC_TONSIL_BCELL_DN**	0.2308
GSE12845	**PRE_GC_VS_DARKZONE_GC_TONSIL_BCELL_DN**	0.2325
GSE23114	**PERITONEAL_CAVITY_B1A_BCELL_VS_SPLEEN_BCELL_DN**	0.2341
GSE12845	**NAIVE_VS_DARKZONE_GC_TONSIL_BCELL_DN**	0.2374
GSE28237	**FOLLICULAR_VS_EARLY_GC_BCELL_DN**	0.2382
GSE13547	**WT_VS_ZFX_KO_BCELL_DN**	0.2389
GSE13547	**2H_VS_12_H_ANTI_IGM_STIM_BCELL_DN**	0.2409
GSE22886	**NAIVE_BCELL_VS_MONOCYTE_DN**	0.2435
GSE12366	**GC_VS_NAIVE_BCELL_UP**	0.2446
GSE15330	**LYMPHOID_MULTIPOTENT_VS_GRANULOCYTE_MONOCYTE_PROGENITOR_DN**	0.2464
GSE22886	**_NAIVE_BCELL_VS_DC_DN**	0.2468
GSE27786	**BCELL_VS_MONO_MAC_UP**	0.2481
GSE13411	**NAIVE_BCELL_VS_PLASMA_CELL_DN**	0.2484

Table S5. Immunological signatures associated with humoral defense obtained by GSEA comparing IBS-D *versus* HV. MiSigDB ID and stardard name assigned by authors are indicated. FDR: False-Discovery Rate.

Gene Name	Description	ES
GREM1	Gremlin 1. DAN family BMP antagonist	0.3576
IGKV1D-16	Immunoglobulin kappa variable 1D-16	0.2870
GREM2	Gremlin 2. DAN family BMP antagonist	0.2856
GGT4P	Gamma-glutamyltransferase 4 pseudogene	0.2611
IGHV3-73	Immunoglobulin heavy variable 3-73	0.2269
IGLV8-61	Immunoglobulin lambda variable 8-61	0.2263
IGLV3-9	Immunoglobulin lambda variable 3-9 (gene/pseudogene)	0.2242
GPR15	G protein-coupled receptor 15	0.2236
ERAP2	Endoplasmic reticulum aminopeptidase 2	0.2204
IGLV7-46	Immunoglobulin lambda variable 7-46 (gene/pseudogene)	0.2165
TYRP1	Tyrosinase related protein 1	0.2101
MT-TT	Mitochondrially encoded tRNA-Thr (ACN)	0.2096
IGKV1D-13	Immunoglobulin kappa variable 1D-13	0.2084
IGLV2-18	Immunoglobulin lambda variable 2-18	0.2061
IGLV3-21	Immunoglobulin lambda variable 3-21	0.2052
IGLV5-45	Immunoglobulin lambda variable 5-45	0.2025
RPL13P12	Ribosomal protein L13 pseudogene 12	0.1984
IGKV6D-21	Immunoglobulin kappa variable 6D-21 (non-functional)	0.19505
IGHG3	Immunoglobulin heavy constant gamma 3 (G3m marker)	0.1939
PSPHP1	Phosphoserine phosphatase pseudogene 1	0.1936
HPCAL4	Hippocalcin like 4	0.1874
IGHV3-20	Immunoglobulin heavy variable 3-20	0.1865
CCL13	C-C motif chemokine ligand 13	0.1856
COL14A1	Collagen type XIV alpha 1 chain	0.1854
IGLV2-11	Immunoglobulin lambda variable 2-11	0.1850
IGKV1-16	Immunoglobulin kappa variable 1-16	0.1833
IGKV1-9	Immunoglobulin kappa variable 1-9	0.1813
IGHV3-49	Immunoglobulin heavy variable 3-49	0.1807
IGHV4-61	Immunoglobulin heavy variable 4-61	0.1806
IGHV1-58	Immunoglobulin heavy variable 1-58	0.1804
SFRP2	Secreted frizzled related protein 2	0.1797
IGLV1-51	Immunoglobulin lambda variable 1-51	0.1763
IGHV3-74	Immunoglobulin heavy variable 3-74	0.1760
CDA	Cytidine deaminase	0.1749
UGT1A4	UDP glucuronosyltransferase family 1 member A4	0.1735
IGLV3-27	Immunoglobulin lambda variable 3-27	0.1733
IGLV2-8	Immunoglobulin lambda variable 2-8	0.1727

Gene Name	Description	ES
LCN2	Lipocalin 2	0.1717
IGLV1-41	Immunoglobulin lambda variable 1-41 (pseudogene)	0.1714
IGHV3-30	Immunoglobulin heavy variable 3-30	0.1696
ACOT12	Acyl-CoA thioesterase 12	0.1686
IGHG2	Immunoglobulin heavy constant gamma 2 (G2m marker)	0.1684
IGKV1-6	Immunoglobulin kappa variable 1-6	0.1668
IGHV7-4-1	Immunoglobulin heavy variable 7-4-1	0.1666
AKR1C1	Aldo-keto reductase family 1 member C1	0.1661
MT1M	Metallothionein 1M	0.1659
IGKV2-24	Immunoglobulin kappa variable 2-24	0.1641
AL356585.2	Immunoglobulin superfamily. member 3 (IGSF3) pseudogene	0.1632
IGHV3-53	Immunoglobulin heavy variable 3-53	0.1614
IGLV2-14	Immunoglobulin lambda variable 2-14	0.1612
PSORS1C3	Psoriasis susceptibility 1 candidate 3	-0.1428
EHMT2-AS1	EHMT2 and SLC44A4 antisense RNA 1	-0.1444
ZNF300P1	Zinc finger protein 300 pseudogene 1	-0.1448
MUC20	Mucin 20. cell surface associated	-0.1457
MROH7	Maestro heat like repeat family member 7	-0.1463
PI4KAP1	Phosphatidylinositol 4-kinase alpha pseudogene 1	-0.1484
AL033380.1	Novel transcript	-0.1493
AC126755.1	Polycystic kidney disease 1 (autosomal dominant) (PKD1) pseudogene	-0.1496
SPNS1	Sphingolipid transporter 1 (putative)	-0.1502
AC021683.6	Novel transcript	-0.1514
NOS2	Nitric oxide synthase 2	-0.1517
ZNF415	Zinc finger protein 415	-0.1521
SMIM5	Small integral membrane protein 5	-0.1528
AC024580.1	TEC	-0.1533
TNFSF15	TNF superfamily member 15	-0.1548
HERC2P3	Hect domain and RLD 2 pseudogene 3	-0.1555
LINC00939	Long intergenic non-protein coding RNA 939	-0.1560
AP006621.5	TEC	-0.1566
SULT1C2P1	Sulfotransferase family 1C member 2 pseudogene 1	-0.1634
ADARB2	Adenosine deaminase RNA specific B2 (inactive)	-0.1639
LINC01235	Long intergenic non-protein coding RNA 1235	-0.1656
AL645608.2	Novel transcript	-0.1658
CACNA1H	Calcium voltage-gated channel subunit alpha1 H	-0.1659

Gene Name	Description	ES
XIST	X inactive specific transcript	-0.1670
SPANXN3	SPANX family member N3	-0.1683
C17orf97	Chromosome 17 open reading frame 97	-0.1700
AC008676.3	Novel protein	-0.1720
PILRB	Paired immunoglobin like type 2 receptor beta	-0.1761
AC120498.6	Novel transcript. antisense to CACNA1H	-0.1770
MIF-AS1	MIF antisense RNA 1	-0.1770
NCCRP1	Non-specific cytotoxic cell receptor protein 1 homolog (zebrafish)	-0.1774
HLA-DQB1	Major histocompatibility complex. class II. DQ beta 1	-0.1782
RNF207	Ring finger protein 207	-0.1782
AC092071.1	Novel transcript	-0.1796
REC8	REC8 meiotic recombination protein	-0.1803
NR1D1	Nuclear receptor subfamily 1 group D member 1	-0.1815
FRMD1	FERM domain containing 1	-0.1864
AKR1B15	Aldo-keto reductase family 1 member B15	-0.1944
HLA-DRB5	Major histocompatibility complex. class II. DR beta 5	-0.1960
BTNL2	Butyrophilin like 2	-0.1969
SLC44A5	Solute carrier family 44 member 5	-0.1992
PRODH	Proline dehydrogenase 1	-0.2018
LINC02520	Long intergenic non-protein coding RNA 2520	-0.2065
SLC11A2	Solute carrier family 11 member 2	-0.2117
CR2	Complement C3d receptor 2	-0.2223
LINC01595	Long intergenic non-protein coding RNA 1595	-0.2267
SULT1C2	Sulfotransferase family 1C member 2	-0.2387
AP000350.6	Novel transcript	-0.2456
FOLH1B	Folate hydrolase 1B	-0.2582
PNCK	Pregnancy up-regulated nonubiquitous CaM kinase	-0.2791

Table S6. Description and enrichment score (ES) of the top 50 genes for IBS-D *versus* HV phenotype by GSEA. These genes are represented in the HeatMap (Figure 4).

11.3. SUPPLEMENTARY DATA CHAPER 3

11.3.1. Participants

Inclusion and exclusion criteria for participants.

	HV	Ileocolectomy patients
Inclusion criteria		
Age: 18-65 years	yes	yes
Acceptance of the study protocol	yes	yes
Exclusion criteria		
Clinical history of food allergy	yes	no
Positivity to SPT to foodstuff	yes	no
Infectious gastroenteritis	yes	no
Gastrointestinal comorbidities	yes	no
Pregnancy	yes	yes
Major psychiatric disorders	yes	no
Use of medication (steroids, immunosuppressive drugs, anti-histaminic and mast cell stabilizers)	yes	no

Table S1: Inclusion and exclusion criteria for participants.

Participant number	Gender	Age (years)	Sample extracted
P1	F	27	Jejunal biopsy
P2	F	26	Jejunal biopsy
P3	F	30	Jejunal biopsy
P4	F	23	Jejunal biopsy
P5	M	25	Jejunal biopsy
P6	F	43	Jejunal biopsy
P7	F	25	Jejunal biopsy
P8	M	-	Jejunal biopsy
P9	M	37	Jejunal biopsy
P10	M	64	Jejunal biopsy
P11	F	34	Jejunal biopsy
P12	F	46	Jejunal biopsy
P13	M	47	Jejunal biopsy
P14	F	-	Jejunal biopsy
P15	F	24	Jejunal biopsy
P16	F	-	Jejunal biopsy
P17	-	-	Ileal specimen
P18	-	-	Ileal specimen
P19	-	-	Ileal specimen
P20	-	-	Ileal specimen
P21	-	-	Ileal specimen
P22	F	28	Blood
P23	F	28	Blood
P24	F	43	Blood
P25	M	32	Blood
P26	F	28	Blood
P27	F	25	Blood
P28	M	38	Blood
P29	F	35	Blood
P30	F	35	Blood

Participant number	Gender	Age (years)	Sample extracted
P31	F	24	Blood
P32	F	36	Blood
P33	F	48	Blood

Table S2. Demographical characteristics and biological samples extracted from each participant. Participants 17-21 were patients undergoing ileocolectomy in General and Digestive Surgery Unit (Vall d'Hebron Barcelona Hospital Campus, Barcelona, Spain) who donated ileal surgical samples for research; we did not hold more information due to privacy policies. Non Available Data is represented with an hyphen. F: Female; M: Male.

11.3.2. Selection of SP treatment concentrations

We selected the concentration of SP to stimulate 126BLCL cells and B cells isolated from blood based on the literature (1), according to the features of its receptor. The highest affinity receptor for SP is TACR1, a G-coupled-protein-receptor, which is rapidly internalized in response to its ligand. Once SP activates TACR1, G-protein-coupled-receptor kinases (GRKs) from the cytosol are translocated into the cell membrane and phosphorylate the receptor. This phosphorylation initiate a desensitization process to SP, where TACR1 interact with β-arrestins and it is internalized and recycled via endocytosis. At low concentrations of SP ([SP]<1nM), a minimal phosphorylation of the receptor can be observed, while at high concentrations ([SP]>10nM), the complex TACR1- β-arrestins last for hours. Therefore, these two concentrations, 1nM and 10nM, have been used to conduct this first approach of stimulation of B cells with SP. Cells were also exposed to SP vehicle (0.05M acetic acid + 0.1%BSA + distilled H_2O), used at the same concentration as SP to discard any potential.

To evaluate the response of these cells to SP, we assessed changes at gene and protein level. Alterations in gene expression usually are fast, within minutes can be detected, contrary to protein expression which requires 24-48h. Previous work from our group, exposing other immune cell lines to SP, concluded 30min and 60min and 24h are suitable time points for a time-course analysis. However, we decided to extend the exposure time up to 4 days, to confirm we could not observe any changes, especially when using low SP concentrations.

11.3.3. Cells isolated from small bowel mucosa

	Sample extracted	Number LPL isolated	Number CD38+ cell isolated
P1	Jejunal biopsy	8.32×10^5	-
P2	Jejunal biopsy	9.28×10^5	-
P3	Jejunal biopsy	3.28×10^5	-
P4	Jejunal biopsy	3.36×10^5	-
P5	Jejunal biopsy	8.32×10^5	-
P6	Jejunal biopsy	3.92×10^5	-
P7	Jejunal biopsy	2.48×10^5	-
P8	Jejunal biopsy	6.88×10^5	-
P9	Jejunal biopsy	4.32×10^5	-
P10	Jejunal biopsy	3.76×10^5	-
P11	Jejunal biopsy	9.76×10^5	-
P12	Jejunal biopsy	2.72×10^5	-
P13	Jejunal biopsy	3.52×10^5	-
P14	Jejunal biopsy	5.28×10^5	-
P15	Jejunal biopsy	6.40×10^5	-
P16	Jejunal biopsy	5.60×10^5	-
P17	Ileal specimen	2.25×10^7	-
P18	Ileal specimen	1.05×10^7	1.07×10^6
P19	Ileal specimen	1.09×10^7	1.36×10^6
P20	Ileal specimen	2.01×10^7	-
P21	Ileal specimen	1.90×10^7	1.76×10^6

Table S3: Cells isolated from jejunal and ileal tissues from participants. CD38+ isolation from LPL could not be conducted in jejunal biopsies due to the low number of LPL obtained.

11.3.4. BIBLIOGRAPHY

1. Mashaghi A, Marmalidou A, Tehrani M, Grace PM, Pothoulakis C, Dana R. Neuropeptide substance P and the immune response. Cell Mol Life Sci [Internet]. 2016;73(22):4249–64.

ANNEX 2

12. ANNEX 2

12.1. ORIGINAL ARTICLES

1. Title: Decreased TESK1-mediated cofilin 1 phosphorylation in the jejunum of IBS-D patients may explain increased female predisposition to epithelial dysfunction.

- Authors: Rodiño-Janeiro BK, Martínez C, Fortea M, Lobo B, Pigrau M, Nieto A, González-Castro AM, Salvo-Romero E, Guagnozzi D, **Pardo-Camacho C**, Iribarren C, Azpiroz F, Alonso-Cotoner C, Santos J, Vicario M.

- Abstract: Disturbed intestinal epithelial barrier and mucosal micro-inflammation characterize irritable bowel syndrome (IBS). Despite intensive research demonstrating ovarian hormones modulation of IBS severity, there is still limited knowledge on the mechanisms underlying female predominance in this disorder. Our aim was to identify molecular pathways involved in epithelial barrier dysfunction and female predominance in diarrhea-predominant IBS (IBS-D) patients. Total RNA and protein were obtained from jejunal mucosal biopsies from healthy controls and IBS-D patients meeting the Rome III criteria. IBS severity was recorded based on validated questionnaires. Gene and protein expression profiles were obtained and data integrated to explore biological and molecular functions. Results were validated by western blot. Tight junction signaling, mitochondrial dysfunction, regulation of actin-based motility by Rho, and cytoskeleton signaling were differentially expressed in IBS-D. Decreased TESK1-dependent cofilin 1 phosphorylation (pCFL1) was confirmed in IBS-D, which negatively correlated with bowel movements only in female participants. In conclusion, deregulation of cytoskeleton dynamics through TESK1/CFL1 pathway underlies epithelial intestinal dysfunction in the small bowel mucosa of IBS-D, particularly in female patients. Further understanding of the mechanisms involving sex-mediated regulation of mucosal epithelial integrity may have significant preventive, diagnostic, and therapeutic implications for IBS.

- Journal: Scientific Reports 2018 Feb 2;8(1):2255. doi: 10.1038/s41598-018-20540-9.

- Impact Factor: 3.998

2. Title: Downregulation of Mucosal Mast Cell Activation and Immune Response in Diarrhoea-Irritable Bowel Syndrome by Oral Disodium Cromoglycate: A Pilot Study.

Authors: Lobo B, Ramos L, Martínez C, Guilarte M, González-Castro AM, Alonso-Cotoner C, Pigrau M, de Torres I, Rodiño-Janeiro BK, Salvo-Romero E, Fortea M, **Pardo-Camacho C,** Guagnozzi D, Azpiroz F, Santos J, Vicario M.

Abstract: Background and goal: Diarrhoea-predominant irritable bowel syndrome (IBS-D) exhibits intestinal innate immune and mucosal mast cell (MC) activation. MC stabilisers have been shown to improve IBS symptoms but the mechanism is unclear. Our primary aim was to investigate the effect of oral disodium cromoglycate (DSCG) on jejunal MC activation and specific innate immune signalling pathways in IBS-D, and secondarily, its potential clinical benefit. Study: Mucosal MC activation (by ultrastructural changes, tryptase release and gene expression) and innate immune signalling (by protein and gene expression) were quantified in jejunal biopsies from healthy (HS; n = 16) and IBS-D subjects after six months of either treatment with DSCG (600 mg/day, IBS-D-DSCG group; n = 18) or without treatment (IBS-D-NT group; n = 25). All IBS-D patients recorded abdominal pain and bowel habits at baseline and in the last 10 days prior to jejunal sampling. Results: IBS-D-NT exhibited significant MC activation and over-expression of immune-related genes as compared to HS, whereas in IBS-D-DSCG MC activity and gene expression were similar to HS. Furthermore, DSCG significantly reduced abdominal pain and improved stool consistency. Conclusions: Oral DSCG modulates mucosal immune activity and improves gut symptoms in IBS-D patients. Future placebo-controlled clinical trials are needed for confirmation of clinical benefit of DSCG for IBS-D.

Journal: United European Gastroenterol J. 2017 Oct;5 (6):887-897. doi: 10.1177/2050640617691690. Epub 2017 Jan 29.

Impact factor: 3.453

12.2. REVIEWS

1. Title: Mucosal RNA and Protein Expression as the Next Frontier in IBS: Abnormal Function Despite Morphologically Intact Small Intestinal Mucosa.

Authors: Rodiño-Janeiro BR*, **Pardo-Camacho C***, Santos J, Martínez C

Abstract: Irritable bowel syndrome (IBS) is one of the commonest gastrointestinal disorders. Although long-time considered a pure functional disorder, intense research in past years has

rendered a very complex and varied array of observations indicating the presence of structural and molecular abnormalities underlying characteristic motor and sensitive changes and clinical manifestations. Analysis of gene and protein expression in the intestinal mucosa has shed light on the molecular mechanisms implicated in IBS physiopathology. This analysis uncovers constitutive and inductive genetic and epigenetic marks in the small and large intestine that highlight the role of epithelial barrier, immune activation, and mucosal processing of foods and toxins and several new molecular pathways in the origin of IBS. The incorporation of innovative high-throughput techniques into IBS research is beginning to provide new insights into highly structured and interconnected molecular mechanisms modulating gene and protein expression at tissue level. Integration and correlation of these molecular mechanisms with clinical and environmental data applying systems biology/medicine and data mining tools emerge as crucial steps that will allow us to get meaningful and more definitive comprehension of IBS-detailed development and show the real mechanisms and causality of the disease and the way to identify more specific diagnostic biomarkers and effective treatments.

Journal: Am J Physiol Gastrointest Liver Physiol. 2019 Jun 1;316(6):G701-G719. doi: 10.1152/ajpgi.00186.2018.

Impact Factor: 3.725

* Shared co-first authorship

2. Title: Epithelial Immunity: Priming Defensive Responses in the Intestinal Mucosa

Authors: **Pardo-Camacho C**, González-Castro AM, Rodiño-Janeiro BK, Pigrau M, Vicario M.

Abstract: As the largest interface between the outside and internal milieu, the intestinal epithelium constitutes the first structural component facing potential luminal threats to homeostasis. This single-cell layer is the epicenter of a tightly regulated communication network between external and internal factors that converge to prime defensive responses aimed at limiting antigen penetration and the maintenance of intestinal barrier function. The defensive role developed by intestinal epithelial cells (IEC) relies largely on the variety of receptors they express at both extracellular (apical and basolateral) and intracellular compartments, and the capacity of IEC to communicate with immune and nervous systems. IEC recognize pathogen-associated molecules by innate receptors that promote the production of mucus, antimicrobial substances, and immune mediators. Epithelial cells are key to oral tolerance maintenance and also participate in adaptive immunity through the expression of immunoglobulin (Ig) receptors

and by promoting local Ig class-switch recombination. In IEC, different types of antigens can be sensed by multiple immune receptors that share signaling pathways to assure effective responses. Regulated defensive activity maintains intestinal homeostasis, whereas a breakdown in the control of epithelial immunity can increase the intestinal passage of luminal content and microbial invasion, leading to inflammation and tissue damage. In this review, we provide an updated overview of the type of immune receptors present in the human intestinal epithelium and the responses generated to promote effective barrier function and maintain mucosal homeostasis.

Journal: Am J Physiol Gastrointest Liver Physiol. 2018 Feb 1;314(2):G247-G255. doi: 10.1152/ajpgi.00215.2016.

Impact Factor: 3.725

3. Title: Mucosal Pathobiology and Molecular Signature of Epithelial Barrier Dysfunction in the Small Intestine in Irritable Bowel Syndrome.

Authors: González-Castro AM, Martínez C, Salvo-Romero E, Fortea M, **Pardo-Camacho C**, Pérez-Berezo T, Alonso-Cotoner C, Santos J, Vicario M.

Abstract: Irritable bowel syndrome (IBS) is one of the most prevalent gastrointestinal disorders in developed countries. Its etiology remains unknown; however, a common finding, regardless of IBS subtype, is the presence of altered intestinal barrier. In fact, signaling and location of cell-to-cell adhesion proteins, in connection with increased immune activity, seem abnormal in the intestinal epithelium of IBS patients. Despite that most research is performed on distal segments of the intestine, altered permeability has been reported in both, the small and the large bowel of all IBS subtypes. The small intestine carries out digestion and nutrient absorption and is also the site where the majority of immune responses to luminal antigens takes place. In fact, the upper intestine is more exposed to environmental antigens than the colon and is also a site of symptom generation. Recent studies have revealed small intestinal structural alterations of the epithelial barrier and mucosal immune activation in association with intestinal dysfunction, suggesting the commitment of the intestine as a whole in the pathogenesis of IBS. This review summarizes the most recent findings on mucosal barrier alterations and its relationship to symptoms arising from the small intestine in IBS, including epithelial structural abnormalities, mucosal immune activation, and microbial dysbiosis, further supporting the hypothesis of an organic origin of IBS.

Journal: J Gastroenterol Hepatol. 2017 Jan;32(1):53-63. doi: 10.1111/jgh.13417.

Impact Factor: 2.251

4. Title: The Intestinal Barrier Function and Its Involvement in Digestive Disease.

Authors: Salvo-Romero E, Carmen Alonso-Cotoner C, **Pardo-Camacho C**, Casado-Bedmar M, Vicario M.

Abstract: The gastrointestinal mucosal surface is lined with epithelial cells representing an effective barrier made up with intercellular junctions that separate the inner and the outer environments, and block the passage of potentially harmful substances. However, epithelial cells are also responsible for the absorption of nutrients and electrolytes, hence a semipermeable barrier is required that selectively allows a number of substances in while keeping others out. To this end, the intestine developed the "intestinal barrier function", a defensive system involving various elements, both intra- and extracellular, that work in a coordinated way to impede the passage of antigens, toxins, and microbial byproducts, and simultaneously preserves the correct development of the epithelial barrier, the immune system, and the acquisition of tolerance against dietary antigens and the intestinal microbiota. Disturbances in the mechanisms of the barrier function favor the development of exaggerated immune responses; while exact implications remain unknown, changes in intestinal barrier function have been associated with the development of inflammatory conditions in the gastrointestinal tract. This review details de various elements of the intestinal barrier function, and the key molecular and cellular changes described for gastrointestinal diseases associated with dysfunction in this defensive mechanism.

Journal: Rev Esp Enferm Dig. 2015 Nov;107(11):686-96. doi: 10.17235/reed.2015.3846/2015.

Impact Factor: 1.858

12.3. CONGRESS PRESENTATIONS

1. Title: Mucosal IgG production and plasma cells-nerves interaction: potential mechanisms of gut-brain axis dysfunction in diarrhoea-predominant irritable bowel syndrome.

Authors: **Pardo-Camacho C,** Melón-Ardanaz E, Albert-Bayo M, González-Castro AM, Expósito E, Martínez C, Nieto A, Pigrau Pastor M, Galán C, Fortea M, Segú H, Rodríguez-Urrutia A, de Torres I, Azpiroz F, Alonso-Cotoner C, Santos J, Vicario M.

Name of the conference: United European Gastroenterology Week (UEG Week)

Date of event: 10/2019

Organizing entity: House of European Gastroenterology

Type of presentation: Poster

2. Title: Mucosal IgG production and plasma cells-nerves interaction: potential mechanisms of gut-brain axis dysfunction in diarrhoea-predominant irritable bowel syndrome.

Authors: Melón-Ardanaz E, **Pardo-Camacho C,** Albert-Bayo M, González-Castro AM, Pigrau Pastor M, Nieto A, Azpiroz F, Santos J, Vicario M.

Name of the conference: NeuroGASTRO

Date of event: 09/2019

Organizing entity: European Society of Neurogastroenterology & Motility (ESNM)

Type of presentation: Poster

3. Title: Acute stress triggers IBS-like miRNA mediated regulation of barrier function in the jejunum of heathy volunteers.

Authors: Rodiño-Janeiro BK, Pigrau M, Nieto A, Salvo- Romero E, Lobo B, González-Castro AM, Fortea-Guillamon M, **Pardo-Camacho C,** de Torres I, Martinez C, Guagnozzi D, Niesler B, Azpiroz F, Vicario M, Santos J, Alonso-Cotoner C.

Name of the conference: Digestive Disease Week

Date of event: 06/2018

Organizing entity: American Gastroenterological Association

Type of presentation: Poster

4. Title: Desmosome associated genes improve predictability of IBS compared to clinical variables.

Authors: Martinez C, Mosquera JL, Rodiño-Janeiro BK, Fortea-Guillamon M, Lobo B, Pigrau M, González-Castro AM, Salvo-Romero E, Pardo-Camacho C, Guagnozzi D, Niesler B, Azpiroz F, Alonso-Cotoner C, Vicario M.

Name of the conference: Digestive Disease Week

Date of event: 06/2018

Organizing entity: American Gastroenterological Association

Type of presentation: Poster

5. Title: Acute stress impacts clock genes and barrier integrity in the intestinal mucosa in health.

Authors: Pigrau M, Rodiño-Janeiro BK, Salvo-Romero E, Nieto A, Hernández-Palet L, Pribic T, Gallart M, Lobo B, González-Castro AM, Fortea-Guillamon M, **Pardo-Camacho C**, Guagnozzi D, Martínez C, Pérez-Berezo T, Iribarren C, de Torrés I, Azpiroz F, Vicario M, Alonso-Cotoner C, Santos J.

Name of the conference: Digestive Disease Week

Date of event: 05/2017

Organizing entity: American Gastroenterological Association

Type of presentation: Poster

6. Title: Integrated multi-omic analysis reveals female predominance of deregulated mucosal actin depolymerization by decreased TESK1-mediated CFL1-phosphorylation in IBS-D.

Authors: Rodiño-Janeiro BK, Martínez C, Fortea-Guillamon M, Lobo B, Pigrau M, González-Castro AM, Salvo-Romero E, **Pardo-Camacho C**, Iribarren C, Guagnozzi D, Azpiroz F, Alonso-Cotoner C, Santos J, Vicario M.

Name of the conference: Digestive Disease Week

Date of event: 05/2017

Organizing entity: American Gastroenterological Association

Type of presentation: Poster

7. Title: Stress induces specific gender-related molecular alterations in barrier regulatory genes in the jejunal mucosa of healthy.

Authors: Rodiño-Janeiro BK, Pigrau M, Nieto A, Pribic T, Hernández- Palet L, Salvo-Romero E, Gallart M, Lobo B, González-Castro AM, Fortea-Guillamon M, **Pardo-Camacho C**, de Torrés I, Martínez C, Guagnozzi D, Pérez-Berezo T, Azpiroz F, Vicario M, Santos J, Alonso-Cotoner C.

Name of the conference: Digestive Disease Week

Date of event: 05/2017

Organizing entity: American Gastroenterological Association

Type of presentation: Poster

8. Title: Increased intestinal mucosal production of immunoglobulin G in diarrhea-predominant irritable bowel syndrome.

Authors: **Pardo-Camacho C,** González-Castro AM, Lobo B, Alonso-Cotoner C, Fortea M, Salvo-Romero E, Casado-Bedmar M, Rodiño-Janeiro B, Azpiroz F, Santos J, Vicario M.

Name of the conference: COST Action BM 1106 GENIEUR Final Conference

Date of event: 03/2016

Organizing entity: COST Action

Type of presentation: Poster

9. Title: Decreased proinflammatory profile and increased corticotropin releasing factor in mucosal eosinophils in association with clinical manifestations diarrhea-predominant irritable bowel syndrome.

Authors: Salvo-Romero E, Martínez C, Lobo B, Pigrau M, Casado-Bedmar M, Sánchez-Chardi A, González-Castro AM, Rodiño-Janeiro B, Fortea M, **Pardo-Camacho C**, Azpiroz F, Alonso-Cotoner C, Santos J, Vicario M.

Name of the conference: COST Action BM 1106 GENIEUR Final Conference

Date of event: 03/2016

Organizing entity: COST Action

Type of presentation: Poster

10. Title: Increased intestinal mucosal production of immunoglobulin G in diarrhea-predominant irritable bowel syndrome.

Authors: **Pardo-Camacho C,** González-Castro AM, Lobo B, Alonso-Cotoner C, Fortea M, Casado-Bedmar M, Rodiño-Janeiro B, Azpiroz F, Santos J, Vicario M.

Name of the conference: United European Gastroenterology Week (UEG Week)

Date of event: 10/2015

Organizing entity: House of European Gastroenterology

Type of presentation: Poster

11. Title: Paired transcriptomic and proteomic profiling analysis of the intestinal mucosa identifies similar biological pathways in diarrhoea.predominant irritable bowel syndrome.

Authors: Rodiño-Janeiro BK, Martínez C, Lobo B, Pigrau M, González-Castro AM, Fortea M, Casado-Bedmar M, **Pardo-Camacho C,** Azpiroz F, Alonso-Cotoner C, Vicario M, Santos J.

Name of the conference: United European Gastroenterology Week (UEG Week)

Date of event: 10/2015

Organizing entity: House of European Gastroenterology

Type of presentation: Oral Presentation

12. Title: Decreased proinflammatory profile and increased corticotropin releasing factor in mucosal eosinophils in association with clinical manifestations diarrhea-predominant irritable bowel syndrome.

Authors: Salvo-Romero E, Martínez C, Lobo B, Pigrau M, Casado-Bedmar M, Sánchez-Chardi A, González-Castro AM, Rodiño-Janeiro B, Fortea M, **Pardo-Camacho C,** Azpiroz F, Alonso-Cotoner C, Santos J, Vicario M.

Name of the conference: United European Gastroenterology Week (UEG Week)

Date of event: 10/2015

Organizing entity: House of European Gastroenterology

Type of presentation: Poster

13. Title: Increased antibody response in the intestinal mucosa of diarrhoea-predominant irritable bowel syndrome in association with psychological stress and abdominal pain.

Authors: González-Castro AM, **Pardo-Camacho C**, Lobo B, Alonso-Cotoner C, Fortea M, Salvo-Romero E, Casado-Bedmar M, Rodiño-Janeiro B, Azpiroz F, Santos J, Vicario M

Name of the conference: International Society of Psychoneuroendocrinology (ISPNE2015)

Date of event: 09/2015

Organizing entity: International Society of Psychoneuroendocrinology

Type of presentation: Poster

14. Title: Caracterización del infiltrado celular, análisis de su ultraestructura y de mecanismos de actividad humoral en la colitis ulcerosa en remisión.

Authors: González-Castro AM, Lobo B, Keita A, Pigrau M, Fortea M, **Pardo C,** Santos J, Söderholm J, Vicario M.

Name of the conference: Semana de las Enfermedades Digestivas (SED 2015)

Date of event: 06/2015

Organizing entity: Sociedad Española de Patología Digestiva (SEPD)

Type of presentation: Poster