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María José Fábrega Fernández

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MODULATION OF INTESTINAL IMMUNE RESPONSE BY OUTER MEMBRANE VESICLES FROM PROBIOTIC AND COMMENSAL *ESCHERICHIA COLI* STRAINS

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Esta Tesis Doctoral ha sido realizada bajo la dirección de la Dra. Laura Baldomà y la Dra. Rosa Giménez, en la sección de Bioquímica y Biología Molecular, Departamento de Bioquímica y Fisiología de la Facultad de Farmacia y Ciencias de la Alimentación de la Universidad de Barcelona.

Barcelona, 24 de noviembre 2017

Dra. Laura Baldomà

Dra. Rosa Giménez

María José Fábrega Fernández

UNIVERSITAT DE BARCELONA

Grupo interacción microbiota intestinal-huésped
Departamento de Bioquímica y Fisiología

Facultad de Farmacia y Ciencias de la Alimentación
Edificio B, 1ª planta
08028 Barcelona
Tel. 934034496
Fax

Dra. **Laura Baldomà Llavinés**, profesora titular, y **Dra. Rosa Giménez Claudio**, profesora agregada, del Departamento de Bioquímica y Fisiología de la Facultad de Farmacia y Ciencias de la Alimentación de la Universidad de Barcelona,

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Que la memoria titulada **Modulation of intestinal immune response by Outer Membrane Vesicles from probiotic and commensal *Escherichia coli* strains** presentada por **MARÍA JOSÉ FÁBREGA FERNÁNDEZ** para optar al Título de Doctor con Mención Internacional por la Universidad de Barcelona, ha estado realizada bajo nuestra dirección en el Departamento de Bioquímica y Fisiología, y considerándola concluida, autorizamos su presentación para ser juzgada por el tribunal correspondiente.

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Barcelona, 24 de noviembre del 2016

Dra. Laura Baldomà
(Directora y Tutora)

Dra. Rosa Giménez
(Directora)

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“Entre las dificultades, se esconde la oportunidad”

Albert Einstein

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RESUMEN GENERAL

El tracto gastrointestinal humano es uno de los ecosistemas microbianos más densamente poblados. Por ello, el sistema inmunitario intestinal está constantemente expuesto a altas concentraciones de antígenos tanto microbianos como alimentarios. La relación simbiótica entre la microbiota intestinal y el huésped es altamente compleja, pero esencial para mantener un correcto balance entre el estado de salud y enfermedad. Estudios realizados con animales "germ-free" han demostrado que la microbiota intestinal es esencial para la correcta maduración y funcionalidad de la barrera intestinal y el sistema inmunitario asociado. Paradójicamente, la microbiota comensal también representa una fuente muy amplia de antígenos, los cuales se encuentran desafiando constantemente la inmunidad innata intestinal, manteniendo un estado constante de estimulación del sistema inmunitario. En individuos sanos, la inmunidad innata es capaz de mantener un balance homeostático multifacético, presentando una baja respuesta frente a la microbiota comensal, pero manteniendo la capacidad de reaccionar frente a un ataque por patógenos. Una de las principales líneas de defensa en la homeostasis intestinal es la barrera que forma el epitelio intestinal, que actúa tanto como barrera física, como colaborador del sistema inmunitario proporcionando la captación de componentes bacterianos. El epitelio intestinal está cubierto por una capa de mucus que forma una cubierta protectora sobre las células epiteliales, las cuales quedan protegidas de daños enzimáticos, químicos o mecánicos. Además, evita el contacto directo de bacterias con el epitelio. Por lo tanto, la compleja comunicación entre la microbiota y las células epiteliales intestinales es mediada a través de factores bacterianos solubles que pueden difundir a través de la capa de mucus y llegar al epitelio. Dentro de estos factores, las bacterias liberan compuestos solubles y vesículas extracelulares. Las vesículas liberadas por bacterias Gram negativas se denominan vesículas de membrana externa (OMVs). Las OMVs representan un mecanismo de liberación a distancia de compuestos bacterianos, protegiendo dichos compuestos de daños externos. Estos compuestos incluidos dentro de las vesículas son capaces de llegar a las células epiteliales y activar diferentes vías de señalización. Como consecuencia, las vesículas liberadas por la microbiota intestinal podrían ser consideradas como un factor clave en la comunicación microbiota-epitelio. Al comienzo de esta tesis, solo

dos aportaciones científicas estaban relacionadas con esta línea de trabajo. Por ello, nuestro grupo tuvo como objetivo estudiar el papel de las vesículas liberadas por la microbiota como moduladores de la homeostasis intestinal, especialmente aquellos parámetros relacionados con la respuesta inmunitaria y función de barrera.

La microbiota intestinal humana está formada por una amplia comunidad bacteriana comensal. Dentro de estas bacterias, algunas cepas presentan propiedades probióticas y proporcionan un beneficio para el huésped. La disbiosis intestinal ha sido asociada con múltiples enfermedades, y la administración de probióticos se ha contemplado como una nueva estrategia terapéutica para modular y restablecer la composición de la microbiota.

En este trabajo, hemos centrado nuestra atención en estudiar las bacterias Gram negativas, en concreto la cepa probiótica *Escherichia coli* Nissle 1917 (EcN) y la cepa comensal ECOR12 (*E. coli* intestinal aislada de un individuo sano). El objetivo de este proyecto fue evaluar la actividad inmunomoduladora de las vesículas extracelulares (OMVs) liberadas por estas cepas de microbiota intestinal. Muchos estudios han mostrado el efecto antiinflamatorio beneficioso de la cepa EcN en la remisión de la colitis ulcerosa. Sin embargo, el papel de las vesículas liberadas por este probiótico era desconocido hasta la fecha. Por ello, el primer objetivo (capítulo 1) propuesto para este estudio fue analizar el potencial de las OMVs, liberadas por las cepas EcN y ECOR12, de modular la respuesta inmunitaria, usando diferentes modelos celulares *in vitro* de barrera intestinal y *ex vivo* con explantes de colon humano. El análisis de expresión génica y cuantificación de proteína de diferentes citoquinas y mediadores inmunitarios (citoquinas proinflamatorias, antiinflamatorias y quimioquinas) confirmaron esta hipótesis. Ambos tipos de vesículas fueron capaces de regular el balance de citoquinas y, específicamente las OMVs de EcN mostraron un mejor perfil antiinflamatorio. Por ello, el siguiente objetivo fue comprobar si las OMVs aisladas de EcN eran capaces de, en ausencia de bacterias vivas, mejorar los síntomas asociados a la colitis (capítulo 2). Para abordar esta hipótesis, se utilizó un modelo *in vivo* de colitis experimental, inducida por DSS (sulfato sódico de dextrano), en ratones. Los resultados demostraron que la administración de vesículas de EcN mejoraron el estado de salud y el daño histológico asociado a la ingesta de DSS. Además, el tratamiento con OMVs contrarrestó la expresión alterada de citoquinas y otros marcadores bioquímicos

relacionados con la inflamación y la función de barrera intestinal. Este estudio demostró, por primera vez, que las vesículas de EcN median efectos antiinflamatorios, los cuales ya fueron previamente demostrados mediante ensayos *in vivo* realizados con la bacteria probiótica. Esta contribución indica que las OMVs liberadas por EcN van ligadas al efecto probiótico de la bacteria, y además, se evidenció que vesículas de microbiota y probióticos tienen un papel importante en la comunicación microbiota-huésped.

Por otro lado, investigaciones previas desarrolladas por nuestro grupo demostraron que las vesículas secretadas por ECOR12 y EcN son internalizadas en células epiteliales mediante endocitosis mediada por clatrina, y son posteriormente redirigidas hasta compartimentos endosómicos. Está descrito que las vesículas de cepas patógenas que son internalizadas interaccionan a través del peptidoglicano con receptores citosólicos tipo NOD, los cuales son reclutados a la membrana de los endosomas, y activan la vía de señalización dependiente de NOD, cuya cascada conduce a la activación del factor de transcripción NF-κB. En este contexto, analizamos si la respuesta inmunomoduladora inducida por vesículas de EcN y ECOR12 dependía de receptores NOD. Para alcanzar este tercer objetivo (capítulo 3), se siguieron dos estrategias experimentales: (i) silenciamiento de células epiteliales intestinales con RNAs de interferencia (siRNAs) dirigidos contra receptores NOD1 o NOD2; e (ii) inhibición de proteínas clave implicadas en la vía de señalización de NOD, usando un inhibidor de la quinasa RIP2, la cual desencadena la cascada de fosforilación que resulta en la activación del NF-κB y su translocación hacia el núcleo. En estas células, la respuesta inflamatoria inducida por OMVs fue evaluada mediante la activación de NF-κB, y cuantificación de la expresión de citoquinas IL-8 e IL-6. Además, empleando microscopía confocal de fluorescencia, se demostró la co-localización (interacción) de NOD1 con vesículas bacterianas y el reclutamiento de NOD1 hacia endosomas tempranos, desencadenado por OMVs. En general, los resultados indicaron que tanto las vesículas de EcN como de ECOR12 fueron capaces de activar NOD1, pero no NOD2, si bien el tiempo necesario para dicha activación fue diferente entre ambos tipos vesiculares. Las OMVs de EcN desencadenaron la agregación de NOD1 (20 minutos) y la degradación de IκBα (90 minutos) antes que las OMVs de ECOR12. Esta es la primera vez que se describe la activación intracelular de receptores NOD1 por vesículas derivadas de microbiota intestinal, confirmando así el papel de estas

vesículas como mediadores clave de los efectos de la microbiota en la homeostasis intestinal.

Finalmente, en base a los efectos inhibitorios de los sobrenadantes de EcN frente a infecciones causadas por patógenos entéricos como *Salmonella*, nos planteamos explorar si este efecto podría ser debido a la liberación de OMVs (capítulo 4). Esta parte experimental de la tesis fue comenzada y llevada a cabo durante una estancia en el grupo de la Dra. Ana Eulalio (Universidad de Würzburg), centrado en el estudio de la regulación de miRNAs del huésped por patógenos bacterianos invasivos. El proyecto fue continuado en el laboratorio de la Universidad de Barcelona. Los resultados demostraron que EcN reduce en un 50% la invasión de *Salmonella*, pero este efecto no es mediado por OMVs, sino por otros factores solubles secretados. La naturaleza de estos factores activos es todavía desconocida, pero nuestros ensayos descartaron que fuera una proteína, una molécula de DNA o RNA. Con respecto al mecanismo de acción de estos factores liberados por EcN, nuestros resultados excluyeron una inhibición de la adhesión de *Salmonella* o de la replicación intracelular. Sin embargo, sí que nos sugieren que estos efectores secretados probablemente estarían actuando interfiriendo con la secreción de factores de virulencia a través del T3SS de *Salmonella*. Sorprendentemente, este efecto no es específico de cepa, si no que otras cepas de *E. coli* mostraron la misma actividad inhibitoria sobre la infección de *Salmonella*.

En conclusión, los datos presentados en esta tesis muestran que las OMVs liberadas por cepas *E. coli* de la microbiota, tienen un papel clave en la modulación de la respuesta inmunitaria del huésped, con una prominente activación de la señalización a través de NOD1. Además, las OMVs liberadas por este probiótico podrían ser las responsables de muchos de los efectos atribuidos al probiótico en sí, en concreto, los efectos antiinflamatorios observados en el modelo *in vivo* de colitis. Sin embargo, las OMVs no contribuyen al efecto inhibitorio mediado por EcN frente a la invasión por *Salmonella*, siendo este efecto mediado por factores solubles secretados. Esta observación demuestra que las vesículas podrían actuar como “comunicasomas” relevantes en la comunicación entre el huésped y la microbiota, controlando parámetros clave de la homeostasis intestinal, lo cual incluye la defensa frente a daños externos (inflamación o patógenos), pero también el desarrollo de la tolerancia del huésped frente a la microbiota comensal.

GENERAL SUMMARY

The human gastrointestinal tract is one of the most densely populated microbial ecosystems. Therefore, the intestinal immune system is constantly exposed to high concentrations of microbial and food antigens. The symbiotic relationship between gut microbiota and the human host is highly complex, but is essential to maintain a delicate balance between health and disease. Studies performed with germ-free animals have shown that gut microbiota is fundamental for the maturation and correct functionality of intestinal wall and mucosal immune system. Paradoxically, commensal microbiota is also a vast source of antigenic materials, which challenge the intestinal innate immunity resulting in a state of constant stimulation of the immune system. In healthy individuals, the innate immune system is able to maintain a multifaceted homeostatic balance, being hyporesponsive to commensal microbiota, but preserving the capacity to react against pathogen's attack. One of the major frontline defences in the intestinal immune homeostasis is the intestinal epithelial layer. This is a key interface, which acts as both a physical barrier and an immune sensor for bacterial components. The intestinal epithelium is covered by a mucus layer that forms a protective coating over host cells, defending it against mechanical, chemical and enzymatic damage and, most importantly avoiding direct access of bacteria to the epithelial layer. Therefore, complex communication between microbiota and intestinal cells is mediated by secreted bacterial factors that can diffuse through the gel-mucus layer and reach the epithelium. Within these secreted factors, bacteria release soluble compounds and extracellular vesicles. Vesicles released by Gram-negative bacteria are called outer membrane vesicles (OMVs). OMVs represents a long-distance secretion mechanism, protected from external conditions, through which bacteria can deliver their own compounds to intestinal cells and activate different signalling pathways. Consequently, vesicles released by gut microbiota could be considered as key factors in the microbiota-epithelium crosstalk. At the beginning of this thesis work, only two scientific contributions were associated with this research area. Hence, research of our group was aimed at studying the role of microbiota released OMVs as modulators of gut homeostasis parameters, especially those related with the immune response and barrier function.

Human gut microbiota is comprised of a vast commensal community. Among these bacteria, some strains display probiotic properties that provide great benefit to the host. As microbiota imbalance (dysbiosis) has been associated with several diseases, administration of probiotics is emerging as a therapeutic strategy to modulate and restore microbiota composition.

In this work, we have focused our attention on the Gram-negative probiotic *Escherichia coli* Nissle 1917 (EcN) and the commensal ECOR12 strain (*E. coli* intestinal isolate from a healthy individual). The aim of this project was to evaluate the immunomodulatory activity of extracellular vesicles (OMVs) released by these microbiota strains. Many studies have shown the beneficial anti-inflammatory effects of the probiotic EcN against ulcerative colitis. However, contribution of EcN OMVs to these properties was unknown. Thus, as first objective (chapter 1), we proposed to analyse the potential of EcN and ECOR12 OMVs to modulate the immune response using different *in vitro* cellular models of intestinal barrier and human colonic explants, as *ex vivo* model. Gene expression and protein quantification analysis of selected cytokines and immune mediators (pro-inflammatory and anti-inflammatory cytokines, and chemokines) confirmed the hypothesis. Both types of vesicles were able to regulate the cytokine balance and, specifically EcN OMVs showed a better anti-inflammatory profile. Therefore, the next objective was to verify whether isolated EcN OMVs were able, in the absence of live probiotic cells, to ameliorate symptoms associated with colitis (chapter 2). To explore this hypothesis, we used an experimental mice model of colitis induced by dextran sodium sulphate (DSS). Results showed that administration of EcN OMVs improved the physical health status and histological damage. In addition, OMVs treatment counteracted altered expression of cytokines and other biochemical markers of inflammation and intestinal barrier function. This study showed for the first time that EcN OMVs mediate the anti-inflammatory effects previously reported for this probiotic in experimental colitis. This contribution indicates that OMVs released by EcN are needed for the probiotic effects, and in addition evidenced that probiotic and microbiota vesicles have an important role in the intercellular communication with the host.

On the other hand, previous research developed by our group showed that OMVs secreted by ECOR12 and EcN are internalized in epithelial cells by clathrin-mediated endocytosis, following the intracellular trafficking through endosomal

compartments. It is known that internalized vesicles from some bacterial pathogens recruit and interact cytosolic NOD receptors at the endosome membrane, which triggers the NOD-dependent signalling pathway that leads to NF-κB activation. In this context, we sought to analyse whether the immunomodulatory response induced by EcN or ECOR12 OMVs depends on cytosolic NOD receptors. To achieve this third objective (chapter 3), different experimental approaches were used. To prove involvement of these receptors we followed two strategies: (i) silencing of intestinal epithelial cells with specific siRNAs targeted against NOD1 or NOD2 receptors, and (ii) blocking the NOD specific signalling pathway with an inhibitor of RIP2 kinase which triggers the phosphorylation cascade that results in NF-κB activation and translocation into the nucleus. In these cells the inflammatory response induced by OMVs was assessed by measuring the activation of NF-κB (by Western blot of IκBα degradation) and the expression of IL-8 and IL-6 cytokines (by ELISA and RT-qPCR). In addition, confocal fluorescence microscopy was used to prove co-localization (interaction) of NOD1 with bacterial vesicles and the recruitment of NOD1 to early endosomes triggered by OMVs. Overall, results showed that both EcN and ECOR12 vesicles activate NOD1, but not NOD2, although activation timing profiles differed between OMVs. EcN OMVs triggered aggregation of NOD1 (20 min) and degradation of IκBα (90 min) earlier than ECOR12 OMVs. This is the first description of activation of intracellular NOD1 receptors by microbiota derived vesicles and it confirms the role of vesicles as key mediators of gut microbiota effects on intestinal homeostasis.

Finally, based on the inhibitory effect of EcN SNs against infections caused by enteric pathogens such as *Salmonella*, we planned to elucidate whether this effect could be due to released OMVs (chapter 4). This experimental part of the thesis was started and carried out during a stay abroad in the group of Dra. Ana Eulalio (University of Würzburg), a leading group in the area of regulation of host miRNAs by invasive bacterial pathogens. The project was continued in the laboratory at University of Barcelona. Results showed that EcN causes 50% reduction of *Salmonella* invasion, but this effect is not mediated by OMVs, but by another soluble secreted factor(s). The nature of this active factor is still unknown, but it neither is protein, DNA nor RNA. Regarding the mechanism of action, results ruled out that the EcN interference factor inhibits *Salmonella* adhesion or intracellular replication. Instead, they suggest that this secreted effector probably acts by

interfering with the T3SS-mediated secretion and delivery of virulence *Salmonella* proteins into the infected host cell. Interestingly, this effect is not strain specific as other *E. coli* strains also display the same inhibitory activity *on Salmonella* infection.

In conclusion, data presented in this thesis show that OMVs released by microbiota *E. coli* strains have a key role in modulating host immune responses, with a prominent activation of NOD1 signalling pathway. In addition, OMVs released by the probiotic EcN could be responsible of most of the effects attributed to the probiotic, especially although not exclusively, the anti-inflammatory effects observed in the experimental colitis mouse model. Nevertheless, OMVs do not contribute to the inhibitory effect showed by EcN against *Salmonella* invasion, being this effect mediated by a soluble secreted factor. This observation gives support that vesicles behave as relevant communicasomes in the host-microbiota crosstalk, controlling key parameters of the intestinal homeostasis, which include defense against external damages (inflammation or pathogens) but also the development of host tolerance to commensal microbiota.

ABBREVIATIONS:

AJs	Adherens Junction
AMP	Antimicrobial Peptide
APC	Antigen-Presenting Cell
DAMP	Danger Associated Molecular Pattern
CD	Crohn´s Disease
Escherichia coli Nissle	EcN
EV	Extracellular Vesicle
GALT	Gut-Associated Lymphoid Tissue
GI	Gastrointestinal
IBD	Inflammatory Bowel Disease
IEC	Intestinal Epithelial Cell
IL	Interleukin
INF	Interferon
IIS	Intestinal Immune System
JAMs	Junctional Adhesion Molecules
LPS	Lipopolysaccharide
M cell	Microfold cell
MAMP	Microbe Associated Molecular Pattern
MMP	Matrix Metalloprotease
MUC	Mucin
MyD88	Myeloid Differentiation primary-response protein 88
NFκB	Nuclear Factor kappa-light-chain-enhancer of Activated B cells
NLR	NOD-like Receptor
NOD	Nucleotide-binding Oligomerization Domain
OMV	Outer Membrane Vesicle
PAMP	Pathogen Associated Molecular Pattern
PG	Peptidoglycan
PRR	Pattern Recognition Receptor
PSA	Polysaccharide
RIP	Receptor-Interacting Protein
SCFAs	Short Chain Fatty Acids
SCV	*Salmonella* Containing Vacuole
sIg	Secretory Immunoglobulin
SPI	*Salmonella* Pathogenicity Island
SN	*Supernatant*
T3SS	Type III Secretion System
TAK1	Transforming growth factor-Activated Kinase 1
TCR	T Cell Receptor
TER	Trans-epithelial Resistance
TFF3	Trefoil Factor 3
TJ	Tight Junction
TGF	Transforming Growth Factor
TIR	Toll-Interleukin-1 Receptor
TIRAP	TIR-Protein
TLR	Toll Like Receptor
TNF	Tumour Necrosis Factor
TRAM	TRIF-related Adaptor Molecule
TRIF	TIR-domain-containing adaptor protein-inducing INF-β
UC	Ulcerative Colitis
ZO	Zonula Occludens

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1. INTRODUCTION

1.1 THE HUMAN GASTROINTESTINAL TRACT

The human gastrointestinal (GI) tract extends from the mouth to the anus, and includes several organs: the oropharyngeal cavity, the oesophagus, the stomach, the small intestine (duodenum, jejunum and ileum) and the large intestine (cecum, colon, rectum and anus). The movement of muscles (peristalsis), along with the secretion of hormones and enzymes, lets the digestion of food, and the absorption and transport of water, electrolytes and nutrients. The surface of the GI tract, which is exposed to the outside environment, is composed of epithelial cells that form the GI epithelium. These intestinal cells create a functional and permeable barrier that is highly regulated to prevent the entry of pathogens and takes part in the host defence through the activation of the Intestinal Immune System (IIS). To reach these purposes, a complex tissue organization and a vast cellular diversity are required.

The intestinal tract wall is structured in four layers: a) the mucosa: the closest layer to the intestinal lumen that is composed of epithelial cells and a layer of connective tissue directly beneath the epithelium termed lamina propria; b) the submucosa: formed by connective tissue; c) the muscularis layer, and d) the serosa: the innermost layer that is continuous with the mesentery and surrounds the organs.

To increase the surface area, the intestinal epithelial cells (IECs) present small projections of their membranes known as microvilli. Moreover, in the small intestine, mucosa and submucosa are organized into irregular evaginations that increase in ten-fold the absorption surface. The resulting structures are called intestinal villi and crypts. The crypts are highly protected because intestinal stem cells are located in this region and they are responsible of mucosal reparation. Newly formed epithelial cells move up from the bottom of the crypt to the villus, where after 4-5 days they are eventually killed by apoptosis and extruded. However, the epithelium of the colonic mucosa does not present intestinal villi, and the crypts are invaginated. This complex organization makes the human GI tract one of the largest interfaces (250-400m^2) between the host and the external environment (Thursby and Juge, 2017; Mowat and Agace, 2014; C. Sasakawa, 2009).

1.1.1 THE GASTROINTESTINAL MICROBIOTA

In the last few decades, there have been increasing and speedy understanding about the microorganisms that live on the surface and inside the human body, thanks to new strategies for genome sequencing and to projects like the Human Microbiome and Metagenomics of the Human Intestinal Tract (MetaHIT). The terminology used to describe these polymicrobial communities is "microbiota", and their genomic content is called "microbiome".

The whole set of microorganisms that colonize the human GI tract is called intestinal microbiota. The microbial diversity in the GI tract is huge, covering all varieties of living species such as archaea, prokarya, eukarya and viruses, which fluctuates lengthwise (mouth to rectum) and cross sectionally (lumen to mucosa). Approximately, 90% of bacteria belong to the phylum *Bacteroidetes* and *Firmmicutes*. The intestinal microbiota is shaped after birth by both genetic and environmental factors like diet, exercise, age, hygiene, pathogens, stress, tobacco, antibiotics or other medications and even surgical practices.

The microbiota covers a wide range of physiological functions that provide benefits to the host such as strengthening the intestinal epithelium integrity, harvesting energy from different sources, protecting against pathogens and modulating host immunity. Thus, alterations in the normal biodiversity have been associated with some diseases like asthma, atopic diseases, obesity, colorectal cancer or inflammatory bowel disease (IBD). Thereby, gut microbiota is important to maintain and protect the intestinal homeostasis (Vindigni *et al*., 2016; Thursby and Juge, 2017).

1.1.1.1 GUT MICROBIOTA COMPOSITION AND DISTRIBUTION

In general, most of the studies done to date indicate that before birth, the intestinal tract is sterile, although recent discoveries ensure that tissues like the placenta contains microorganisms. However, the main and fastest initial colonization takes place during childbirth, in which the new-born is exposed to faecal and vaginal microbiota of the mother. In the following months, with life events such as breastfeeding, illness, diet or even antibiotic treatments, a stable commensal microbiota is established. It is considered that at two or three years of age, the composition and metabolism of the child intestinal microbiota resembles

that of the adult. In adulthood, the gut microbiota is relatively stable, but in the elderly (over the age of 65), the microbial community changes again, with a reduction in abundances of species that produce short chain fatty acids (SCFAs), such as butyrate (Thursby and Juge, 2017).

The intestinal microbiota is a complex ecosystem that includes a vast range of microbes, including approximately 1000 species of bacteria, 5 genera of archaea, 66 genera of fungi, viruses (mainly bacteriophages) and parasites (Shen *et al*., 2017). Surprisingly, the average ratio of number of microorganisms compared with eukaryotic cells is 1.3:1 in a healthy human (Kverka and Tlaskalová-Hogenová, 2017).

Although the presence of commensal bacteria significantly contributes to nutrient digestion, vitamin synthesis and tissue maturation, their high number represents a permanent challenge to the integrity of the epithelial surface, keeping the local immune system constantly on alert. For this reason, the intestinal epithelium is overlaid by a mucus layer that represents the first barrier between the intestinal lumen and the mucosal tissue (Zhang *et al*., 2015). This separation allows the inner mucus layer and the crypts being protected from faecal stream and accessible only to certain microorganisms. The intestinal microbiota inhabits the surface of the mucus layer (Donaldson *et al*., 2015).

Along the human lower GI tract (small and large intestine), diverse physiological and structural conditions can be found, such as chemical composition, nutrient richness, different intestinal architecture, as well as diverse existence of IIS complexes. These facts will condition the bacterial composition through the GI tract. Few years ago, the composition and classification of the intestinal microbiota was done through faecal material. However, what is detected in stools may not be representative of what is taking place within the host. Thus, recent high-throughput-omics techniques allow quantifying microbiota composition from samples derived from various locations along the GI tract. This approach lets to know the global spatial organization of gut microbiota and how it varies in health or disease conditions (Donaldson *et al*., 2015; Tropini *et al*., 2017). For example, the MetaHit project allowed to map 3.3 million non-redundant microbial genes from faecal samples of 124 European individuals and nowadays this information is being used to study how different illnesses like type-2 diabetes affects to human microbiota (Dusko Ehrlich and MetaHIT consortium, 2010; Forslund *et al*., 2017).

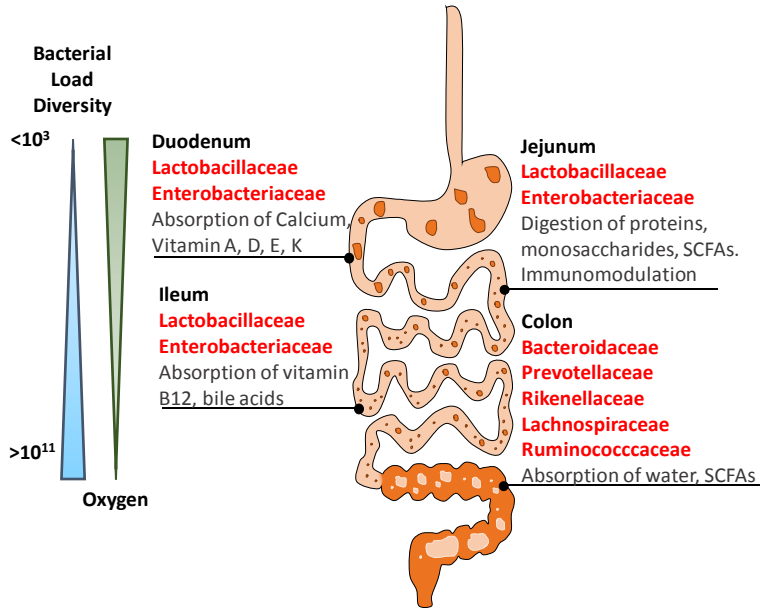

Figure 1. Bacterial biogeography in the gut. Distribution of the predominant bacterial phyla that integrate the microbiota along the gut. Image adapted from (Donaldson *et al*., 2015).

Thanks to the combination of old and modern techniques, today we know that intestinal microbiota increases in quantity and complexity along the bowel. For example, in the small intestine the most abundant cultivable species are *Bifidobacterium*, *Enterobacteria*, *Bacteroides* and *Fusobacteria*. The limiting factors in this region are the peristaltic movements, the high levels of oxygen and antimicrobials, and the secretion of pancreatic and biliary juices. On the other hand, in the caecum and colon resides the largest and most diverse number of microorganisms of any part of the human organism. Lower oxygen environment, concentrations of antimicrobials, slower peristaltic movements and the lack of simple carbon sources enable the presence of strict anaerobic populations such as *Bacteroides*, which is one of the most abundant. Moreover, these microbial populations co-exist with other Gram-positive bacteria belonging to the genera *Eubacterium*, *Bifidobacterium*, *Peptostreptococcus* and *Ruminococcus*, or with sporulated Gram-positive bacteria like the genus *Clostiridium*. Less abundant are facultative anaerobic populations like *Enterobacterium*, *Enterococcus*, *Lactobacillus* and *Streptococcus* (Donaldson *et al*., 2015). Inside the *Enterobacterium* family, *Escherichia coli* strains are found as part of normal human

gut microbiota, and this species is frequently the first bacterium to colonize the intestinal tract in childhood and it is a lifelong colonizer in adulthood (Conway and Cohen, 2015). The general distribution of the gut microbiota is graphically represented in Figure 1.

Despite of the microbiota composition in terms of species is specific to everyone; it is highly conserved between individuals in terms of composition at phyla level and large phylogenetic groups (Landman and Quévrain, 2016).

1.1.1.2 FUNCTIONS

The intestinal ecosystem is characterized by dynamic and reciprocal epithelium-microbiota-immune system interactions (Shanahan, 2011). The genetic information codified in the microbiome complements that encoded by the human genome, contributing with trophic, metabolic and protective signals that are beneficial to the host. As mentioned before, the gut microbiota provides great beneficial properties to the human body, especially due to its enormous genomic and metabolic potential. Most important microbiota roles (shown in Figure 2) are maintaining epithelial barrier, providing nutrients and vitamins, protecting against pathogens and stablishing an appropriate immune function (Thursby and Juge, 2017).

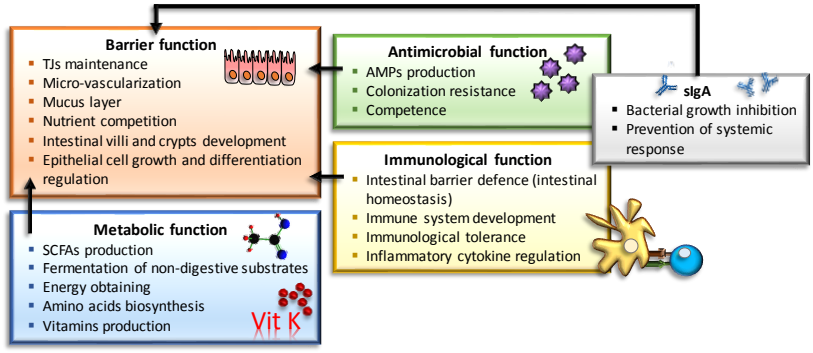

Figure 2. Roles of gut microbiota. Beneficial functions of the human gut microbiota can be divided into four major categories: antimicrobial function, immunological function, metabolic function, and structural and barrier function. All are interconnected and the relationship between them are completely necessary to maintain the beneficial effect exerted by microbiota.

To carry out all these functions, the microbiota maintains a symbiotic relationship with the gut mucosa. Gut microbiota is considered a "hidden organ" by itself as it plays a fundamental role in human health and intestinal physiology, with a huge metabolic capability and functional plasticity (Jandhyala *et al*., 2015).

Considering the broad and essential role of intestinal microbiota in the immune response as well as in different crucial metabolic pathways of the host, we can easily predict the functional impact of microbiota imbalance on the development of various immune and metabolic pathologies (Landman and Quévrain, 2016).

1.1.1.2.1 ANTIMICROBIAL FUNCTION

In the intestinal lumen, pathogens and commensal bacteria compete for nutrients and epithelial adhesion sites. This is a challenging situation because the human body needs to be tolerant to the beneficial commensal bacteria and prevent overgrowth of pathogens. In this continuous fight, the first and most simple protection mechanism is the presence of the mucus layer, but in places like the small intestine this adherent mucus layer is discontinuous. In this context, the role of the microbiota is essential.

Most beneficial and pathogen microorganisms are able to secrete antibacterial compounds such as lactic acid, hydrogen peroxide and antimicrobial peptides (AMPs), which inhibit bacterial growth (bacteriostatic effect) or cause bacterial death (bactericide effect). AMPs produced by bacteria are called bacteriocins, which are small and heat-stable peptides that bacteria use to compete against other bacteria. Recently, new specific terminologies have been evolved to classify bacteriocins : (i) microcins and colicins (bacteriocins secreted by *Enterobacteria* with small and high molecular weight respectively) and (ii) lantibiotics (bacteriocins secreted by Gram-positive bacteria) (Duquesne and Destoumieux-Garzón, 2007; Rev *et al*., 2001; Sang and Blecha, 2008).

Moreover, other metabolites or structural components of microbiota can induce the synthesis of AMPs by host specific cells, like Paneth cells, via pattern recognition receptor (PRR). Among the defence peptides released by the host there are cathelicidins, C-type lectins and pro(defensins). Among commensal strains, *Bacteroides* and *Lactobacillus* are key individual genus for antimicrobial protection. For example, *Bacteroides thetaiotaomicron* induces the expression of matrix metalloproteases (MMPs) that cleave pro-defensins into defensins (active form);

and *Lactobacillus spp*. produce lactic acid that increase the antimicrobial activity of host lysozyme by disrupting the outer membrane of bacterial cell wall.

Another mechanism that the gut microbiota has evolved is to prevent the overgrowth of pathogenic strains by inducing local production of immunoglobulins. For instance, Gram-negative bacteria like *Bacteroides* induce the expression of secretory immunoglobulin A2 subclass (sIgA2) in the intestinal mucosa, which coats microbiota strains providing a resistance mechanism against degradation by bacterial proteases. This mechanism restricts the translocation of gut microbes from the intestinal lumen to the circulation and thereby prevents a systemic immune response (Landman and Quévrain 2016; Jandhyala *et al*., 2015).

1.1.1.2.2. IMMUNOLOGICAL FUNCTION

The huge number of immune cells in the body resides at sites colonized by commensals such as the skin or the GI tract. Furthermore, the correct development of gut microbiota is strictly related to the healthy maturation of the immune system, and microbiota constitutes a stimulus that drives the correct development of the immune system. Thus, after birth, the neonate immune system is immature, and this enables microbiota colonization without over-reaction or gut over-inflammation. Like in an adult system, the host-commensal communication is through recognition of damages or conserved microbial or pathogen associated molecular patterns (MAMPs/PAMPs/DAMPs) by Toll Like Receptors (TLRs) present in naïve innate cells. However, and contrary to the adult, the microbiota and the undeveloped immune system work together generating a hypo-response and making possible the development of the intestinal homeostasis. Thus, microbiota plays a fundamental role in the post-natal development and maturation of the host immune system, in the defence role of the intestinal epithelial barrier and therefore to its containment (Belkaid and Hand, 2014). For instance, germ-free mice exhibit numerous immunologic deficiencies (hypoplastic Peyer´s patches, intraepithelial lymphocyte decrease, deficiency in certain T lymphocyte populations, intestinal IgA and cytokines decrease…), that could be reversed after inoculation with healthy murine commensal microbiota (Macpherson and Harris, 2004). Microbiota is a critical and active inducer of regulatory responses that drive

to the immunological host tolerance against microbes and other orally ingested food antigens.

One of the most important immunological points in the control of intestinal homeostasis focuses on the balance between effector T lymphocytes (mainly Th17 that are present in an inflammatory environment) and regulatory T lymphocytes (Treg). The Treg cells maintain both peripheral and mucosal homeostasis in the GI tract in response to specific antigens derived from the commensal microbiota. For example, studies in mice revealed that the bacterial PSA from *Bacteroides fragilis* protects from colitis via TLR2 signalling (T cells), limiting Th17 responses and inducing the activation of Treg cells which in turn, release anti-inflammatory mediators like IL-10 (Telesford *et al*., 2015). Moreover, commensal-specific Treg responses can induce IgA class switching in an antigen-specific manner thereby controlling the host relationship with the microbiota through multiple mechanisms (Chorny *et al*., 2010). In other cases, Treg stimulation takes place via short chain fatty acids (SCFAs) produced by microbiota metabolism (Smith *et al*., 2013). The presence of stable microbial communities in the gut allows the induction and maintenance of a set of activated Treg cells that contribute to control host-microbiota relationship and regulate the systemic immune responses (Belkaid and Hand, 2014).

1.1.1.2.3. METABOLIC FUNCTION

Intestinal microbiota survives in the GI tract because it can obtain energy by metabolizing carbohydrates and proteins, which are components of the host diet and cannot be digested by the human being. When these components reach the colon, they are bio-transformed into different substrates that allow microbiota growth. This metabolic activity results in the secretion of metabolites that are mostly absorbed and used by the host (Landman and Quévrain, 2016).

- Carbohydrate metabolism: Approximately, in a healthy individual, between 10 to 60 grams of carbohydrates reach the colon every day. Some colonic microbiota groups, mainly the genera *Bacteroides, Bifidobacterium, Ruminococcus and Roseburia*, produce extracellular hydrolases and stablish a trophic chain using and degrading these carbohydrates into SCFAs, the final fermentative products (acetate, pyruvate and butyrate) (Landman and

Quévrain, 2016) that are rapidly absorbed; only 5% is excreted through faeces. Butyrate, released mainly by *Firmicutes* phylum, is the principal energy source for colonocytes and enterocytes. Apart from this, butyrate has anti-inflammatory properties by inhibiting NF-κB and the expression of pro-inflammatory cytokines such as IL-12 and TNFα, protects the epithelial layer by inducing mucin synthesis, attenuating bacterial translocation and enhancing tight junction (TJs), and exerts anti-cancer activities.

On the other hand, propionate and acetate are mainly released by *Bacteroidetes* phylum. Propionate is mainly captured in the liver, while acetate goes directly into peripheral tissues (Lin and Zhang, 2017).

- Gas metabolism: The main gas produced during fermentation is hydrogen, which can be excreted by rectal emission or by pulmonary route. However, most of the hydrogen is transformed *in situ* by hydrogenotrophic microbiota into methane, acetate, and sulphides (Lucas *et al*., 2017).
- Protein metabolism: Some microbiota species (like *Veillonella*, *Fusiobacterium* or *Clostridium*) depend on this metabolism because they obtain the energy from amino acids, which are also used as a nitrogen source. The main proteolytic bacteria belong to the genera *Bacteroides*, *Clostridium*, *Propionibacterium*, *Fusobacterium*, *Streptococcus* and *Lactobacillus*. They hydrolyse proteins into small peptides and amino acids. Like in carbohydrate fermentation, this metabolic activity also produces SCFAs (Landman and Quévrain, 2016).
- Lipid metabolism: lipids that are present in the luminal colon are mainly non-absorbable lipids, which come from bacteria or desquamated colonocytes. These lipids are metabolized by some bacteria existing in the microbiota. Colonic cholesterol mainly comes from the bile. Around 5% of bile acids secreted in the bile reach the colon, where they are degraded by microbiota and excreted in stools. This metabolic activity avoids cholesterol accumulation and reduces cardiovascular risk and colonic carcinogenesis. In addition, other compounds like steroid hormones and xenobiotics are also metabolized by colonic microbiota. Some of the most representative genus involved in lipid metabolism are *Bacteroides*, *Bifidobacterium* and *Clostridium* (Ramakrishna, 2013; LeBlanc *et al*., 2017; Landman and Quévrain, 2016).

- Minerals and vitamins: some microbes, especially *Lactobacillus* and *Bifidobacterium*, can produce B-group vitamins (B1, B2, B12 or B9). Other types of vitamins like vitamin K, biotin, folic acid and pantothenic acid have been also associated with microbiota (LeBlanc *et al*., 2017). Moreover, microbiota help to the absorption of some minerals such as calcium, phosphorous, magnesium and specially iron, necessary for host physiologic functions (Ramakrishna, 2013; O'Hara and Shanahan, 2006).

1.1.1.2.4. STRUCTURAL AND BARRIER FUNCTION

Microbiota improves intestinal barrier function primarily through regulation of synthesis of key proteins that contribute to the physical epithelial barrier maintenance such as TJ proteins. For instance, *Escherichia coli* Nissle secreted TcpC protein, which induces upregulation of the tight junction-associated protein claudin-14 and reinforce the intestinal epithelial barrier (Hering *et al*., 2014). Moreover, microbiota can secrete proteins that prevent cytokine induced apoptosis of the IECs and, thus, barrier break. Other Gram-negative bacteria like *Akkermansia muciniphila* can increase the levels of endocannabinoids that regulate gut barrier functions by decreasing metabolic endotoxemia. Furthermore, microbiota is involved in the development of the intestinal microvasculature by inducing the transcription factor angiogenin-3. (Jandhyala *et al*., 2015).

On the other hand, microbiota is important for the development of an impenetrable mucus layer that separates gut microbes from the epithelium, contributing to barrier function (Rodrıguez-Pineiro and Johansson, 2015). Some evidences indicate that intestinal microbiota modulates the production of mucins and host defence peptides. For instance, some microbiota strains induce human β-defensine-2 (HBD-2), which in turns upregulates expression of mucins MUC2 and MUC3 in an intestinal epithelial cell model (Robinson *et al*., 2015).

1.1.2. INTESTINAL BARRIER

The GI tract is the largest organ of the human body that represents the widest contact surface with the outside. Thus, it is a potential way of entry for different antigens or pathogens. However, the physical barrier linked with the associated immune system has developed different mechanisms to prevent the access of

harmful molecules to the organism. The first line of defence that bacteria and antigens find is the lumen itself, where the presence of low pH and pancreatic and biliary juices kill bacteria and degrade dangerous compounds (Keita and Soderholm, 2010). Moreover, another line of defence is formed by commensal bacteria, which helps to protect the host by diminishing the contact of pathogenic bacteria with the intestinal surface. The different mechanisms that microbiota has to fight against pathogens include secretion of specific antimicrobial factors, competence for binding sites and reduction the intraluminal pH (Emami *et al*., 2009). Another important strategy that the GI system uses to avoid injury is to coat the intestinal epithelium by a mucus layer that physically separates the enterocytes from the external lumen. Mucus provides limited access of the microbiota to the apical side of the epithelium (Rescigno, 2011). On the other hand, the induction of secreted IgA (sIgA), mainly found into the mucus, provides defence against intestinal pathogens and has an important antimicrobial role (Caballero and Pamer, 2015). Along with these strategies, the epithelial barrier itself forms a physical structure that acts as a protection mechanism, avoiding and controlling the pass of pathogens or molecules, which could break the intestinal homeostasis. The epithelial architecture is based on one single monolayer of cells that are closely linked to each other by sets of proteins and molecules that form different complex structures. The most relevant are the TJs and desmosomes (Keita and Soderholm, 2010). Finally, the gut peristaltic movements are particularly important because this function prevents prolonged bacterial stasis in the intestine, reducing their attachment and potential penetrance into the mucus layer (Emami *et al*., 2009).

1.1.2.1. THE MUCUS LAYER

To maintain tissue homeostasis, IECs are covered by a viscous gel layer that lubricates the surface helping to the movement of luminal contents and protecting the epithelium from physical damages during peristalsis, acidic digestion, absorption of ingested food and microbial colonization. Constantly, Globet cells secrete and renew mucin glycoproteins to form and maintain the mucus layer. Moreover, this layer has the potential of trapping digestive enzymes, AMPs and sIgA. Globet cells are the main source of secreted mucin, whose production is

upregulated by TLR signalling after degradation caused by commensals or other mechanical sources (Faderl *et al*., 2015; Wells *et al*., 2017).

The gelatinous and polymer-like structure of the mucus is due to the presence of secreted mucins. They are glycoproteins that contain up to 80% carbohydrates, mainly organized as high-density clusters of O-linked oligosaccharides, which are connected with a large macromolecular complex via cysteine-rich domains at both the amino and the carboxy termini (Wells *et al*., 2017). Mucins are grouped into two families: secretory and transmembrane mucins. The first group includes *MUC2 (intestine)*, *MUC5AC (stomach surface)*, *MUC5B (salivary glands)* and *MUC6 (stomach glands).* The membrane-associated group contains *MUC1 (small amounts in the intestine and more abundant in the stomach)*, *MUC3*, *MUC4*, MUC12, *MUC13* and *MUC17* (Pelaseyed *et al*., 2014).

MUC2 is the predominant secretory mucin synthesized by Globet cells. This is a dimeric and highly glycosylated multidomain protein, secreted by exocytosis, which forms a compact gel-like hydrated layer with approximately 50 µm in distal colon of mice and about 200 to 300 µm thickness in the human colon. Transmembrane mucins consist in a cytoplasmic tail, a single transmembrane domain and a highly glycosylated extracellular domain and are the major components of the epithelial glycocalyx that covers the brush border of the IEC. These mucins help secreted mucins to set up the mucus layer, providing an anchor function (Faderl *et al*., 2015). However, some specific mucins like human MUC17 participate inhibiting cell apoptosis and have a relevant contribution in epithelial restitution and mucosal healing (Luu *et al*., 2010).

The organization of the mucus layer varies along the digestive tract and its thickness, density and composition differ between the stomach, small intestine and colon (Figure 3A). The small intestine, whose function is the absorption of nutrients, is covered with a single and loose mucus layer. However, the mucus layer in the colon, which mainly protects host from the dense microbial colonization, is organized in two different layers. The inner dense mucus layer is closely linked to the IEC glycocalyx. Under health conditions, it limits or prevents bacterial penetration, mainly because of its high-density structure and also due to the presence of AMPs, such as the antibacterial lectin REgIIIγ (regenerating islet-derived 3 γ). In addition to this protective role, the inner mucus layer also

contributes to maintaining the outer mucus layer, which is highly dynamic and in close contact with microbiota.

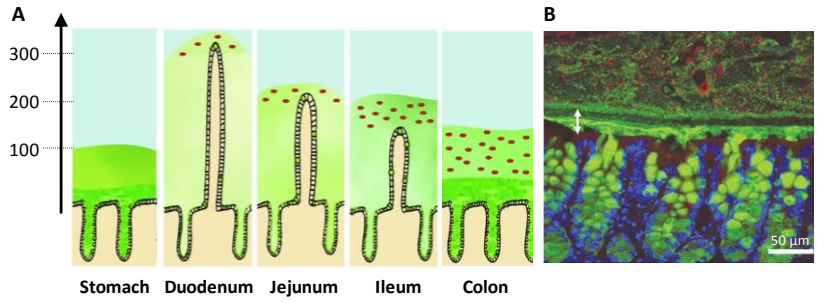

Figure 3. Mucin layer structure. A) Mucins layers through the GI tract in mice. The mucus is illustrated in different shades of green depending on mucus density and bacteria are showed in red (Ermund *et al*., 2013). B) Immunostaining of mice colon section. MUC2 is green-stained with specific anti-MUC2 antibodies, bacteria are visualised in red by using a general 16S rDNA red-probe, and cellular DNA is shown in blue (Hoechst). Double arrows pointed inner mucus layer (Rodrıguez-Pineiro and Johansson, 2015).

This external mucus layer can be degraded by specific bacteria of the gut microbiota and thus, needs to be constantly renewed. This fast turnover is important for preserving the inner mucus layer free from bacteria. As in the colon, the stomach is coated by a double-layer mucus structure consisting of an inner layer firmly attached to the epithelial cells and an outer layer, whose main function is to protect from the high concentration of hydrochloric acid, rather than from bacterial colonization. Due to the low pH, the stomach is home of very few microorganisms (unlike colon that harbours more than a kilogram of bacteria). In the stomach the outer layer is more strongly attached to the epithelial cells than the same layer in the colon (Pelaseyed *et al*., 2014; Faderl *et al*., 2015; Rescigno, 2011).

The different mucin layer structures that are present along the GI tract sections, have been visualized by fluorescence microscopy techniques, using anti-mucin antibodies and fluorescence *in situ* hybridization (FISH) to detect bacterial 16S rRNA (Figure 3B).

1.1.2.2. INTESTINAL EPITHELIAL CELLS

1.1.2.2.1. CELL TYPES

The IECs form a biochemical and physical barrier that maintains segregation between luminal microbial communities and the immune system in the underlying

mucosa. In health conditions, this surface is preserved by pluripotent intestinal epithelial stem cells (IESCs) located in the base of crypts (Peterson and Artis, 2014). The intestinal monolayer is formed by different specialized cell types that are joined with neighbouring cells through TJs, forming a semi-permeable physical barrier. The IECs are the absorptive enterocytes (the most abundant), microfold cells (M cells), Globet cells and Paneth cells.

The M cells are specialized epithelial cells that lack surface microvilli and display on their surface specific glycoprotein receptors by which recognize and interact with bacterial structures (Mowat, 2003). In this way, they detect and capture luminal antigens and intact microorganisms and transport them to antigen-presenting cells such as macrophages or dendritic cells (DCs). M cells are localized above Peyer`s patches or isolated lymphoid follicles (ILFs). Both are part of the lymphoid tissues known as gut-associated lymphoid tissues (GALT) composed mainly of B cells follicles surrounded by DCs.

Globet cells contribute to the formation and maintenance of the intestinal mucus layer that coats the epithelial monolayer (Faderl *et al*., 2015). Thus, these cells have an important role in maintaining the mucus layer that protects the epithelium from inflammation. Moreover, Globet cells are also specialized in producing AMPs, like trefoil factor 3 (TFF3) that binds to mucin and help to maintain the mucus integrity layer as well as promoting epithelial repair, IECs migration and apoptosis resistance. Another AMP is the resistin-like molecule-β (RELMβ) which promotes MUC2 secretion and contributes to inflammation by modulating macrophage and adaptive T cell responses. Moreover, it has been also described that Globet cells could contribute to antigen uptake, delivering luminal soluble antigens to subepithelial DCs, just as M cells do (Mcdole *et al*., 2012). Paneth cells, which are mainly located in the crypts, are characterized by the presence and secretion of dense granules that contain AMP including α-defensins, cathelicidins, REgIII β/γ and lysozymes. These cells also help to maintain normal crypt stem cell activity through production of molecules like pro-epidermal growth factor, and are crucial for intestinal homeostasis (Mowat and Agace, 2014).

Apart from Paneth cells, the common enterocytes can also produce some types of AMPs like RegIII β/γ, although their key function is directly related with the absorption of nutrients and the transport of molecules, especially of sIg produced by plasma cells in the lamina propria. Dimeric IgA complexes stablish contact with

the polymeric immunoglobulin receptor located on the basolateral membrane of IECs and cross the epithelial barrier. This relationship between epithelial cells and immune cells controls the adaptive immune response that in turn regulates the microbiota and the intestinal homeostasis (Peterson and Artis, 2014).

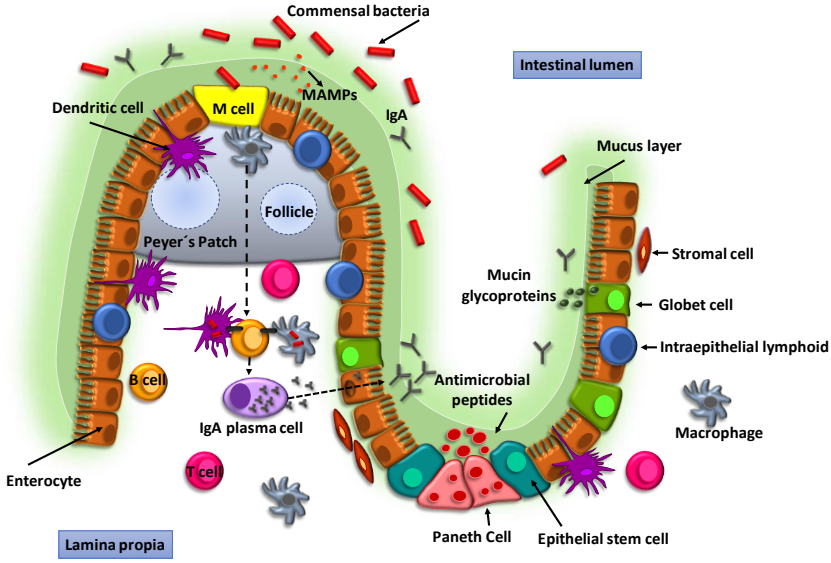

Figure 4. Immune and intestinal epithelial cells. The Image shows the different intestinal epithelial cell types. The secretory Globet cells that overlie the Peyer`s patches, the Paneth cells that release antimicrobial proteins, and the M cells that transport luminal antigens across the epithelial barrier to intestinal DCs. The DCs containing live bacteria induce IgA production to kill the bacteria (Abreu, 2010)

Among IECs there are also enteroendocrine cells, which are less abundant. These cells are highly specialized and specifically release hormones that regulate the digestive function. They represent a link between the central and the enteric neuroendocrine systems, being a special partner in the microbiota gut-brain axis.

1.1.2.2.2. INTERCELLULAR JUNCTIONS

Under normal conditions, the intestinal epithelial barrier maintains monolayer continuity because the IECs are attached to each other sealing the gaps between them. This process is controlled by intercellular junctions that include apical TJs, subjacent adherens junction (AJs) and desmosomes involved in cell-cell adhesion and intracellular signalling (Figure 5 A). IECs are interconnected by TJs and AJs to

create a physical barrier that separates the luminal material from the underlying immune cells and controls intestinal permeability. These intercellular junctions restrict the paracellular passage of bacteria or macromolecules, but they are permeable to small non-charged solutes. In this process pH has an essential role discriminating the passage of ions, water or small compounds through this space. The backbone of TJs and AJs is formed by transmembrane proteins in the cell membrane that associate with cytoplasmic plaque forming proteins such as zonula occludens-1 (ZO-1) or junction-associated molecule A. These cytoplasmic proteins constitute scaffolds for TJ assembly to intracellular actin-myosin cytoskeleton complexes, and in addition, are involved in the modulation of multiple cell processes such as cell proliferation and differentiation, as well as regulation of barrier permeability (Figure 5B). The tightness of the barrier depends on the tissue and the health status of the organism, with variations along the intestinal tract (Pelaseyed *et al*., 2014; Emami *et al*., 2009; Förster, 2008).

a) Tight junctions

TJs are the most known intercellular junctions. They are essential to control the paracellular passage and play pivotal role in organizing diverse processes as morphogenesis, cell polarity, cell proliferation and differentiation. The three main families of transmembrane proteins that form the TJ backbone are: claudins, TJ-associated MARVEL domain-containing proteins, and junctional adhesion molecules (JAMs). Function of these proteins requires an effective coordination through different signals where the cytosolic TJ plaque is one of the control sites.

a1) Transmembrane proteins:

- The claudin family consists of around 27 proteins, identified in different species. Claudins consist of four transmembrane regions, two extracellular loop domains and cytoplasmic N- and C- terminal domains. Claudins are classified in two groups: claudins that increase barrier tightness (claudins 1, 3, 5, and 18) and claudins that increase barrier permeability (claudins 2, 10 and 15). In addition to strengthen the epithelial barrier, some claudins may participate in signalling events that control epithelial homeostasis. The intracellular C-terminal domain of claudins binds to TJ-associated PSD95-DlgA-ZO1 homology (PDZ)-containing proteins such as ZO proteins and multi-PDZ domain protein 1 (MPDZ) facilitating the recruitment of structural and signalling proteins to TJs. Claudins are expressed in a tissue-specific manner and most IECs express more than two isoforms. This allows

multiple isotype combinations creating specific barrier functions. Claudins can be specific targets for pathogenic bacteria, causing TJ disassembly and barrier disruption, thus, claudins have an important role controlling intestinal homeostasis (Luissint *et al*., 2016).

- The *TJ-associated MARVEL domain-containing proteins* include three types of proteins: MARVELD1 (occludin), MARVELD2 protein (tricellulin) and MARVELD3 proteins. Occludin was the first TJ protein identified. The transmembrane domain spans the membrane four times and let a short N-terminal and a long C-terminal cytosolic domain. Its role in the control of paracellular permeability is not clear yet, but it is known that occludin is not essential for TJ formation, and by itself is not sufficient to form the TJ network. MARVELD3 proteins coupled with TJs affect cell behaviour and survival. MARVELD2 contributes to barrier regulation (Förster, 2008).

- Junction-associated adhesion molecules (JAMs). This class of integral membrane proteins is a member of the immunoglobulin superfamily. JAMs include three isoforms JAM-A, JAM-B and JAM-C, formed by a single transmembrane domain with two extracellular immunoglobulin-like loops and a cytoplasmic tail containing C-terminal PDZ-binding motif. JAM-A (F11 receptor) is the most studied protein and through its cytoplasmic motive, it establishes contact with PDZ domains of TJ scaffold proteins. JAM-A is involved in immune cells transmigration through endothelial cells and in cell adhesion. In general, JAM proteins positively modulate intestinal homeostasis, permeability, reinforcement of the barrier, cell proliferation, polarization and migration (Luissint *et al*., 2016).

a2) Cytosolic proteins:

- TJ plaque proteins or scaffold proteins. The first TJ-associated protein identified in 1989 was ZO-1 (Stevenson *et al*., 1986) and since then, two groups of proteins have emerged, including more than 30 different types. The first category contains peripherally associated scaffolding proteins such as ZO-1, ZO-2, AF6/afadin, cingulin and 7H6 antigen that join the transmembrane TJ proteins to the actin cytoskeleton and to other cytosolic proteins. The second class are signalling proteins (ZONAB, RhoA, RalA or Raf-1), implicated mostly in gene transcription, junction assembly and barrier regulation.

b) Adherens junction (AJs) and desmosomes.

The preservation of the intestinal epithelial barrier is not limited to TJs. Thus, processes like cell-cell contact and modulation of intestinal permeability are also managed by AJs complexes, which are formed by endothelial cadherins, nectins and catenins. The most important protein is E-cadherin, that assembles through α and β-catenin to actin filaments. This transmembrane protein also mediates calcium-dependent intercellular adhesions. Another type of intercellular junctions is desmosomes, which provide mechanical strength to the epithelium. This group includes cadherin-like proteins such as desmoglein and desmocollin (Luissint *et al*., 2016) (Förster, 2008).

In addition to these physical intercellular junctions, the immune system has also a central role in the modulation of the barrier function through the release of cytokines and other regulators.

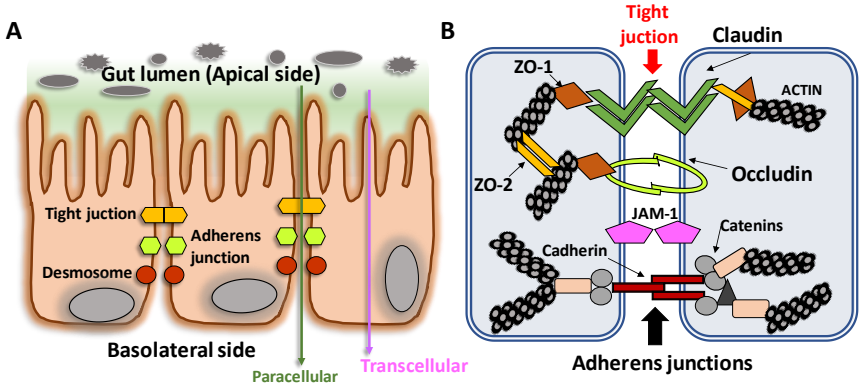

Figure 5. Intercellular connection between IECs. A) Localization of the diverse cellular junctions in adjacent epithelial cells. B) Schematic TJs and AJs representation that shows the transmembrane protein composition that connect cytosolic scaffold proteins with actin cytoskeleton to form intermolecular and intercellular connections in the paracellular space (Wells *et al.*, 2017).

1.1.2.3. RECEPTORS

The gut is continuously exposed to a huge density of commensal microorganisms. In addition, the intestine is faced by colonization of newcomers, including pathogens, and even by bacterial components that are spilled on the mucosal surface. Therefore, IECs must be in constant alert and awareness of what is happening in the intestinal lumen, defending the barrier and controlling the host health status. For that, IECs are equipped on their surface with innate immune

receptors, known as PRRs. The PPRs identify microbial-associated molecular patterns (MAMPs), also referred as pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), peptidoglycan (PG) or teichoic acids, which are components of the bacterial cell wall or components from fungi, yeasts or protozoa. PPRs also recognize endogenous signals, called danger-associated molecular patterns (DAMPs), which are produced by cells in response to ischemia or tissue damage. The detection of PAMPs or DAMPs can occur at both membrane and cytosolic levels. Thus, PRRs are classified in two subgroups in bases to their subcellular localization:

a) Membrane receptors. This group includes 2 families:

a1) Toll-like receptors (TLRs). These are classic innate PPRs, which include 10 different types in humans and 12 in mice. They belong to a family of type-I transmembrane glycoproteins composed of: (i) a N-terminal domain with leucine-rich repeat motifs that dictates ligand specificity, (ii) a hydrophobic transmembrane domain, and (iii) a C-terminal toll-interleukin-1 receptor (TIR) domain that triggers intracellular signalling. They can be localized in the cell membrane or in intracellular membranes of the endosomal compartments. The types TLR1, TLR2, TLR4, TLR5 and TLR6 are found mainly in the plasma membrane, but types TLR3, TLR7, TLR8, TLR9 (humans) and TLR11, TLR12, TLR13 (murine) are found in intracellular endosomal compartments (membranous vesicular structures like the endoplasmic reticulum, endosomes, lysosomes and endolysosomes). In relation to the function that TLRs perform, TLR1, TLR2 and TLR6 recognize lipoproteins and lipopeptides; TLR3 double-stranded RNA; TLR4 lipopolysaccharides; TLR5 structural proteins of bacterial flagellum, and TLR7, TLR8 and TLR9 recognize nucleic acids (Table 1). Besides being present in IECs, TLRs are expressed in a wide variety of cell types like DCs, macrophages, neutrophils, monocytes, T and B cells, epithelial cells, endothelial cells, fibroblasts and even neural cells. Nevertheless, the TLR composition depends on the cell type. In the intestine, the TLRs mainly expressed are TLR4 and TLR5 (Marques and Boneca, 2011; Tang *et al*., 2012; Muñoz-Wolf and Lavelle, 2016; Oviedo-Boyso *et al*., 2014). TLR4 receptor was the first TLR identified. It recognizes Gram-negative LPS and myeloid differentiation factor 2 (MD2) on the cell surface, but it can also detect viral envelope proteins. TLR5 is mainly expressed at the basolateral side of IECs and by DCs located in the intestinal lamina propria. This receptor recognizes flagellin from flagellated bacteria and triggers protection

responses, such as the induction of B cells to produce IgA and the differentiation of T naïve cells into Th17 and Th1 (antigen-specific) (Takeuchi and Akira, 2010; Kawai and Akira, 2009).

a2) C-type lectin receptors (CLR). This important family of receptors plays a major role in antimicrobial immunity. These receptors are mainly expressed in myeloid cells and recognise carbohydrates such as mannose, fucose and glucans, thus allowing recognition of most types of pathogens (Takeuchi and Akira, 2010).

b) Cytosolic receptors. These receptors can be divided into three subgroups:

b1) Nucleotide-binding oligomerization domain leucin-rich repeat-containing receptors or NOD-like receptors (NLRs). Nowadays, 22 and 34 NLR types have been identified in humans and mice respectively. Structurally, these receptors are organized in three functional domains: (i) the central nucleotide-binding or NACHT domain required for oligomerisation, (ii) the C-terminal leucine-riche repeat (LRR) for specific ligand recognition, and (iii) an N-terminal protein-protein interaction domain, which manages intracellular signal induction. NLRs can be divided into four subsets depending on the N-terminal effector region. These subsets are: NLRA (only one member is included, and it is involved in transcriptional modulation of MHC class genes), NLRB (with baculovirus inhibitor repeat or BIR domain), NLRC (with CARD domains) and NLRP (with pyrin domain or PYD). These receptors sense a wide range of cytosolic PAMPs in the cytosol albeit they can also be stimulated by cytosolic DAMPs. Diverse intracellular stresses lead to the generation of DAMPS such as loss of cell membrane integrity induced by bacterial toxins, ion imbalance, radical oxygen species (ROS) or extracellular ATP sensing. NLRs are key orchestrators of inflammation as components of a protein complex known as inflammasome, although they also participate in many other inflammasome-independent functions, in which NOD-1 (CARD4) and NOD-2 (CARD15) members have special relevance (Table 1). NOD1 is ubiquitously expressed, whereas NOD2 is mainly expressed in innate immune cells and IECs. The main biological role of NLRs has been related to host defence against infection, through activation of NFκB and MAPK signalling pathways that induce production of pro-inflammatory cytokines and antimicrobial molecules (Marques and Boneca, 2011; Tang *et al*., 2012; Muñoz-Wolf and Lavelle, 2016; Oviedo-Boyso *et al*., 2014).

PRRs and ligands			
PRRs	**Localization**	**Ligand**	**Origin**
TLRs			
TLR1	Plasma membrane	Triacil lipoprotein	Bacteria
TLR2	Plasma membrane	Lpp	Bacteria, virus and parasites
TLR3	Endolysosomal	dsRNA	Virus
TLR4	Plasma membrane	LPS	Bacteria and virus
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacil lipoprotein	Bacteria and virus
TLR7 (Human TLR8)	Endolysosomal	ssRNA	Bacteria and virus
TLR9	Endolysosomal	CpG-DNA	Bacteria, virus and protozoa
TLR10	Endolysosomal	Unknown	Unknown
TRL11	Plasma membrane	Profilin type molecule	Protozoa
NLRs			
NOD1	Cytoplasm	m-DAP	Bacteria
NOD2	Cytoplasm	MDP	Bacteria

Table 1. PRRs classification. This table summarize the main PRRs found in human cells, based on their localization, ligand and origin. (Takeuchi and Akira, 2010; Arroyo *et al*., 2015).

b2) Retinoic acid inducible gene I (RIG-1)-like receptors (RLRs). This small group of cytosolic receptors are constitutively expressed in a wide array of immune and non-immune cells and act as sensors of RNA helicases that recognize viral cytosolic RNA (Wells *et al*., 2011).

b3/b4) AIM2-like receptors (ALRs). This is a newly described PRR family, which members act as intracellular sensors for nucleic acids (Tang *et al*., 2012; Muñoz-Wolf and Lavelle, 2016).

Another important family of innate immune response receptors are the Scavenger Receptors (SR). This family comprises transmembrane glycoproteins of the cell surface that are present in IECs but also in immune cells such as macrophages and DCs. SRs are divided into eight classes based on the domain structure, and the most representatives are SR-A, MARCO and CD36. They play an

important role in innate immune defence recognizing PAMPs (LPS, lipoteichoic acid-LTA, bacterial CpG DNA and yeast zymosan/β-glucan) and act as co-receptors of TLRs, modulating the inflammatory response against TLR agonists. In DCs and macrophages these receptors mediate direct phagocytosis (Areschoug and Gordon, 2009).

The activation of PRRs leads to a signalling cascade that triggers a transcriptional program that activates the expression of modulators, like pro-inflammatory mediators or AMPs, and in B cells induces IgA class switching. These effects are on the basis of the crosstalk between the intestinal epithelium layer and the adjacent immune cells. These interactions are completely necessary to maintain intestinal homeostasis. A failure in this crosstalk triggers an abnormal mucosal response and leads to a pathological status (Marques and Boneca, 2011; Tang *et al*., 2012; Muñoz-Wolf and Lavelle, 2016; Oviedo-Boyso *et al*., 2014).

1.1.3. INTESTINAL IMMUNE SYSTEM

The immune system is the most complex machinery in the human body for protection against harmful external influences, and it is essential for survival. It is made up of different organs, cells and proteins spread throughout the body, and they work together to form alliances against pathogens. Just before birth, the neonatal immune system is immature, but with the organism development and the direct contact with external components, this system will go learning, maturing and developing by itself. This system acquires special relevance at the GI tract as the GI surface is the largest area of the whole body in direct contact with the outside. Moreover, the intestinal tract is the first organ that after birth must harbour a huge number of external microorganisms. Most of them come from the mother and establish the intestinal microbiota of the new-born. Thus, GI balance between health and disease is principally leaded by the IIS. Besides of direct protection, IIS is necessary for the modulation of many other physiological and signalling processes whose purpose is maintaining the welfare state of the organism. For instance, the production of mucus is controlled by immune mediators such as leukotrienes, interferon-γ (INFγ) or interleukins.

Most of immunological processes take place in the intestinal mucosa. Cells of the innate and adaptive immune system are also present in this layer or mixed with

epithelial cells, receiving the name of intraepithelial lymphocytes (IEL) (Mowat and Agace, 2014). The set of intestinal immunological molecules that works in the intestine is called GALT, and it provides host protection against harmful pathogens and has a fundamental role in the development of induced tolerance against innocuous products from food and commensal microbiota, all of which are potentially immunogenic.

Anatomically, GALT can be divided into two immunological areas. The first one includes organized tissues like isolated lymphoid follicles, Peyer´s patches and mesenteric lymph nodes, which are responsible for the induction of the adequate immune response. A second level of organization is formed by isolated immune cells, including IgA-secreting plasma cells, T cells located in the lamina propria and T cells distributed throughout the epithelium compartment (Figure 4).

The Peyer´s patches are macroscopic lymphoid aggregates located in the submucosa and separated from the intestinal lumen by a single layer of columnar epithelial cells known as the follicle-associated epithelium (FAE). Internally, they are formed by collections of B-cell follicles and intervening T-cell areas. The FAE is characterized by the presence of B cells, T cells, macrophages, DCs and the specialized enterocytes M cells that are the main inducers of the intestinal immune response. M cells capture luminal antigens and microorganisms by transcytosis (endocytosis or phagocytosis) but they are unable to process them by themselves because they do not express Major Histocompatibility Complex (MHC) class II molecules. Instead, they transport the antigenic molecules to the antigen-presenting cells (APCs) such as macrophages and DCs, located mainly in the Peyer´s patches. After this, APCs, which express MHC in their surfaces, go to the T-cell areas and B-cell follicles and present the processed antigens through MHC to naïve lymphocytes, which pass through the draining lymphatic system to the MLNs where they are differentiated. Moreover, in the GALTs, the presence of antigens reduced L-selectin expression and increased expression of integrins, which facilitates the passage of lymphocytes from the blood stream to the intestinal mucosa (Mowat, 2003).

All this complex network that combines innate and adaptive immunological processes are integrated by the central and the IIS to ensure the health equilibrium in the GI tract, which must adapt to and function within a constantly changing environment.

1.1.3.1. INNATE IMMUNITY

The host innate immune system provides the first line of defence against infection by pathogens. The innate immune system is a non-memory non-antigen specific defence mechanism against microorganisms (Mowat, 2003). A great variety of anatomical, physic, chemical and biological barriers is involved in the innate response. First, both mucus and intestinal epithelial layers create a protective frontier that limits the access of luminal antigens to the host. In addition, bowel peristaltic movements difficult pathogen adhesion to the intestine. The chemical barriers include acidic pH, bile salts, SCFAs, hydrolytic enzymes, etc., that restrict bacterial growth. Finally, the beneficial commensal and probiotic microbiota create a biological wall, which try to compete and block the pathogen entry into the gut mucosa. All these mechanisms are external strategies to avoid infection, but if the pathogen manages to overcome all of them, the innate immune system has other internal defence mechanisms to destroy the infectious agent. The main components of these internal mechanisms are divided in two groups: (i) cellular components such as phagocytic cells (macrophages), natural killer (NK) cells, eosinophils and innate lymphoid cells (ILC γδ); and (ii) soluble factors like C reactive protein, cytokines or the complement system.

When the pathogen gets to reach the adjacent area of the intestinal epithelium, three possible mechanisms get underway: phagocytosis or endocytosis (pinocytosis or receptor-mediated endocytosis). In phagocytosis, when the antigenic particle touches the cell surface, the target cell emits pseudopodia that encompass the particle. Endocytosis consists in the ingestion of soluble material of the extracellular fluid by small endocytic vesicles (Okumura and Takeda, 2017; Richards and Endres, 2014; Neu *et al*., 2010).

However, the major signalling process that activates the innate immune system is through immune receptors. With this strategy, bacterial-wall components like LPS or PG or other pathogen derived products known generally as PAMPs alert the innate immune system by interacting with cell membrane or cytosolic PRRs expressed by IECs and DCs (Mowat, 2003). This recognition plays an importer role initiating the innate immune response. The transmission of information activates the effector cells to produce pro-inflammatory cytokines (TNF-α, INF-γ and IL-12) and chemokines (IL-8) through NFκ-B activation to recruit and activate more cells of the innate immune system including neutrophils, NK cells, and macrophages.

Activation of these immune cells in turn triggers production of antimicrobial mediators such as reactive oxygen or nitrogen species (ROS or RNS) that suppress bacterial growth. Moreover, Paneth cells contribute to innate immunity secreting a wide array of antibacterial products (lysozyme, α-defensins, phospholipase A2) that try to control the infection (C. Sasakawa, 2009). On the other hand, IECs express antimicrobial molecules such as REgIIIγ that binds to bacterial peptidoglycan, reducing bacterial colonization of the inner dense mucous layer that covers IECs. Recently, a specific group of innate lymphocytes derived from common lymphoid progenitors, designated as ILCs, have been identified. These cells play essential roles in defence against intestinal bacteria that cross through the IECs layer and reside in GALTs. They represent an important defence of the innate immune system mainly orchestrating antimicrobial defences. Three different phenotypes have been characterized: ILC1 (T-bet dependent and INF-γ producer), ILC2, (GATA-3 dependent and IL-4 / IL-13 producer), and ILC3 (RORγt dependent and IL-17 / AIL-22 producer) (Caballero and Pamer, 2015).

Immature DCs in peripheral and lymphoid tissues are highly phagocytic and able to recognize microbes via PRRs on their surface. Upon contact DCs mature and migrate to lymph nodes and present antigenic peptides to immune cells (C. Sasakawa, 2009).

In addition, some cytosolic receptors of the NLR family proteins can sense cytosolic PAMPs, and at the same time activate the assembly of caspase-1. Caspase-1 is activated through interaction with the apoptosis-associated speck-like protein containing a carboxi-terminal (ASC). This tripartite association is entailed in the regulation of innate immune responses contributing to the antimicrobial defence. The term coined for this tripartite complex is inflammasome. Activation of caspase-1 through an auto-proteolytic process allows the secretion of the pro-inflammatory cytokines IL-1β and IL-18. IL-1β generates fever as a mechanism to combat infection, activates lymphocytes and promotes infiltration of immune cells at sites of infection. IL-18 induces INF-γ and contributes to Th1 polarization (Eisenbarth and Flauell, 2009; Martinon *et al*., 2002).

1.1.3.2. SIGNALLING PATHWAYS

During gestation and later development of the organism, cells acquire evolutionarily conserved pattern-recognition receptors (PRRs) and scavenger receptors (SRs), located at cytoplasmic level, at cell surface or at intracellular cell membranes to protect against pathogen invasion and to maintain intestinal homeostasis. As stated above, these receptors are key components of the innate immune response and their natural ligands are conserved molecular structures found in bacteria, viruses and fungi, generally denominated as PAMPs. The nomenclature and classification of the different receptors and their ligands have been presented in the section "Intestinal epithelial receptors" and summarized in table 1. However, far from a simple characterization, pharmacological and genetic approaches have been tackled to understand the intracellular signalling mechanisms that trigger the final response.

TLR-associated pathways. TLRs constitute, together with NLRs, the most studied immune receptors. TLRs are key players of the inflammatory and innate immune responses that control important gut functions, such as epithelial cell proliferation, IgA production, maintenance of TJs, antimicrobial peptide expression and recognition of pathogens. To achieve these processes, TLRs must transmit the received information to an intracellular signalling cascade, in which five different TIR-domain adaptor molecules come into play: myeloid differentiation primary-response protein 88 (MyD88), TIR- protein (TIRAP), TIR-domain-containing adaptor protein-inducing INF-β (TRIF), TRIF-related adaptor molecule (TRAM) and sterile-α- and armadillo-motif-containing protein 1 (SARM1).

The MyD88 pathway is one of the most important signalling routes that guide translocation of the NFκB transcription factor into the nucleus to control the expression of a diverse array of genes related with immunity, cell cycle, and anti-apoptotic or stress responses. The cascade is initiated by recruitment of MyD88 to the TIR binding domain of TLR. MyD88 interacts with IL-1R-associated kinase (IRAK), which in turn associates with TRAF6 that activates the transforming growth factor-activated kinase 1 (TAK1). TAK1 activates by phosphorylation I kappa B kinase (IKK) complex (formed by two subunits, IKKα and IKKβ), which in turn phosphorylates IκB proteins. This phosphorylation triggers ubiquitination and degradation of IκB, which allows the translocation of NFκB into the nucleus. In the cytosol, NFκB is inactive when associated to IκB proteins. Once in the nucleus, NFκB

activates transcription of genes related with inflammation (TNF-α, IL-1β, IL-6 and IL-12p40), cell adherence and recruitment of chemokines, growth factors and anti-apoptotic signals. Moreover, activated TAK1 also stimulates the mitogen-activated protein kinase (MAPK) pathway, which results in the phosphorylation of the extracellular signal-regulated kinase (ERK), the c-Jun N-terminal kinase (JNK) and the p38 kinase. Then, JNK phosphorylates c-Jun that binds to c-Fos forming the AP-1 complex, which goes to the nucleus and stimulates transcription of inflammatory genes (Figure 6). In addition, p38 kinase can phosphorylate and activate the transcription factor cAMP response element-binding protein (CREB), which induces transcription of pro-inflammatory mediators such like cyclooxygenase 2 (COX-2) and TNF-α.

Another important signalling pathway is controlled by the TIR-domain adaptor molecule TRIF, which acts in collaboration with MyD88 to activate NF-κB and AP-1 transcription factors. It is known that TLR4 and TLR3 signals activate the TRIF pathway using different domains. The C-terminal domain contacts with the receptor-interacting protein 1 (RIP1) kinase, which becomes ubiquitinated and recruits TRAF6 and TAK1. In this tripartite complex, TAK1 is activated and, as previously described for the MyD88 pathway, generates the active nuclear form of NFκB and AP-1 transcription factors (Figure 6). The TRIF N-terminal region, through IKKs and TRAF activation, activates interferon regulatory transcription factor (IRF), which translocate into the nucleus and upregulates the transcription of type-I interferons (INF-I), like INF-β. The INF-I pathway plays a key role in antiviral responses and control of cell survival and differentiation of haematopoietic cells.

Interestingly, TLR4 is the only TLR capable of activate both MyD88 and TRIF cascades.

NLR-associated pathways. Intracellular NLRs have two main signalling routes depending on the final function: the inflammasome pathway and the inflammasome-independent pathway. Some members of NLR family link to an adaptor protein called ASC which is equipped with two domains: the pyrin domain that interacts with NLRs, and the caspase activation and recruitment domain or CARD, which recruits caspase-1 to induce self-cleavage and activation. Activated caspase-1 will transform pro-IL-1β and pro-IL-18 into active forms. The active inflammasome complex has key roles in immunologic processes such as antimicrobial or autoimmune responses. Regarding the non-inflammasome

pathways, it is remarkable the functions of some NLRs member like NOD-1 and NOD-2. Both receptors recognize PG derived peptides. NOD-1 recognises the γ-D-glutamyl-meso-diaminopimelic acid (iEDAP) and TriDAP (like iEDAP with an extra D-alanine residue). These structures are present in Gram-negative bacteria and in a restricted number of Gram-positive bacteria. Thus, many studies report the involvement of NOD-1 in the recognition of *Helicobacter pylori*, *Pseudomonas aeruginosa* or *Shigella flexneri* (Gram negatives), *Listeria monocytogenes* and *Streptotoccus pneumoniae* (Gram-positives) and parasites like *Trypanosoma cruzi*. However, NOD-2 recognises the muramyl dipeptide (MDP), a compound widely present in many microorganisms including both Gram-positive and Gram-negative bacteria. NOD2 recognizes pathogens as *Mycobacterium tuberculosis*, *Listeria monocytogenes* and *Toxoplasma gondii*. Both NOD1 and NOD2 receptors are constitutively expressed by many cell types, albeit factors like cytokines, TLR ligands and bacteria can also induce their expression, especially that of NOD2. The signalling pathway begins with the presence of intracellular PG that has been introduced into the target cell by means of invasive pathogens or through endocytosis. In the endocytic pathway PG reaches the endosomal compartments, where by means of specific endosomal channels can access the cytosol, where NOD recognizes the ligand. Upon recognition, oligomerization of NOD is activated and in this form, recruits the adaptor protein RIP2 that establishes contact with the CARD domains of NOD. RIP2 suffers polyubiquitination and recruits TAK1, which lead to the activation and nuclear translocation of NFκB. In addition, NEMO is also recruited to the NFκB activating complex (Figure 6). RIP2 also activates the MAPK pathway. On the other hand, NOD2 also recognizes viral single-stranded RNA and through the IRF pathway induces INF-I transcription factor that activates INF-β production. NOD-1 also triggers INF-I activation through iEDAP recognition (Muñoz-Wolf and Lavelle, 2016**;** Kawai and Akira, 2009; Marques and Boneca, 2011; Oviedo-Boyso *et al*., 2014)**.**

In most cases this complex network of signalling pathways is not enough to clear the infection, but help to control the pathogen until more specific responses from the adaptive immune come into action

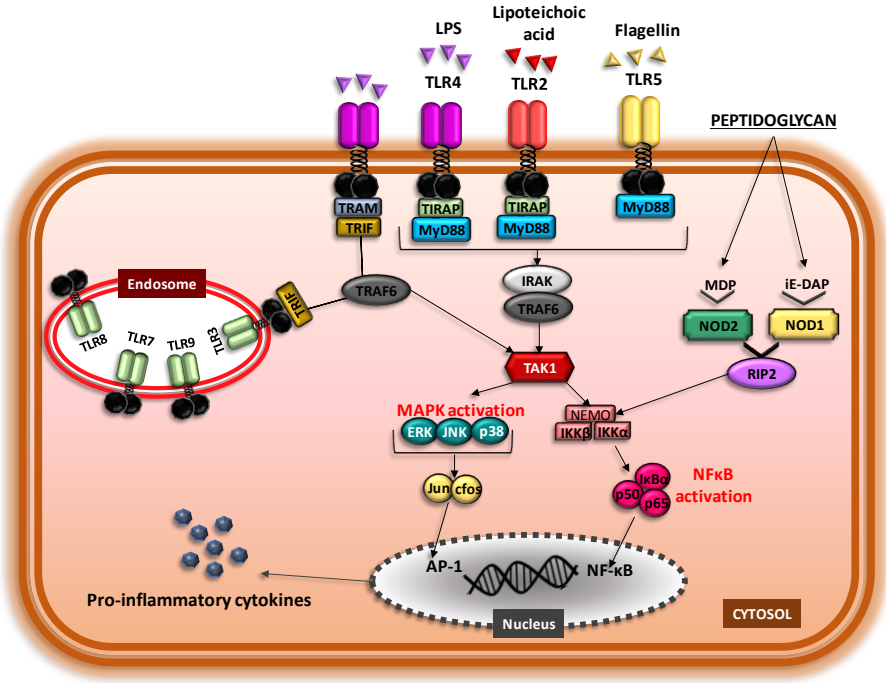

Figure 6. Diagrammatic representation of TLR and NLR signalling pathways in intestinal epithelial cells. Microbial pathogens and pathogen-derived components can activate host receptors, which activate the recruitment of different adaptor proteins (MyD88, TIRAP, TRIF or TRAM) to the cytoplasmic domains. MyD88 activates by phosphorylation IRAK and promotes its association with TRAF6, which recruit and activate the kinase TAK1. TAK1 activates the IKK complex, which in turn phosphorylates and subsequent degrades IKb, allowing nuclear NF-κB translocation to activate the expression of multiple genes. Moreover, TAK1 can also activate the MAP kinase signalling pathway, which triggers the final activation of the AP-1 transcription factor. On the other hand, bacterial PG can also activate cytosolic NOD receptors, which in turn activate NF-κB through IKK complex activation (Knight *et al*., 2008; Bermudez-Brito *et al*., 2012b).

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1.1.3.3. ADAPTIVE IMMUNITY

Unlike innate immunity, the adaptive immunity is antigen-specific, and its execution is slower. Each antigen is specifically recognized and memorized. Thus, adaptive responses increase after repeated exposures to the same agent.

The key players in adaptive immunity are APCs, B and T cells, which control the humoral and cellular responses. Especially, APCs (macrophages and DCs) play a fundamental role in the connexion between innate an adaptive immunity because they are responsible for processing and presenting the antigens to T cells through MHC (Chorny *et al*., 2010).

After the intestinal innate responses take place via PRRs-PAMPs and ASCs activation, lymphocytes enter the mucosa, redistribute into distinct compartments and trigger an adaptive immune response with the participation of specific cells: a) B-cells, producers of antibodies or immunoglobulins that stay in the lamina propria, b) $CD4^+$ T cells, redistributed in the villus-crypt unit, are important in local tolerance to environmental antigens, and c) $CD8^+$ T cells that migrate to the epithelium and have cytotoxic activity (Mowat, 2003).

One important adaptive immune defence mechanism at the intestinal level is secretory IgA. This immunoglobulin is secreted at the gut lumen by differentiated plasma cells (derived from naïve B cells), mostly located in the lamina propria and Peyer´s patches, in response to intestinal microorganisms. Secretory IgA (sIgA) binds to basolateral receptors in the intestinal epithelium and is translocated across IECs to the lumen. In the mucus layer, sIgA traps bacteria and dietary antigens, neutralizes pro-inflammatory PAMPs and blocks bacterial binding sites in the epithelium. Thus, sIgA that surrounds the pathogen neutralizes its pathogenic potential and helps antigen recognition to mediate a specific response. Thus, sIgA plays an important role in providing defence against pathogens (antimicrobial activity) and maintaining microbiota homeostasis (Caballero and Pamer, 2015; C. Sasakawa, 2009).

T cells are the most relevant players of the adaptive immunological responses. Normally, mucosal T cells are classified based on T cell receptor (TCR) and co-receptor expression. Conventional T cells or "type a" express the TCRαβ receptor and the two co-receptors CD4 and CD8αβ (heterodimer). The non-conventional T cells or "type b" contain on their surface the two receptors TCRαβ and TCRγδ, and CD8αα as co-receptor. The type a T cells are abundant in the lamina propria, whereas the type b T cells are more prevalent in the mucosal epithelium.

Referring to type a T cells, they can be classified based on their function in three different linages: (i) T helper o Th cells (TCRαβ $CD4^+$) that are mainly found in the lamina propria, (ii) memory T cells, and (iii) T cytotoxic o Tc cells (TCRαβ $CD8^+$), which kill cancerous, damaged or virally infected cells by inducing apoptosis (Van Wijk and Cheroutre, 2010; Charles A Janeway *et al.*, 2001). More recently, two new T cell linages have been identified: Foxp3-expressing regulatory T cells or Treg cells ($CD4^+$ CD25), which are essential in preventing exaggerated responses against gut microbiota (tolerance or hypo-responsiveness), and pro-inflammatory IL-17

producer cells or Th17 cells ($CD4^+$), which play a critical role in host defence against bacterial and fungi when the other T cells linages cannot confer complete protection.

T cells maturation and bifurcation of the $CD4^+$/$CD8^+$ linages happen in a multi-layered process that is thymus and MHC-dependent (Tanaka and Taniuchi, 2013).

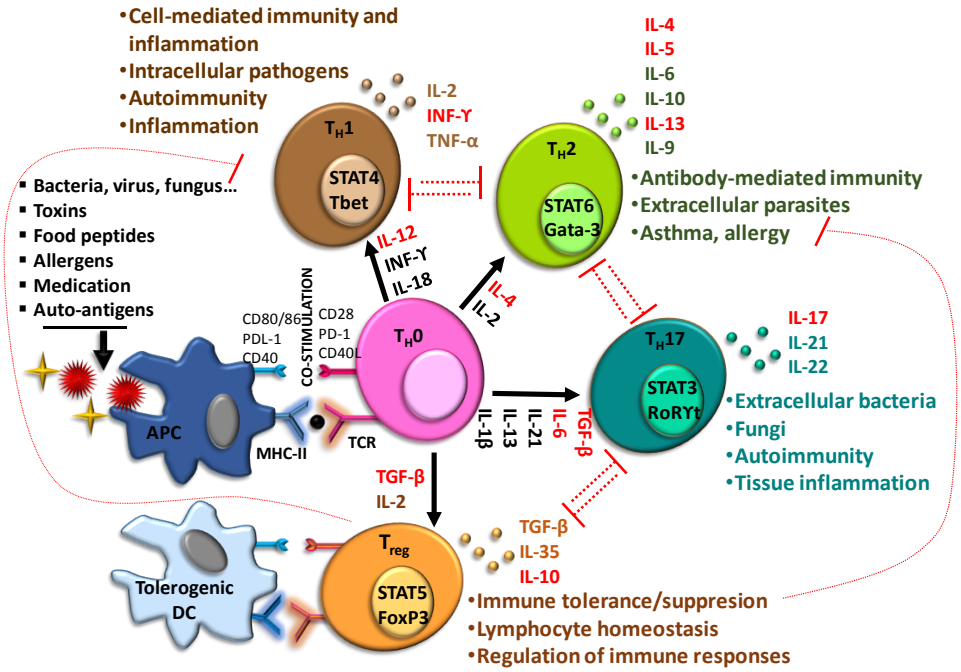

Figure 7. Differentiation into different effector $CD4^+$ T cell linages. Differentiation to T helper (Th1, Th2, Th17) and T regulatory (Treg) cells is initiated through interaction of uncommitted (naïve) CD4+ T helper cells with APC cells. The effector cell types are characterized by synthesis of specific cytokines and their immuno-regulatory functions. Differentiation along different linages involves different cytokines, and activation of distinct signalling pathways and transcription factors (Jetten, 2009; Ait-Oufella *et al.*, 2014).

However, interestingly, some studies have discovered an epigenetic regulation stablished by gut microbiota in which some conventional intestinal $CD4^+$ T cells can be reprogrammed and acquire the cytotoxic features that $CD8^+$ lymphocytes have when they become IELs. Moreover, it has been reported that TGFβ can induce $CD8^+$ expression on conventional $CD4^+$ T cells (Konkel *et al.*, 2011; Mucida *et al.*, 2013).Upon activation, lymphocytes Th naïve differentiate into Th (TCRαβ $CD4^+$) or

Treg ($CD4^+$ CD25) depending on the stimulus. These subpopulations can be further classified according to the released cytokines (Figure 7)

Regarding Th cells four subsets have been established, namely Th1, Th2, Th17 and Treg. Th1 cells secrete IL-2 and INF-γ and cooperate in the development of T and B cells, as well as in cell mediated immunity by stimulating phagocytosis of intracellular microorganisms. Th2 cells secrete IL-4, IL-5, IL-6, IL-10 and IL-13 and contribute to the humoral immunity (immunoglobulin secretion by B cells). These cells mediate allergic reactions and provide defence against infections caused by helminths and arthropods. Th17 subset releases mainly IL-17 and it has related to IBD pathogenesis. However recently, Th17 effect has been argued, and most of the authors propose a protective effect of Th17 on the GI tract under steady-state homeostatic regulation, but a pathogenic effect under dysbiosis and uncontrolled inflammation. In general, they also participate helping to clear fungal and extracellular bacterial infections when Th1 and Th2 responses are not efficient enough. On the other hand, Treg cells are divided into two subgroups: Th3 cells that release TGFβ and Treg cells that secrete IL-10. Both cell types display suppression activity during inflammatory and infectious processes and contribute to food and microbiota tolerance. (Neu *et al*., 2010; Hirahara and Nakayama, 2016)(Owaga *et al*., 2015).

1.1.3.4. INFLAMMATORY MEDIATORS

At this point, it is interesting to highlight the crucial role of cytokines and chemokines mediating immune-inflammatory responses of innate or adaptive type. Cytokines are small soluble factors with pleiotropic functions that are produced by many cell types, predominantly leukocytes, as part of a gene expression pattern that regulate immunity (innate and acquired), inflammation and haematopoiesis. They interconnect a network of signals to alert the whole organism of a potential threat, discriminating between the proper and the strange to maintain intestinal homeostasis. Perturbations on this regulatory network of cytokines by genetic, environmental or microbial elements have deleterious consequences. This group of inflammatory mediators encompasses a multitude of types with different functions, which are summarized in table 2 (Appendix). This wide group of immune mediators have been classified in three categories: pro-inflammatory cytokines, anti-inflammatory cytokines and chemokines (Khan, 2008;

Martin-Viñas and Quigley, 2016; Zuber Shaikh, 2011). The mediators that have a more relevant role in inflammation and are directly linked with this work, are explained with more detail as follows.

Pro-inflammatory cytokines

-Interleukin-1 superfamily. It mainly includes two proteins, IL-1α and IL-1β, which play a central role in both innate and adaptive responses. The major sources of IL-1 are activated mononuclear phagocytes. IL-1 exerts its effects through cell surface receptors (IL-1R1 to IL-1R9). The main physiologic effect is fever, as IL-1 enhances prostaglandin E_2 (PGE_2) in bacterial and viral infections. This cytokine induces the acute phase response against infection, inducing tissue inflammation and the release of histamine from mast cells that generates vasodilation. Activation of Th cells and B cells is also mediated by IL-1.

This family also includes another pro-inflammatory member, IL-18, which plays a role in cancer and various infectious diseases. It is produced by macrophages, DCs and IECs. IL-18 bears structural homology to IL-1α and IL-1β and depends on IL-1β to become mature. Moreover, caspase-1 also contributes to its processing. IL-18 plays an important role in host defence against bacterial, viral, fungal and protozoan infections. A relevant mechanism of IL-18 action is the induction of INF-γ, which activates nitric oxide synthesis, among other mediators, and eliminates the invading pathogen (Khan, 2008; Martin-Viñas and Quigley, 2016; Zuber Shaikh, 2011).

-Interleukin-6: IL-6 is synthesized by a wide variety of cells such as mononuclear phagocytes, T cells (Th1), B cells, endothelial cells and fibroblasts. Its biological functions include: stimulation of acute phase protein synthesis (primary inducer of fever), maturation of B cells, activation and differentiation of T cells, and induction of hormones and IL-2. IL-6 is induced by bacterial LPS along with TNF-α and IL-1. All these functions classify IL-6 as a pro-inflammatory cytokine. Nonetheless, in some circumstances, IL-6 acts as an anti-inflammatory agent, downregulating the production of pro-inflammatory cytokines like GM-CSF or INF-γ (Khan, 2008; Martin-Viñas and Quigley, 2016; Zuber Shaikh, 2011).

-Interleukin-12: IL-12 is produced by activated B cells, macrophages and other APCs and has immunoregulatory effects on NK and T cells. IL-12 plays an essential role in cell-mediated immunity enhancing the Th1/Th2 balance into Th1 or T cytotoxic responses. Thus, IL-12 regulates the production of INF-γ from macrophages, which in turns inhibits IL-4 and IL-10. Recently, IL-23 has been shown to display a similar structure to IL-12 and analogous biological functions (Khan, 2008; Martin-Viñas and Quigley, 2016; Zuber Shaikh, 2011)

-Interleukin-17 family: This family consists of a subset of cytokines that mediate in both acute and chronic pro-inflammatory responses. Six members have been identified, from IL-17A to IL-17F, which are commonly referred as IL-17. We will focus on IL-17A, secreted mainly by Th17 cells against pathogen invasion and fungal infection at epithelial and mucosal barriers. IL-17 mediates their immunological effects by inducing pro-inflammatory cytokines, AMPs and chemokine secretion, which triggers the recruitment of neutrophils and macrophages to eliminate the pathogen. However, a dysregulated IL-17 production can result in excessive pro-inflammatory cytokine expression and chronic inflammation, which lead to tissue damage. Signal transduction of IL-17A is mediated through NF-κB and MAPK activation (Jin and Dong, 2013).

-Interleukin-22: IL-22 is a glycoprotein belonging to the IL-10 related cytokine family. It is released by NK cells, innate and adaptive immune cells, albeit, specifically, it is highly expressed by Th17 cells. IL-23 is a potent inducer of IL-22 expression in NK cells. Moreover, IL-6 and TNF-α are the major driving cytokines for IL-22 release. The IL-22 receptor is highly expressed within tissues such as epithelial cells of the GI tract. It is known that IL-22 leads to the activation of proliferative and anti-apoptotic pathways to maintain the epithelial barrier during inflammation. In this context, IL-22 induces pro-inflammatory cytokines, mucins and AMPs to control the epithelial integrity during inflammation and fighting against pathogens. Recent evidences show a correlation between IL-17 and IL-22, which play a central role in IBD. Both mediators are upregulated in the intestinal mucosa of IBD patients. However, IL-22 has been described as a master regulator in intestinal barrier regeneration, promoting a protective role in IBD (Dudakov *et al*., 2015; Eyerich *et al*., 2017; Sanjabi *et al*., 2010).

-Tumour necrosis factor (TNF): Five members are included in this group, being TNF-α and TNF-β the most studied proteins. They both are produced by activated monocytes/macrophages, fibroblasts, mast cells and NK cells. Specifically, TNF-α is mainly produced by activated macrophages, NK cells, B and T cells (Th1), and the most potent inducer is LPS. TNF shares with IL-1 the function of producing fever, and with IL-6 the ability to induce acute phase reactive proteins. Thus, LPS infection triggers the widespread secretion of TNF-α (and subsequently IL-1 and IL-6) by macrophages. The main role of TNF-α and TNF-β can be summarised in endothelial activation, tumour cell killing, apoptosis and mediation of inflammation (Khan, 2008; Martin-Viñas and Quigley, 2016; Zuber Shaikh, 2011).

-Interferon (INF): This group is subdivided into INF-α, produced by leukocytes, INF-β by fibroblasts and INF-γ by activated T cells and NK cells. The most studied type is INF-γ, which is a potent activator of phagocytes and produced by a vast number of cell types. In the innate defence response, APCs and NK cells stimulate the production of INF-γ, while in acquired responses T lymphocytes are the major source. Its production is modulated by IL-12 and IL-18 (secreted by APCs) but inhibited by IL-4, IL-10 and TGF-β (Khan, 2008; Martin-Viñas and Quigley, 2016; Zuber Shaikh, 2011).

Anti-inflammatory cytokines

-Interleukin-10: It is the main anti-inflammatory cytokine within the human immune response. IL-10 is secreted by a large range spectre of cells including macrophages, mast cells and even T cytotoxic cells to inhibit viral infections. However, the main source comes from monocytes and Th2 cells. The major immune biological effect is the regulation of the Th1/Th2 balance. IL-10 inhibits the synthesis of many pro-inflammatory cytokines (IL-1, IL-2, IL-3, IL-4, IL-6, IL-18, GM-CSF, TNF-α, MIP-1α and INF-γ). Thus, IL-10 promotes Th2 responses, inhibiting INF-γ production from Th1 cells through IL-12 suppression. Additionally, IL-10 increases the production of anti-inflammatory compounds. Endogenous concentrations of IL-10 are essential to protect the host from systemic inflammation (for example, it limits the inflammatory response to gut-associated bacteria), but it can render the host susceptible to lethality from infection. For this reason, the balance pro-

inflammatory/anti-inflammatory cytokines is essential for maintaining health conditions (Khan, 2008; Martin-Viñas and Quigley, 2016; Zuber Shaikh, 2011).

-Transforming growth factor (TGF)-ß: This family includes approximately 30 members, that have effects on many cells types, playing important roles in inflammation, cell proliferation control, cell differentiation and formulation of the extracellular matrix. Nevertheless, TGF-β1 is the most studied and abundant. TGF-β1 is produced by leukocytes and is involved in the suppression of T and B cell proliferation and differentiation, limiting IL-2 and TNF production. However, like other cytokines, TGF-β has dual pro and anti-inflammatory activities, which pivot on the presence of other growth factors, cytokine composition in different inflammatory environments and the differentiation state of the target cells. Additionally, TGF-β1 is the representative cytokine of Treg cells. In general, this family is involved in development, immune regulation, immune tolerance, carcinogenesis, tissue repair and differentiation of many cell types (Khan, 2008; Martin-Viñas and Quigley, 2016; Zuber Shaikh, 2011).

Chemokines

Chemokines form a large family of small heparin-binding chemotactic cytokines, which role is attracting neutrophils at sites of inflammation, but also activate migration of other cell types such as monocytes, lymphocytes, basophils or eosinophils. The primary signals produced by innate responses are key stimuli for the secretion of chemokines. Some of them are involved in illness conditions like allergic asthma, arthritis, psoriasis and chronic inflammatory disorders. This group of cytokines includes around 27 members and they are produced by a wide variety of cell types (phagocytes, T cells, endothelial and epithelial cells and neutrophils). Some chemokines have pro-inflammatory potential and their release requires specific stimulus like viral infection, bacterial products or other agents like IL-1 and TNF-α.

-Interleukin-8 (CXCL8): IL-8 is the most studied chemokine, and it is characterized by having a dual role as pro-inflammatory cytokine and chemokine. IL-8 is produced by and targets an extensive variety of cells types including leukocytes and non-leukocytes. Its main role is to act as chemoattractant for neutrophils at the inflamed tissue and stimulate granulocyte activity. For that, IL-8 upregulates the

expression of cell-surface molecules like the intracellular adhesion molecule (ICAM-1), which allows neutrophils adherence to endothelial cells and their extravasation through vessels walls. (Khan, 2008; Martin-Viñas and Quigley, 2016; Zuber Shaikh, 2011).

-MIP-1α-(CCL3): Macrophage inflammatory protein 1α (MIP-1α) is a member of the C-C subfamily of chemokines, a large superfamily of low-molecular weight that exhibit a variety of pro-inflammatory activities. MIP-1α is a chemoattractant of macrophages, monocytes, neutrophils and T cells. This chemokine is produced by a variety of cells, including lymphocytes, monocytes/macrophages, mast cells, basophils, epithelial cells and fibroblast (Cook, 1996; Ramos *et al*., 2005).

Taken together, the immune system encompasses a complex network which includes a great number of immunological mediators, immune cells and non-related immune cells. Specifically, intestinal host-microbial interface forms a collaborative intimate alliance between the innate-adaptive immune components and the host microbiota, whose final and key function is maintaining an intestinal host balance, characteristic of a health status. In this equilibrium, the protector role of host microbiota (commensal and probiotics) is essential. However, in some cases, threatening events such as uncontrolled bacterial colonization, epithelial barrier disruption or dysregulated immune stimulation, can lead to break the homeostatic balance. In this case, if the host multi-responses are not efficient or enough to solve the problem, an unhealthy status can be developed, leading to the disease.

1.2. INTESTINAL DISORDERS AND MICROBIOTA IMBALANCE

As previously described, the host GI tract coexists with a complex community of over 100 trillion microbial cells, identified as intestinal microbiota. However, because of their key influence on human wellbeing is also considered as a “hidden organ”. Changes in microbiota population can have harmful impact on human health. Many factors (genetics, environment, diet, reduced physical activity…) may lead to imbalances or shifts in microbiota composition, a concept known as dysbiosis. This condition has been linked to GI disorders caused by bacterial or viral infections, and also to metabolic (obesity, insulin resistance, hepatic steatosis) and

immunological diseases (allergies, autoimmune diseases or inflammatory diseases) (Ghoshal *et al*., 2017; Guinane and Cotter, 2013). Here, we will focus on IBD and bacterial infections.

1.2.1. IMMUNOLOGICAL DISEASES

Within illnesses associated with IIS, one of the most studied is the IBD, a collection of idiopathic inflammatory disorders, which pathophysiology (chronic and reverting inflammation of the GI tract) is mainly linked with variations in the normal gut microbiota. Since the middle of the twentieth century, IBD incidence is rising dramatically in industrialized countries. Currently, the prevalence of IBD in the Western world is up to 0.5% of the general population, and it is estimated that over one million residents in the USA and 2.5 million in Europe have IBD (Kaplan, 2015).

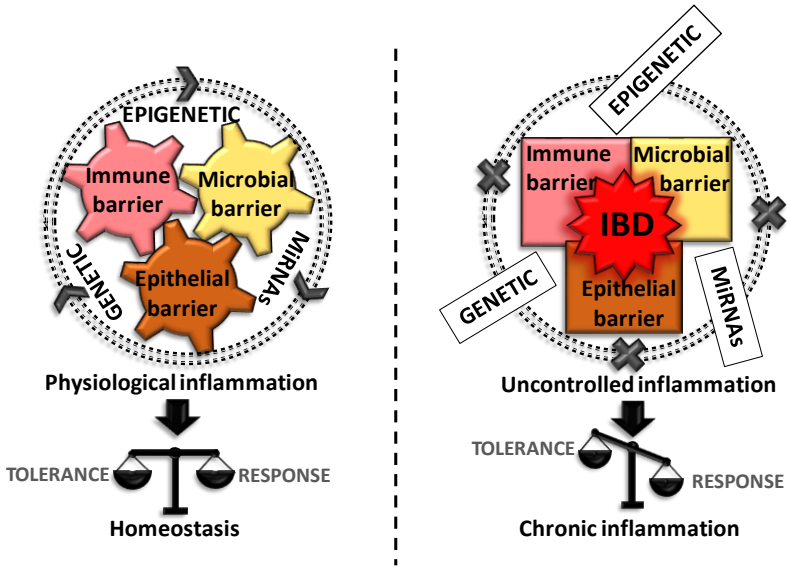

Figure 8. Factors involved in inflammatory bowel diseases. Pathogenesis of IBD results from the combined effects of a complex interaction between three basic components: microbiota, immune system and intestinal epithelium, which in turn are controlled by genetic and epigenetic factors, such as miRNAs modulation. The consequence of these altered parameters (right panel) is the intestinal disease, characterized by an uncontrolled inflammation . (Loddo and Romano, 2015; Zhang *et al*., 2017b).

The aetiology of IBD remains largely unknown, albeit, there is a general consensus that a combination of four factors is involved in the IBD pathogenesis:

environmental influences, genetic variations, intestinal microbiota alterations and disturbances in the innate and adaptive immune responses (Figure 8) (Triantafillidis *et al*., 2011). Studies done in monozygotic twins have provided evidence for genetic predisposition to IBD, showing concordance rates of 36% for Crohn's disease (CD) and of 16% for ulcerative colitis (UC). However, this hereditable component is not responsible for the whole illness, indicating that the pathogenesis of IBD is due to an unappropriated and persistent inflammatory response to commensal gut microbes in genetically susceptible individuals (Aguilera and Melgar, 2016). Integration of all this information using modelling systems could guide to a better understanding of the origin of IBD and the wide spectrum of associated manifestations (Loddo and Romano, 2015; Balzola *et al*., 2011).

The two major subtypes of IBD are UC and CD, which have received special attention because their incidence is increasing. Although they are common disturbances, and their symptoms are well-studied (diarrhoea, abdominal pain, rectal bleeding, fever, nausea and vomiting), its aetiology remains poorly understood. They can seriously impair quality of life and require prolonged medical and/or surgical interventions (Zand *et al*., 2015). In general, histologically and macroscopically, IBD is characterized by cell infiltration associated with oedema, loss of Globet cells, fibrosis, erosions and intestinal ulcers. However, UC and CD mainly differ in the distribution along the GI tract and the associated damages.

In CD, inflammation may affect to any part of the GI tract and the intestinal epithelium can be affected in a transmural or a segmental way (Baumgart and Sandborn, 2007). In this pathogenesis, Th1 and Th17 responses are involved, which induce a severe inflammation through INF-γ, IL-12 and IL-17, (Wang *et al*., 2016). Unlike CD, UC is restricted to the colon, specifically to the mucosa and submucosa with extensive epithelial damage, cryptitis and crypt abscesses (Baumgart and Sandborn, 2007). UC is characterized by continuous, diffuse and superficial inflammation of the colon, accompanied by bloody and mucous diarrhoea (Lucas López *et al*., 2017). In this case, the cytokine profile in inflamed areas seems to exhibit a Th-2 profile, with high presence of IL-4 and IL-13 (Xu *et al*., 2014).

An effective cure for IBD does not exist yet, and thus, patients often require lifelong medication. Conventional treatment for IBD involves the use of immunosuppressive agents, corticosteroids, TNF, antibodies and antibiotics, but not all patients are responsive to drug therapy (Denis *et al*., 2016). As commented

above, microbiota is important in the pathogenesis of IBD. In fact, recent studies have reported a marked change in microbiota composition (quantitative and qualitative) in CD and UC, being reduced microbial diversity a common characteristic in comparison with healthy individuals (Frank *et al*., 2007). Studies in IBD patients have shown a reduction in *Bacteroidetes* and *Firmicutes* and an increase in *Proteobacteria* (Lupp *et al*., 2007; Frank *et al*., 2007, 2011; Sartor and Mazmanian, 2012), pointing to the hypothesis of dysbiosis as a main mechanism involved in IBD pathogenesis.

Along medical evolution, antibiotic treatment in IBD was the first option. Nowadays, it is routinely used, albeit in some cases, like in immunocompromised patients, this treatment is not recommended, as antibiotics destroy unconsciously commensal microbiota, thus increasing the risk of barrier loss and severe infections. In addition, discontinuation of antibiotic therapy is associated with bacterial resistance (Surette and Wright 2017) (S. B. Levy and McMurry 1974; N. Wang *et al*. 2017). Regarding these limitations, another therapeutic strategy for IBD has emerged, based on manipulation of the gut microbiota and focused on beneficial bacteria intake (Becker *et al*., 2015). For instance, clinical assays in patients with IBD have shown that intake of VSL#3 (a mix of beneficial bacteria) induces the remission rate of the illness with no severe adverse effects (Sood *et al*., 2009). Moreover, the probiotic *Escherichia coli* Nissle 1917 is an efficient treatment against UC in children and adolescents, and in preventing relapse of CD (Henker *et al*., 2008).

Interestingly, due to the important role of microbiota on health, new treatment approaches, focused on restoring the whole microbiota balance, have evolved in the last few years. This is the use of faecal microbiota transplantation (FMT). The first controlled trial using FMT to treat IBD was published in 1989. Since then the number of new FMT trials has considerably increased, showing good remission rates (Lopez and Grinspan, 2016). Nowadays, FTM is commonly used in clinical trials as treatment of *Clostridium difficile* infections and as a mechanism to understand links between microbiota and obesity (Paramsothy *et al*., 2017). For instance, recent *in vivo* mice experiments have shown that transferring microorganisms from the gut of obese mice to lean mice, and *viceversa*, affect the way in which microorganisms metabolize food, thereby reversing or inducing obesity respectively (Kulecka *et al*., 2016). However, more well-designed controlled

studies of FMT potential are needed because long-term durability and safety remain unclear.

1.2.2. GASTROINTESTINAL INFECTIONS: *SALMONELLA*

Worldwide, one of the main problems of human mortality is food-borne infections by *Salmonella* spp., usually initiated by ingestion of contaminated food or water. This Gram- negative intestinal pathogen, is a member of *Enterobacteriaceae* family that has developed some specific mechanisms to overcome the resistance to colonization (mediated by gut microbiota) and to mislead the hots immune system, giving as a result intestinal dysbiosis and infection. The central clinical manifestations of salmonellosis are gastroenteritis and typhoid fever (Ahmer and Gunn, 2011).

Salmonella virulence genes are mainly organized in clusters, which receive the name of *Salmonella* pathogenicity islands (SPI). In *Salmonella*, five SPIs have been described (SPI-1, SPI-2, SPI-3, SPI-4 and SPI-5). SPI-1 encodes a type III secretion system (T3SS-1 or SPI-1 T3SS) that injects into the target cell several bacterial effector proteins (SptP or SopE) involved in pathogen internalization. SPI-2 encodes another type III secretion system, which is required for bacterial survival into epithelial cells and macrophages. SPI-3 encodes genes required for intracellular bacterial growth under limiting Mg^{2+}concentration, a condition characteristic of phagosomes, and also encodes an adhesin required for intestinal persistence. SPI-4 has been described to be essential for intestinal infection and epithelial adherence by SiiE, co-interacting with SPI-1. SPI-5 has been less studied, but it is believed to encode effector proteins that are secreted by the SPI-1 and SPI-2 T3SS (Main-Hester *et al*., 2008).

Salmonella spp. encompasses six different serotypes in bases on the agglutinating properties of the somatic O, flagellar H and capsular Vi antigens (*Abortusovis, Choleraesuis, Dublin, Enteritidis, Gallinarum* and Typhimurium), being the serotypes *Thyphimurium* and *Enteritidis* the major cause of human GI salmonellosis. All of them contain a virulence plasmid named *Salmonella* plasmid virulence (*spv*), which harbours five genes *spv* RABCD. The whole set of virulence genes encodes proteins involved in intracellular multiplication, regulation of host proteins or ADP-ribosylation of actin to disrupt the cytoskeleton of IECs. Moreover,

Salmonella spp. also produces endotoxins (lipid A of the outer membrane lipopolysaccharide) and exotoxins (cytotoxins, enterotoxins and salmolysin). Additionally, *Salmonella* spp. also expresses fimbriae, which are filamentous surface structures involved in cell adhesion and invasion. Besides, most of *Salmonellae* present flagella, which confer motility and also contribute to the infection process (Van Asten and Van Dijk, 2005).

When *Salmonella* spp. overcomes the commensal bacteria layer, the pathogen can invade IECs at the apical side, migrate to the basolateral membrane, and through exocytosis reach the interstitial space of the lamina propia, where they are captured by APCs and rapidly spread. The recognition between APCs (mostly macrophages) and the bacteria is an important point in *Salmonella* pathogenicity. Two molecular mechanisms have been proposed as cell invasion pathways: (i) a receptor-mediated entry pathway, in which bacteria are strongly linked to the host cell membrane and only few cytoskeletal rearrangements are necessary, and (ii) a non-dependent receptor pathway, in which T3SS-1 of *Salmonella* is indispensable for host cell invasion.

Here, we will explain in detail the role of T3SS-1 in *Salmonella* invasion (Figure 9). The T3SS-1 is main pathogenicity multi-subunit protein complex by which the pathogen injects, like a needle system, effector proteins directly from *Salmonella* into the host cell cytoplasm. When the pathogen interacts with the host cell surface through cholesterol affinity, the injectisome or needle-type complex is assembled on the host cell membrane allowing the entry of translocases (SipB, SipC and SipD) and virulence effectors in a programmed order. The five major effectors to manipulate the host cytoskeleton are SopE, SopE2, SopB, SipA and SipC. The first three proteins (SopE, SopE2, SopB) activate Rho family members (Cdc42 and Rac), which in turn activate protein complexes (N-WASP, Scar/WAVE-Arp2/Arp3), that promote actin remodelling. SipA and SipC can link actin by themselves, promoting actin polymerization at the bacterial attachment point. All these invasion effectors affect normal host cell processes related with TJs integrity, membrane trafficking, cell division, apoptosis, bacterial killing, cytokine and chemokine production (Figure 9) (Velge *et al*., 2012).

The secretion system named T3SS-2 (SPI-2 encoded) is localized in *Salmonella*-containing intracellular vacuoles (SCV), thus allowing the release of effectors that are necessary for bacterial intracellular survival and replication (Velge *et al*., 2012).

Furthermore, experiments in mice indicate that a novel type VI secretion system (T6SS) is also required for full *Salmonella* virulence (Liu *et al*., 2013).

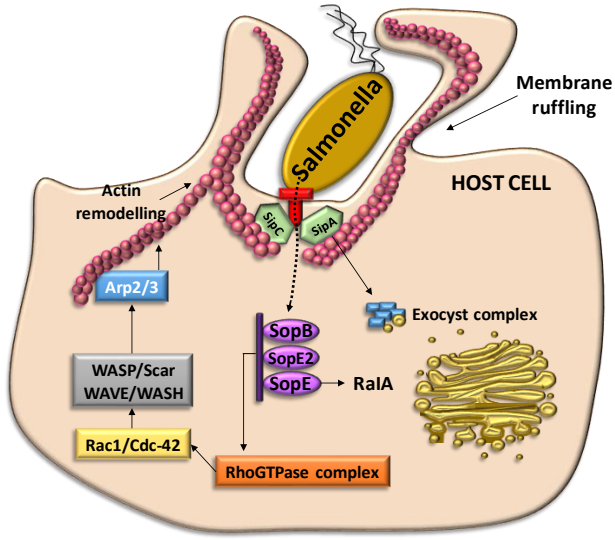

Figure 9. Proposed T3SS-1 mechanism used by *Salmonella* to infect cells. *Salmonella* infects nonphagocytic cells by targeting several signalling pathways that induce transient actin-rich membrane ruffles to engulf the bacteria. This process is carried out by bacterial effector proteins that are injected into host cells through T3SS-1. Thus, *Salmonella* invasion proteins (Sip C and Sip A) stabilize the needle system and help to the actin polymerization machinery to produce membrane ruffling. This activity is supported by other bacterial effectors such as SopB, SopE2 and SopE, that pass through the channel T3SS into the host cells and they contribute to the actin remodelling and host cell exocytosis (Velge *et al.*, 2012)**.**

Recently, increasing evidence suggests an important role of micro-RNAs (miRNAs) in different pathologies, like salmonellosis. miRNAs are non-coding small molecules of RNA, of about approximately 22 nucleotides in length, that control post-transcriptional downregulation of target mRNAs in a great number of biologic processes. Modulation of miRNAs is one of the strategies adopted by bacterial pathogens to survive inside host cells. miRNAs are key components of the molecular cross-talk between bacteria (either microbiota or pathogens) and intestinal mucosa cells. *In vitro* studies indicate that *Salmonella* regulate miRNAs in both epithelial cells and macrophages (Schulte *et al*., 2011; Sharbati *et al*., 2012). For instance, *Salmonella* LPS activates NF-κB through TLR4, and NF-KB in turn activates the expression of miR-146a and miR-155, which target TRAF-6 and TAB2 respectively, to counteract the inflammatory response (Carlyon *et al*., 2016; Maudet *et al*., 2014).

1.3. PROBIOTICS

As already presented, gut microbiota is an essential component of human physiology, with a main role in modulating immune responses. The intestinal microbiota contains an immense variety of microorganisms where bacteria are a key piece. This group includes commensal and probiotic bacteria, responsible for the maintenance of the intestinal homeostasis. In this context, it is important to emphasize the beneficial impact of probiotic bacteria on intestinal function (Ghoshal *et al*., 2017).

Although humans have been consuming probiotics for centuries in fermented food, the first legal definition for probiotics was done in 2001 by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) committee as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (WHO and FAO, 2006). The word "probiotic" is derived from the Greek language and it can roughly be translated into "for life" (Sonnenborn and Schulze, 2009). However, this term should be reconsidered since dead probiotics or bioactive molecules produced by them (proteins, peptides, nucleotides and polysaccharides) also have beneficial effects. Thus, the terms "live is not an exclusive requirement for the probiotic activity (Ghoshal *et al*., 2017).

Beyond this definition, probiotics must present certain multifactorial characteristics to fulfil the criteria to be classified as a probiotic. The main probiotic features are: not to have human pathogenic effects, be able to survive in the intestinal tract (resist to the acidic pH in the stomach and also to bile salts and digestive enzymes in the small intestine), be a good colonizer of the intestinal mucosa, diminish pathogen adherence and invasion to cell surface (for example changing gut environment or competing for nutrients), and present antimicrobial properties against pathogens (such as bacteriocins). Besides, all these characteristics must be accompanied by beneficial effects on the host at different levels, especially the intestinal epithelial barrier function, IIS and the microbiota itself (Ghoshal *et al*., 2017).

Enhancement of the epithelial barrier. The intestinal wall is the main defence mechanism to protect the host from the environment. It is known that probiotics help to maintain the epithelial barrier, and one of mechanisms used is the

regulation of TJ proteins. For instance, *Escherichia coli Nissle* 1917 (EcN) prevents the disruption of the mucosal barrier caused by the enteropathogenic *E. coli* (EPEC) and restore the mucosal integrity by enhancing ZO-2 expression (Zyrek *et al*., 2007). *Lactobacillus* spp positively modulates the epithelial barrier by influencing the phosphorylation and abundance of adherence junction proteins (Hummel *et al*., 2012). Similarly, VSL#3 probiotic cocktail upregulates expression of TJ proteins through activation of ERK pathway (Dai *et al*., 2012). The ability of *Bifidobacterium* spp. to strengthen the epithelial barrier has been attributed to secretion of metabolites like acetate (Hsieh *et al*., 2015). In addition to TJ regulation, probiotics can exploit other mechanisms to enhance the barrier function, like promoting mucus secretion. For example, the probiotic cocktail VSL#3 increases the expression of MUC2, MUC3 and MUC5AC in a cellular model (Otte and Podolsky, 2004)

Increased adhesion to intestinal mucosa. This is a prerequisite for the subsequent colonization and interaction with the host IECs. Normally it is done by specific interaction of probiotic surface proteins with components of the mucus and epithelial layer, leading to the competitive exclusion of pathogens. In this context, *Lactobacillus* and *Bifidobacterium* have been reported to produce surface proteins, like adhesins, which mediate attachment to the mucous layer (van Tassell and Miller, 2011). For instance, *Lactobacillus reuteri* 1063 secretes a mucus-targeting bacterial adhesin named mucus-binding protein (MUB) (Roos and Jonsson, 2002). However, these probiotic mechanisms act in combination with host defence mechanisms, such as production of chemical defences or AMPs by IECs that help to prevent colonization by pathogenic bacteria and barrier disruption. For example Paneth cells secrete lysozyme, which attack bacteria cell membranes (Bel *et al*., 2017). Interestingly, some probiotics up-regulate the expression of host AMPs. This is the case of *Escherichia coli Nissle* 1917, which induces the secretion of defensins by epithelial cells contributing to stabilize the gut barrier (Möndel *et al*., 2009).

Competitive exclusion and production of anti-microbial compounds. Probiotics can inhibit the attachment of pathogens by means of steric interference at IECs receptors. However, probiotics also have other mechanisms to avoid pathogen

colonization, such as creation of a hostile environment, production and secretion of antimicrobial products (AMPs) like lactic acid, or competition for available nutrients. Some AMPs released by probiotics induce pore formation or inhibit cell wall synthesis in the target bacterial cells, thus destroying and killing pathogens (Bermudez-Brito *et al*., 2012b). For instance, lactacin F and nisin, two bacteriocins produced by lactic acid bacteria, form transient channels in bacterial membranes that lead to membrane injury (Dalmau *et al*., 2002).

Modulation of the immune system. Probiotics can exert immunomodulatory effects. This is because DCs interact directly with bacteria that have gained access via M cells. The interactions are usually through TLRs on DCs, which start a Treg differentiation (Bermudez-Brito *et al*., 2012a). Thus, probiotics can up-regulate expression and production of protective cytokines or inhibit the release of pro-inflammatory cytokines, diminishing in this way cytokine-induced epithelial damage. This leads to the strengthening of the epithelial barrier and to the establishment of a tolerant state (Gómez-Llorente *et al*., 2010). For instance, *Lactobacillus rhamnosus* secretes two proteins called p75 and p40, which inhibit cytokine-induced epithelial apoptosis and promote cell epithelial growth (Yan *et al*., 2007). Moreover, probiotics can supress intestinal inflammation via downregulation of TLR expression, secretion of metabolites that inhibit TNF-α from incoming blood mononuclear cells and inhibition of NF-κB signalling in enterocytes (Gómez-Llorente *et al*., 2010).

However, it is important to note that not all these properties are found in all probiotics. Thus, each probiotic has individual mechanisms of action that differ from other strains, and confer specific characteristics. Thus, probiotic effects are strain-specific. However, it has been proposed that some mechanisms could be highly widespread among the probiotic genera, others could be frequently present among probiotic species, and others may be observed only in a few strains (Figure 10).

The original observation that specific bacterial strains provide health benefits was proposed by the Russian Èlie Metchnikoff at the Pasteur Institute, who 110 years ago observed that yogurt ingestion (containing *Lactobacillus* spp.) positively influences longevity of certain human population in Bulgaria.

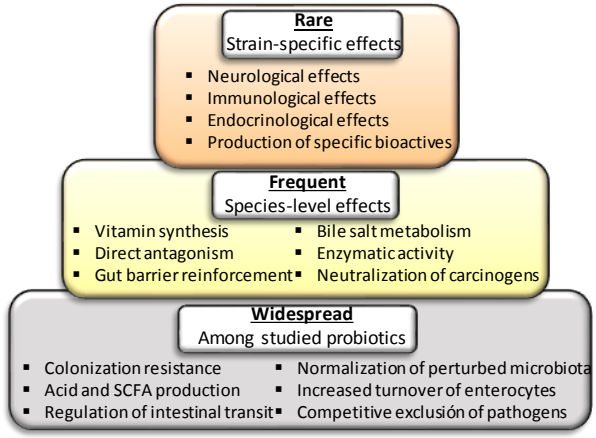

Figure 10. Distribution of beneficial mechanisms among probiotics. Probiotics commonly present mechanisms that are generally found among other genera. In addition, frequently, they can also have mechanisms that are distributed mainly within same species. However, there are also mechanisms strain-specific, which are less abundant within probiotic community (Hill *et al*., 2014).

In 1907, he suggested that "The dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes" (Surette and Wright, 2017; WHO and FAO, 2006). Nowadays, the ingestion of probiotics is enormously extended in human population, and a new industry has arisen around it. Thus, among the most common probiotic bacterial strains that have clinical uses are: *Lactobacillus* spp., *Bifidobacterium* spp., *Escherichia coli Nissle* 1917 and *Streptococcus thermophilus*. Moreover, other nonbacterial microorganisms are also included as probiotics, like the yeast *Saccharomyces boulardii*. All of them have usually been studied as possible treatments against GI diseases. For instance, *Lactobacillus casei* Shirota has been described to reduce the incidence of diarrhoea associated with *Clostridium difficile* (Wong *et al*., 2014); *Saccharomyces boulardii* is used to prevent and improve antibiotic-associated diarrhoea symptoms (Zhang *et al*., 2017a); a specific probiotic cocktail named VSL#3 (mixture of *Streptococcus thermophilus*, *Lactobacillus* and *Bifidobacterium* strains) was effective for UC remission (Sood *et al*., 2009), and *Bifidobacterium longum* in preventing the same disease (Wei *et al*., 2016). In addition to their use in intestinal disorders, probiotics have been explored to treat other pathological conditions such as respiratory tract infections, urinary

tract infections, bacterial vaginosis, allergic rhinitis and asthma, diabetes mellitus, obesity etc.

Although probiotics generally have an exceptional and established safety record, the truth is that they are not completely absent from risk, especially in some population groups such as premature infants, immunocompromised individuals, critically ill patients and those with central venous catheters. This is in part due to their condition of being alive bacteria, and some of them have evolved from pathogenic strains that after evolution have lost or inactivated their virulence factors (Ghoshal *et al*., 2017).

1.3.1. *ESCHERICHIA COLI* NISSLE 1917

Escherichia coli Nissle 1917 (EcN) was discovered by the physician and bacteriologist Alfred Nissle Gesellschaft from Freiburg, Germany. He observed that some *E. coli* bacteria in a faecal sample, isolated from a soldier who survived to an outbreak of diarrhoea during The First World War, showed antagonistic *in vitro* activity against pathogenic microorganisms. Dr. Nissle assumed that this *E. coli* strain had protected the soldier from a *Shigella* infection that killed most of his companions. This was the first time, before the antibiotic era, that an agent with anti-infective action was described. Since then, this probiotic was patented in Germany with the name of Mutaflor® (Sonnenborn and Schulze, 2009).

EcN (O6:K5:H1 serotype) is a Gram-negative bacterium that belongs to the *Enterobacteriacea* family. Its genome contains 5324 genes and 190 are strain-specific, being also responsible of the probiotic characteristics of EcN. This strain is included in the *E. coli* B2 phylogenetic subgroup, and has received the GRAS (Generally Recognized As Save) status. EcN is one of the most studied probiotics and widely used in clinical treatments because of its anti-inflammatory properties. In 1989, it was reported by the first time that treatment with EcN improved symptoms in patients suffering enteric disorders (group of 1074 patients). However, the greater effectiveness of EcN has been associated with the remission of UC. Clinical trials have shown that EcN was as effective as mesalazine (the clinical standard treatment) in the remission of UC (Reister *et al*., 2014; Wassenaar, 2016).

Although the anti-inflammatory and beneficial properties of EcN on intestinal inflammatory diseases have been widely proven, the mechanisms by which EcN

exerts these positive effects are not always known. It is assumed that efficacy of this probiotic can be attributed to a combination of factors that mediate probiotic fitness in the intestinal tract, interference with pathogens and immunomodulatory activities (Sonnenborn and Schulze, 2009; Reister *et al*., 2014; Trebichavsky *et al*., 2010).

Fitness factors

Unlike pathogenic bacteria, which release virulence factors, EcN is a non-pathogenic bacterium that expresses a great variety of fitness factors that contribute to its colonization efficiency, survival within the host, competition with other strains and communication with the host (Figure 11) (Trebichavsky *et al*., 2010). Thus, EcN produces three types of fimbriae (F1A, F1B and F1C), which facilitate its adhesion to IECs and the colonization of the gut. In particular, studies done in infant mice have shown that F1C fimbriae is necessary for biofilm formation, adherence of EcN to IECs and the consequent colonization of the gut (Lasaro *et al*., 2009). Besides, EcN O6 polysaccharide side-chain is very short due to a point mutation that introduces a stop codon in the gene *wzy* encoding the O6 antigen polymerase. The lack of this enzyme gives a semi-rough phenotype when EcN is grown in solid media. The O6 antigen represents the outer part of the LPS, and this particular change makes EcN serum-sensitive (Grozdanov *et al*., 2002). Moreover, the presence of an extracellular capsular antigen K5, endows EcN with a capsule that mediates adhesion and colonization. The K5 capsule also stimulates TLR5, increasing the expression of antimicrobial responses and chemokines by epithelial cells (Hafez *et al*., 2009, 2010). EcN also possesses flagella (serotype H1), which allow bacterial movement in the viscous intestinal mucus layer and induce antimicrobial responses through TLR5 activation.

However, probably the most remarkable EcN fitness factor is the expression of multiple siderophores (iron-chelating compounds) that allow this probiotic to uptake iron. Most of bacteria require iron for survival, but its acquisition is generally difficult because iron has low solubility and high toxicity. In this context, EcN expresses at least five different iron uptake systems that allow survival under limiting iron conditions, namely aerobactin, enterobactin, salmochelin, yersiniabactin and hemin-citrate dependent iron acquisition systems. Moreover,

EcN can capture ferrous compounds by an EfeU system (Sonnenborn and Schulze, 2009).

Interference factors

EcN releases interference factors that delay invasion of pathogens in human IECs. Among the interfering strategies used by EcN there are bacteriocin production, intracellular ATP depletion, increasing membrane permeability, sensitization to lytic action, weakening of flagellar motility and prevention of bacterial internalization (Trebichavsky *et al*., 2010). For example, EcN produces microcins MccH47 and MccM that help to compete with bacteria in the GI tract. They are AMPs that have some bactericidal properties against phylogenetically related bacteria (Wassenaar, 2016), but the producer strain is itself immune against them. For instance, *in vitro* experiments showed the antimicrobial potential of EcN, directly inhibiting enterohemorrhagic *E. coli* (EHEC) growth (Mohsin *et al*., 2015). Metabolically, EcN produces SCFAs, mainly acetic acid and formic acid but also propionic and butyric acids, as end products of carbohydrate metabolism. SCAFs may inhibit growth of certain pathogens (Sonnenborn and Schulze, 2009).

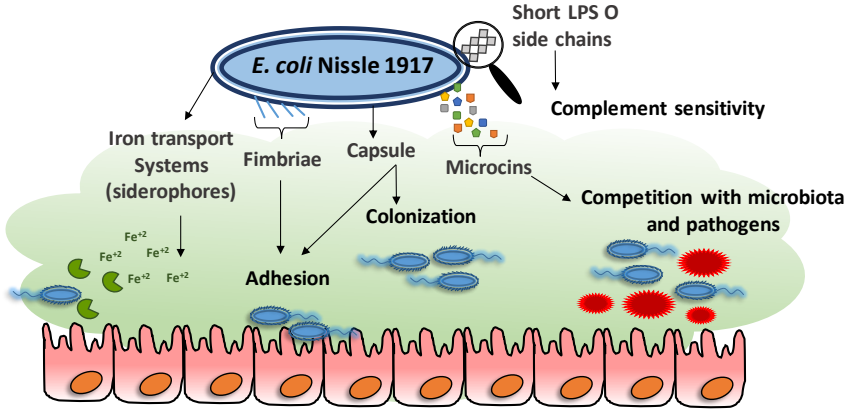

Figure 11. Main fitness and interference factors of EcN. This figure shows the multiple fitness properties that EcN exploits to colonize the gut and compete with resident microbiota and pathogenic bacteria, making EcN a safe microbe for hosts (Behnsen *et al*., 2013).

On the other hand, EcN activates IECs to secrete AMPs, such as cathelicidins, lipocalin-2 that binds to siderophores and sequesters ferric enterochelin, and zinc and manganese chelating agents, thus limiting availability of these ions to other

bacterial strains (Sonnenborn and Schulze, 2009). In this context, flagellin is also able to activate in IECs the expression of β-defensin-2, an antimicrobial peptide that inhibits a huge range of microorganisms (Gram-negative, Gram-positive, yeast and virus) (Behnsen *et al*., 2013; Deriu *et al*., 2013).

Modulatory factors

EcN is also able of exert beneficial effects to control intestinal homeostasis. This includes induction of alert molecules (IL-8 and other chemokines), anti-inflammatory and immuno-stimulating factors, as well as regulation of TJs.

Regarding immunomodulatory factors, EcN increases the secretion of IgA and mucin, and modulate the balance between anti- and pro-inflammatory cytokines in the host by regulating T cell activation, ensuring an immunological homeostasis (Guzy *et al*., 2008; Becker *et al*., 2014). This activity has been evidenced in studies using different *in vitro* epithelial and immune models. Thus, EcN limits intestinal inflammation by diminishing the secretion of pro-inflammatory cytokines such as IL-2, TNF-α, IFN-γ, IL-6, IL-1β, GM-CSF or G-CSF (Sturm *et al*., 2005; Jiang *et al*., 2014), and increasing anti-inflammatory cytokine production such as IL-10 (Helwig *et al*., 2006) and downregulating the expansion of recruited T cells (Sturm *et al*., 2005). However, EcN is also capable to activate, through K5 capsule and TLR5/MyD88 pathway, the expression of pro-inflammatory chemokines (MCP-1, RANTES, MIP-2a, MIP-2B, IL-8 and IP-10) that attract innate immune cells to initiate the local inflammation and protect against pathogens (Hafez *et al*., 2009; Hafez *et al*., 2010; Lammers *et al*., 2002). Besides, EcN also reduces the expression of cyclooxygenase-2 (COX-2) and prostaglandin E_2 (PGE_2) (which are involved in colorectal carcinogenesis (Otte *et al*., 2009)), and promotes a lower activation of inflammasome, than pathogen *E. coli* strains (Becker *et al*., 2014).

At the *in vivo* level, studies using experimental colitis mice and pig models have proven that EcN diminishes tissue damage and inflammation associated with the activation of NFκB via TLR4 and TLR2, reducing the expression of pro-inflammatory cytokines and INF-γ (Grabig *et al*., 2006b; Vlasova *et al*., 2016). Recently, the *in vivo* anti-inflammatory effect of EcN has been associated to the synthesis of colibactin, a genotoxic polyketide that EcN secretes (Olier *et al*., 2012).

Additionally, the probiotic EcN also positively modulates the intestinal epithelial barrier through both increased expression of secreted antimicrobial

factors such as β-defensin-2 (Schlee *et al*., 2007) and upregulation and redistribution of the TJ proteins ZO-1 (Ukena *et al*., 2007), ZO-2 (Zyrek *et al*., 2007) and claudin-14 (Hering *et al*., 2014). Upregulation of claudin-14 in HT-29/B6 cells has been attributed to TcpC (Hering *et al*., 2014), an immunomodulatory protein secreted by uropathogenic *E. coli* strains that interferes with the host immune defence by inhibiting MyD88/Toll-like receptor 4 signalling cascade (Yadav *et al*., 2010; Snyder *et al*., 2013). Likewise, EcN can modulate the intestinal barrier through miRNAs regulation (Veltman *et al*., 2012).

Taken together these studies prove that the EcN immunomodulatory effects result from an equilibrated modulation (activation/inactivation) of mechanisms that control the immune response, leading to a positive balance between pro and anti-inflammatory local cytokines. In addition, the fitness properties that enhance EcN survival in the gut (healthy or inflamed) play an important role in the beneficial effects of this probiotic (Behnsen *et al*., 2013).

1.4. ROLE OF BACTERIAL SECRETED FACTORS IN THE HOST - MICROBIOTA CROSSTALK AT THE INTESTINAL MUCOSA

In the mucosal luminal interface, crosstalk between microbiota and the host is essential for the survival of both organisms, and microbiota - host signals are indispensable to maintain the intestinal homeostasis. This communication involves a complex network of signalling pathways (Fischbach and Segre, 2016), and extracellular factors secreted by probiotic and commensal bacteria, which have a prominent role. Unlike bacteria that cannot normally access to the intestinal epithelium, secreted factors can spread and cross through the mucus layer and directly interact with IECs, modulating cell signalling pathways and responses such as secretion of cytokines or AMPs, mucus production, regulation of intestinal TJs and immunomodulatory effects (Sánchez *et al*., 2008, 2010). The study of microbiota released factors is especially relevant to understand host-microbiota interactions. Bacterial effectors can be released to the medium as free soluble components (proteins for instance) or through secretory vesicles.

1.4.1. OUTER MEMBRANE VESICLES

All archaea and bacteria, either commensal or pathogenic strains, have evolved different systems to contact and communicate with host cells. One mechanism is

the formation of membrane vesicles that can deliver the cargo to distant targets in the host. In fact, membrane vesicles are considered intercellular communicasomes. Bacterial membrane vesicles are spherical membranous structures that are produced during the normal growth of bacteria. They enable a protected secretion of proteins, lipids, RNA, DNA and other effector molecules in a protected environment. Many studies with Gram-negative pathogens conducted in the last decade have shown that membrane vesicles are internalized in host cells and contribute to virulence by delivering cytotoxic factors, as well as mediators that interfere with the host immune system. Regarding non-pathogenic bacteria, reports are scarce. However, there is evidence that vesicles produced by microbiota promote immunomodulatory effects that contribute to the immune tolerance and to stablish a beneficial symbiotic relationship with the host. For this reason microbiota released vesicles are foreseen as key players in signalling processes in the intestinal mucosa (Brown *et al*., 2015).

We will specifically focus on extracellular vesicles released by Gram-negative bacteria, which are known as outer membrane vesicles (OMVs). These vesicles were first visualized by electron microscopy in 1960. They are spherical bilayered nanostructures with an average diameter between 20 to 300 nm in size, which bud and detach from the cell during active growth. Production of OMVs takes place under different environmental conditions either in *in vitro* cultures or in natural environments, such as host tissues and biological fluids. Vesiculation can be altered by external factors such as temperature, nutrient availability, oxidation, quorum sensing and antibiotics (Schwechheimer and Kuehn, 2015). OMVs are equipped with a big range of bacterial compounds such as LPS, outer membrane proteins (OmpA, OmpC and OmpF), periplasmic proteins (like alkaline phosphatase), cytoplasmic proteins, nucleic acids (RNA, DNA), and lipids (like SFACs, glycerophospohlipids, phosphatidylethanolamine, cardiolipin, phosphatidylglycerol, etc). In the case of OMVs from pathogens, they also contain virulence factors (like adhesins, invasins and toxins).

When first discovered, it was thought that these membrane vesicles corresponded to cell debris resulting from cell lysis (Knox, Vesk, and Work 1966) and that their production was a random event. Nowadays, there is evidence that OMVs are the product of a physiological and evolutionary process (Reister *et al*.

2014), although the pathways by which OMVs include their bacterial cargo remain unknown (Schwechheimer and Kuehn, 2015; Kim *et al*., 2015).

1.4.1.1. BIOGENESIS

Gram-negative OMVs are originated from the outer membrane bacterial envelope, whose architecture is basic to understand vesicle biogenesis.

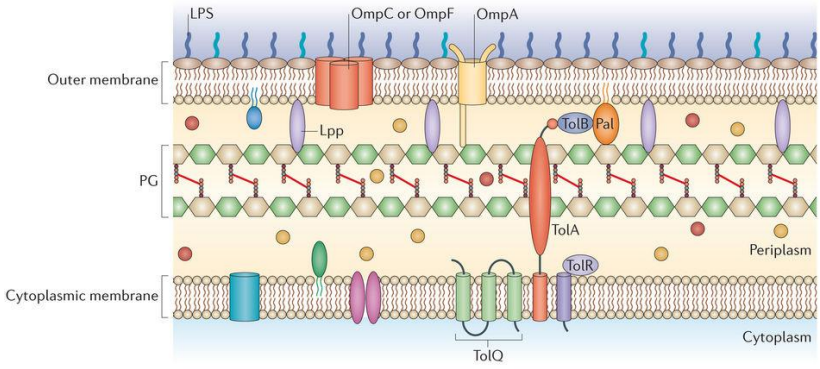

Figure 12. Composition of the Gram-negative cell envelope. The envelope of Gram negative bacteria is formed by 2 walls: the cytoplasmic membrane and the outer membrane. Both membranes are separated by the periplasm, which contains periplasmic proteins and a layer of PG. The cytoplasmic membrane is a phospholipid bilayer, whereas the outer membrane is asymmetric, with an internal line of phospholipids and an external line of LPS. The stability of this structure is due to some crosslinks: Braun´s lipoprotein (Lpp) in the outer membrane with PG, PG with the porin OmpA, and PG with the Tol-Pal (peptidoglycan-associated lipoprotein) complex, composed by TolA, TolB, TolQ, TolR and Pal (Schwechheimer and Kuehn, 2015).

The envelope of Gram-negative bacteria is formed by two membrane layers, the outer membrane and the cytoplasmic membrane, separated by the periplasmic space. These membranes contain a great variety of proteins, which have key roles such as nutrient acquisition, adhesion, secretion, signalling and protection against external environment (Schwechheimer and Kuehn, 2015). The components of the cell envelop of Gram-negative bacteria are schematized in detail in Figure 12.

The mechanisms that lead to OMVs biogenesis are still poorly understood, although it is known that multiple processes seem to be involved, being destabilization of the membrane integrity a crucial factor (Pathirana and Kaparakis-Liaskos 2016). A common feature in all proposed models is that they rely on initial decoupling of the outer membrane from the PG layer by disruption of the crosslinks between PG and the lipoproteins Pal and Lpp. This breakage promotes the curvature of the outer membrane, leading to vesicle formation.

Different curving mechanisms have been proposed for OMVs generation (Figure 13) (Bohuszewicz *et al*., 2016) and they are the following:

1. Accumulation of toxic or misfolded proteins in the periplasm can induce OMVs formation by increasing turgor pressure and inducing membrane bulging (Pathirana and Kaparakis-Liaskos, 2016).
2. Selective crowding of cargo proteins, like virulence factors, in the outer membrane (Bohuszewicz *et al*., 2016).
3. Some types of quorum sensing molecules, like the *Pseudomonas* Quinolone Signal (PQS), localized in the outer membrane increase the surface area of the outer leaflet relative to the inner leaflet by a wedging effect (Jan, 2017).
4. Outer membrane asymmetric enrichment of LPS, fatty acids or phospholipid subtypes that may be further modified altering the charge surface, and thus induce curvature in the outer membrane (Pathirana and Kaparakis-Liaskos, 2016).
5. A recent proposed mechanism based on a decreased expression of the highly conserved phospholipid transporter (VacJ/Yrb ABC), which result in an accumulation of curvature-inducing phospholipids in the outer membrane vesicles (Pathirana and Kaparakis-Liaskos, 2016) (Bohuszewicz *et al*., 2016).

Recently, two novel models for OMVs biogenesis have been reported for specific species:

- In *P. aeruginosa* biogenesis of OMVs can be produced via phage-mediated explosive cell lysis, resulting in membrane fragments that rapidly generate vesicles by curling and self-annealing (Pathirana and Kaparakis-Liaskos, 2016).
- In *S. thyphimurium* OMVs biogenesis can takes place through lipid A diacylation that results in outer membrane remodelling (Pathirana and Kaparakis-Liaskos, 2016).

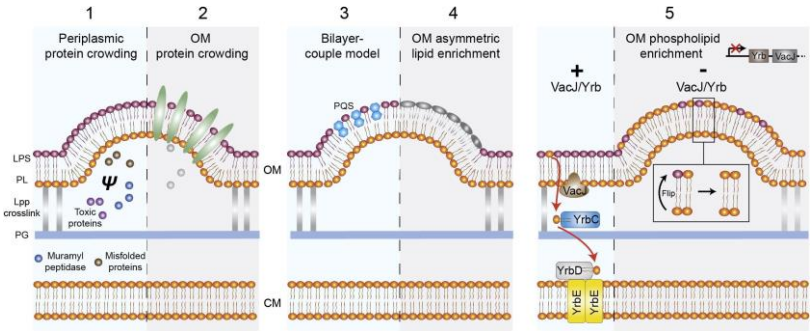

Figure 13. Mechanisms of OMVs biogenesis. This image describes 5 possible membrane curving mechanisms by which outer membrane decoupling is induced to vesiculation (Bohuszewicz *et al*., 2016).

1.4.1.2. FUNCTIONS

OMVs are involved in numerous biological functions, which may be different dependent on their specific cargo (Jan, 2017). The wide array of OMVs functions can be classified in two groups: those linked to bacteria-bacteria interaction and those related with bacteria-host communication.

Regarding bacteria-bacteria interactions, OMVs allow bacteria to resist in the environment by competing with or killing other bacteria. For instance, OMVs *from Pseudomonas aeruginosa* and *Moraxella catarrhalis* can transfer beta-lactam antibiotic resistance. The presence of transport systems for siderophores, amino acids or fatty acids in OMVs helps to nutrient acquisition and bacterial survival. In this context, OMVs can harbour other kind of enzymes, like proteases, which allow to bacterial communities the degradation and the acquisition of nutrients from the outside. Besides, OMVs can carry metal ions that are indispensable for bacterial growth, leading to inter and intra-species competition. Under stress conditions, like the presence of environmental contaminants or low nutrients, OMVs formation is increased. Released OMVs can then sequester and degrade the contaminants or act as nutrient sensors and scavengers of essential nutrients to increase bacterial survival. In addition, stress conditions (desiccation, starvation or antimicrobial agents) stimulate bacteria to produce biofilm, a matrix made of polysaccharides, lipids, nucleic acids and proteins that protects bacteria from the outside. In biofilms, OMVs mediate the delivery of essential molecules that favour the biofilm formation and bacterial survival. OMVs also carry specific degradative enzymes

(such as murein hydrolases or endopeptidases) which are transferred to some types of bacteria, making them less vulnerable to death. (Lee *et al*., 2008; Jan, 2017).

On the other hand, OMVs also participate in bacteria-host interactions, promoting adhesion to host cells and inflammatory responses by delivering virulence factors or immunomodulatory mediators. OMVs can trigger an immune response through activation of PRRs in host cells. In addition, OMVs can increase the adhesion of bacteria to the epithelial cells because they carry proteins such as adhesins or porins that are involved in the adhesion to the host. In pathogenic bacteria, OMVs also harbour virulence factors or toxins which aid bacteria to attack host cells and subverts the host machinery to avoid the host immune responses. These virulence factors include toxins like cytolisin A or digestive enzymes like alkaline phosphatase, which can modulate immune responses into the host (Lee *et al*., 2008; Jan, 2017).

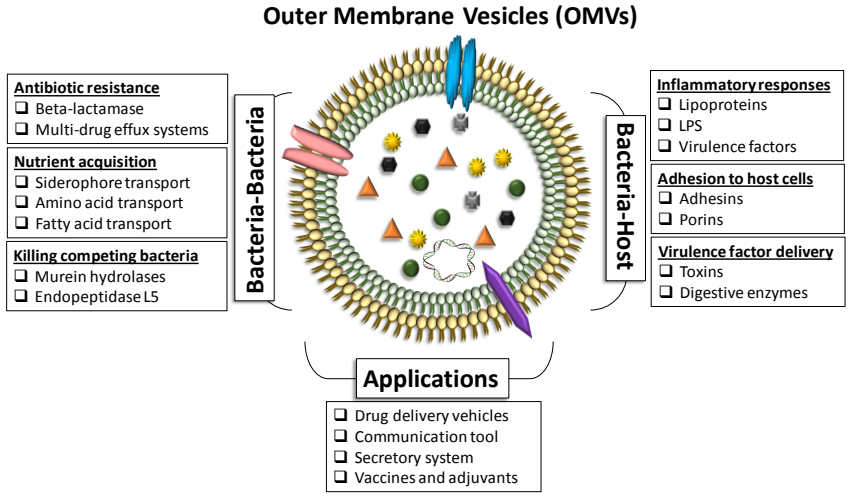

Figure 14. Pathophysiological functions of Gram-negative OMVs and their potential applications. Functions of Gram-negative bacterial OMVs can be divided into two groups: those that affect bacteria-host interaction, and those that are involved in bacteria-bacteria interaction. (Lee *et al*., 2016) (Jan, 2017).

OMVs release is a costly process that requires energy expenditure. However, this secretion mechanism has been selected along evolution because OMVs have

key roles conferring bacteria self-protection or promoting interaction and communication with the host. Thus, OMVs have developed multiple mechanisms to enter into the host cell and induce intracellular signalling events that control host functions. For this reason, OMVs are well-adapted and become essential for inter-kingdom communication.

In addition, due to their important properties as inherent long-distance targeting vehicles and by their intrinsic biocompatibility, bacterial vesicles are being explored as promising novel therapy applications (Figure 14). In fact engineered OMVs are being developed as new vaccines and adjuvants (Olsen and Amano, 2015) or specialized drug delivery vehicles for the treatment of emerging and new diseases caused by bacteria or virus (Fuhrmann *et al*., 2017).

1.4.1.3. ENTRY INTO HOST CELLS

Once OMVs are released from the bacterial surface, they can deliver a wide range of chemically diverse molecules to host cells over long distances, while they protect vesicular contents from external environment. Upon contact with the host cell membrane, OMVs uptake is mediated by endocytosis. Four main endocytic pathways have been described for OMVs entry. Depending on their cargo, OMVs may use more than one route of entry.

-Macropinocytosis. This process does not depend on OMVs cargo. It implies the formation of large protrusions from the host cell membrane through actin remodelling, which allow the sampling and internalization of extracellular medium, including vesicles up to 1 μm in diameter. This pathway is not probably involved in specific delivery processes.

-Clathrin-dependent endocytosis. This process allows the uptake of particles up to 12O nm. This pathway typically depends on ligand binding to a specific receptor located on clathrin- coated pits at the host membrane. Upon binding, invagination of the membrane leads to the formation of an internalized clathrin-coated vesicle that enters in the endosomal trafficking route. Dynamin molecules help to seal the cytoplasmic membrane and the site of internalization. Through this endocytic pathway some OMVs cargo can be returned to the cell surface or degraded in the

lysosomes. OMVs trafficking by this pathway take around 15 minutes to be completed. The ligands that trigger recognition of OMVs by host receptors are not always known. Specific proteins or toxins present in the OMVs cargo can interact with receptors to facilitate the entry, or even recognize adaptor proteins required for clathrin-mediated internalization. This endocytic pathway is specifically inhibited by drugs that target clathrin (chlorpromazine) or dynamin (dynasore) (Figure 15).

-Endocytosis via lipid rafts. Lipid rafts are cell membrane domains enriched in sphingolipids and cholesterol, which form a more ordered and compact region in the cell membrane that function or as platforms for membrane signalling and trafficking. These domains allow the invagination of the host membrane and the entry of particles with 90 nm in diameter. This pathway is specifically inhibited by compounds that sequestrate cholesterol from the cell membrane (fillipin), thus disrupting lipid packing (Figure 15).

-Caveolin-mediated endocytosis. This pathway involves the formation of caveolae, which are cave-shaped invaginations on the cell membrane in domains enriched in cholesterol, sphingolipids, and the proteins caveolin and dynamin. This entry pathway is used for molecules with approximately 60 nm in diameter, and the efficiency of cargo delivery is very high, albeit the internalization timing is slower than the clathrin pathway. This route is used for many pathogens to survive in the cytosol, because caveolae avoid the fusion with lysosomal compartments.

Caveolin-mediated endocytosis is associated with lipid rafts and thus inhibited by cholesterol sequestering agents. In addition to lipid rafts, this pathway requires interaction between OMVs-ligands and host cell receptors (Figure 15).

Apart from endocytosis, OMVs can be internalized by host cells through membrane fusion, which is a rapid and efficient temperature dependent mechanism (O'Donoghue and Krachler, 2016). Structural differences between the outer membrane (from vesicles) and the eukaryotic cell membrane seem not to be an impediment (Figure 15).

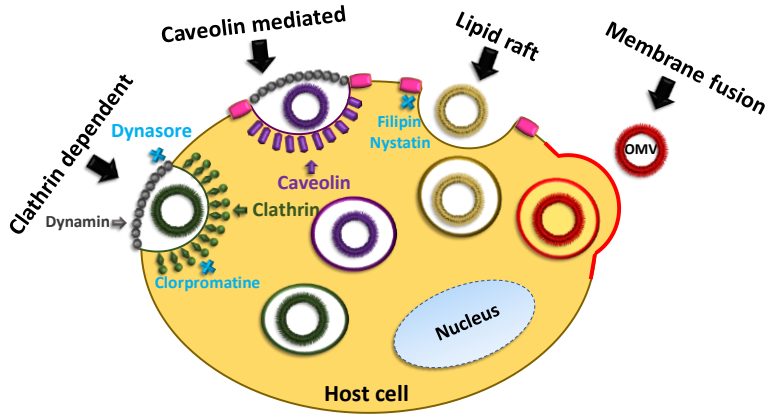

Figure 15. Mechanisms of OMVs entry into host cells. OMVs enter host epithelial cells using various endocytic routes including lipid raft-dependent endocytosis, clathrin dependent entry, caveolin dependent pathway and fusion membrane. (O'Donoghue and Krachler, 2016).

1.4.1.4. INTERACTIONS WITH HOST CELLS: SIGNALLING

OMVs contain many PAMPs such as RNA, DNA, lipoproteins, LPS and PG, which can be recognized by PRRs and induce an innate immune response. Studies performed with OMVs released by pathogenic bacteria have shown that OMVs internalized by epithelial cells can interact with the cytoplasmic receptors NOD1 and NOD2, activating the downstream signalling pathways that trigger immune responses (Pathirana and Kaparakis-Liaskos, 2016). For example, OMVs released by *H. pylori* and *P. aeruginosa* induce in IECs autophagosome formation and inflammatory IL-8 responses in a NOD-1 and RIP2-dependent manner (Irving *et al*., 2014).

Moreover, it has also been described that OMVs can interact with TLRs located on the host cell membrane and mediate inflammatory responses. For example, OMVs from the *P. gingivalis* contribute to pathogenicity by inducing strong TLR2 and TLR4 responses in addition to NOD1 and NOD2 responses (Cecil *et al*., 2016). Besides activation of innate receptors, OMVs can deliver LPS to the host cell cytosol, which leads to elicit the inflammasome pathway through caspase 11 activation. This mechanism has been reported for EHEC vesicles (Vanaja *et al*., 2016).

On the other hand, OMVs can also modulate the adaptive immunity. In this case, the responses are dependent of the OMVs cargo and the bacterial origin. For instance, *H. pylori* OMVs stimulate IL-6 and IL-10 production by mononuclear cells

through inhibition of $CD4^+$ T cell proliferation and induction of T cell apoptosis (Winter *et al*., 2014).

Recently, OMVs have been proposed to act as modulators of the gut-brain axis, as they can reach the systemic circulation and pass through the blood brain barrier. This field could be explored as a novel treatment against psychopathy disorders (Ahmadi Badi *et al*., 2017; Kelly *et al*., 2015; Jordan *et al*., 2016).

Furthermore, it is known that vesicles derived from eukaryotic cells (exosomes) mediate transference of regulatory microRNAs (miRNAs) to neighbour cells. In this context, inter-kingdom crosstalk between human and bacteria in the gut could be also mediated by RNAs contained in OMVs. These small bacterial RNAs might target and align to host mRNAs, affecting part of the epigenetic processes (Celluzzi and Masotti, 2016; Romano, 2017). In fact, a study performed in kidney cells showed that bacterial microRNAs can regulate the expression of specific target human genes (Shmaryahu *et al*., 2014). Moreover, human miRNAs have been identified in bacterial vesicles isolated from faeces (Liu *et al*., 2016). This study evidenced that host miRNAs (miR-515 and miR-1226) enter bacteria and co-localize with bacterial nucleic acids, regulating the expression of specific bacterial genes and thus, affecting microbiota growth. Interestingly, these miRNAs are essential to protect against colitis mice (Liu *et al*., 2016).

1.4.1.5. COMMENSAL AND PROBIOTIC- DERIVED VESICLES

In the last 15 years, research developed in the OMVs field was mainly focused on Gram-negative pathogens. Studies dealing with vesicles released by commensal or probiotic strains are few, being the first publication in 2012. This study revealed that OMVs released by the commensal *Bacteroides fragilis* ameliorate DSS-induced colitis in a mouse model, and that the capsular PSA activates DCs through TLR2 signalling (Shen *et al*., 2012). Similarly, *Akkermansia muciniphila* secretes OMVs, which impair the progression of the leaky gut in an animal model (Kang *et al*., 2013). Regarding Gram-positive probiotics, recent studies provided evidence that released membrane vesicles can mediate beneficial effects on the host: (i) An *in vivo* study showed that proteins present in extracellular vesicles released by *Bifidobacterium longum* KACC 91563 alleviate food allergy through mast cell suppression (Kim *et al*., 2016); (ii) *Lactobacillus plantarum* WCFS1 vesicles up-

regulate host defence genes and protect the host against *Enterococcus* infection in Caco-2 and *Caenorhabditis elegans* models (Li *et al*., 2017); (iii) stimulation of DCs with vesicles released by *Bifidobacterium bifidum* LM13195 induces a immunomodulatory Treg response, pointing to a potential use of these vesicles as adjuvants in immunotherapy (López *et al*., 2012).

In addition, beneficial effects other than immunomodulation have also been described for probiotic vesicles. For example, vesicles released by the probiotic *Lactobacillus rhamnosus* GG increase the apoptotic index in carcinogenic hepatic cells without cytotoxic effects on the HepG2 cell line (Behzadi *et al*., 2017).

Our group has been focused on the study of OMVs released by commensal and probiotic Gram-negative bacteria, specifically *E. coli* strains. Our results provide evidence on the key role of OMVs from *E. coli* intestinal isolates in controlling parameters of intestinal homeostasis. We reported the first vesicular proteome of a probiotic strain, namely EcN (Aguilera *et al*., 2014). We also elucidated that OMVs released by the probiotic EcN and the commensal ECOR12 enter IECs by clathrin-dependent endocytosis. Internalized OMVs are sorted to lysosomal compartments as shown by their colocalization with clathrin and specific markers of endosomes and lysosomes. In addition, cell viability assays confirmed that commensal and probiotic OMVs do not affect cell viability, but reduce proliferation of HT-29 cells (Cañas *et al*. 2016). We have also proven for the first time the role of microbiota vesicles on intestinal epithelial barrier reinforcement. In this context, both cell free SNs and OMVs collected from EcN and the intestinal isolate ECOR63 increase the transepithelial resistance of T84 or Caco-2 monolayers. This is due to the ability of both secreted fractions (OMVs and soluble factors) to upregulate the expression of ZO-1 and claudin-14 and downregulate claudin-2, thus enhancing the intestinal epithelial barrier through strengthening TJ proteins (Alvarez *et al*. 2016).

2. OBJECTIVES

It is widely proven that the probiotic *Escherichia coli* Nissle 1917 (EcN) ameliorates intestinal inflammatory disorders through anti-inflammatory and immunomodulatory properties. These effects have been confirmed in *in vivo* models of colitis induced by DSS and TNBS. Additionally, EcN exhibits antagonistic effects against intestinal pathogens such as *Shigella* or *Salmonella* spp. protecting against infection. However, the probiotic factors that are involved in most of these beneficial effects are not entirely known. In the context of microbiota-host crosstalk, bacterial secreted factors have a relevant role, as they can diffuse through the mucus layer and interact with epithelial and immune cells at the intestinal mucosa.

Nowadays, there is evidence that bacteria-derived extracellular vesicles have crucial roles as intercellular communicasomes in bacteria–host interactions. Recent studies carried out by our group have shown that outer membrane vesicles (OMVs) released by commensal and probiotic *E. coli* strains are internalized in IECs via clathrin-mediated endocytosis and have a protective effect on the epithelial intestinal barrier through regulation of TJ proteins.

The main objective of this thesis is focused on the study of the immunomodulatory potential of EcN OMVs in different intestinal *in vitro* and *in vivo* models, and their implication in maintaining intestinal homeostasis. This study also includes vesicles isolated from commensal *E. coli* strains (ECOR12) for comparison.

The specific objectives are listed according to each of the four chapters that are presented in the thesis:

1. To study the ability of OMVs released by the probiotic EcN and the commensal ECOR12 to modulate cytokine/chemokine responses in different *in vitro* and *ex vivo* models of intestinal barrier (Chapter 1).

2. To determine whether EcN OMVs can ameliorate intestinal inflammation in a mouse model of colitis induced by dextran-sodium sulphate (Chapter 2).

3. To study involvement of NOD intracellular receptors in the inflammatory response activated by internalized EcN and ECOR12 OMVs in intestinal epithelial cells (Chapter 3).

4. To assess whether EcN OMVs or other secreted factors can exert inhibitory effects against *Salmonella enterica* serovar Thyphimurium infection (Chapter 4).

3. CHAPTER 1

Activation of immune and defense responses in the intestinal mucosa by Outer Membrane Vesicles of Commensal and Probiotic *Escherichia coli* strains.

María José Fábrega, Laura Aguilera, Rosa Giménez, Encarna Varela, María Alexandra Cañas, María Antolín, Josefa Badía and Laura Baldomà.

RESUMEN

La microbiota intestinal presenta una alta influencia sobre la salud humana. Por ello, desajustes en la estructura del microbioma han sido asociados con diversas enfermedades. La modulación en la composición de la microbiota a través de la terapia con probióticos es una forma de aprovechar los efectos beneficiosos de la microbiota comensal. Sin embargo, aunque existe un amplio conocimiento de la respuesta inducida por la microbiota intestinal, los factores microbianos que median dichos efectos no son del todo conocidos. Las bacterias Gram negativas liberan vesículas de membrana externa (OMVs) como mecanismo de secreción de factores microbianos, los cuales tienen un papel importante en la comunicación intercelular. En este trabajo, nosotros hemos analizado si las OMVs de la cepa probiótica *Escherichia coli* Nissle 1917 (EcN) y la cepa comensal *E coli* ECOR12 son capaces de desencadenar una respuesta inmunitaria, utilizando varios modelos celulares: (i) células mononucleares de sangre periférica (PBMCs) como modelo de barrera intestinal dañada, (ii) estimulación apical de un co-cultivo formado por Caco-2/PBMCs como modelo de barrera intestinal intacta, y (iii) explantes de mucosa colónica como modelo *ex vivo*. También se realizaron estimulaciones con lisados bacterianos. Los resultados obtenidos mostraron que en PBMCs, tanto las OMVs como los lisados activaron la expresión y secreción de varias citoquinas y quimioquinas, si bien en el modelo de co-cultivo, solo las OMVs fueron capaces de inducir la secreción basolateral y una regulación positiva del ARN mensajero de estos mismos mediadores. Además, hemos demostrado que las OMVs son internalizadas en células Caco-2 polarizadas, y que estas células epiteliales activadas son capaces de activar a su vez, una respuesta en las células inmunocompetentes subyacentes. Asimismo, estos efectos asociados a las OMVs fueron corroborados con el modelo *ex vivo*. Este estudio experimental muestra que las OMVs son una estrategia efectiva usada por bacterias beneficiosas del intestino, para comunicarse y modular respuestas del huésped, activando diferentes vías de señalización a través de la barrera epitelial intestinal.

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Activation of Immune and Defense Responses in the Intestinal Mucosa by Outer Membrane Vesicles of Commensal and Probiotic *Escherichia coli* Strains

*María José Fábrega[1†], Laura Aguilera[1†], Rosa Giménez[1], Encarna Varela[2], María Alexandra Cañas[1], María Antolín[2], Josefa Badía[1] and Laura Baldomà[1]**

[1] Departament de Bioquímica i Biologia Molecular, Facultat de Farmàcia, Institut de Biomedicina de la Universitat de Barcelona, Universitat de Barcelona, Barcelona, Spain, [2] Department of Gastroenterology, Digestive System Research Unit, Institut de Recerca Vall d'Hebron, CIBER EHD, Instituto de Salud Carlos III, University Hospital Vall d'Hebron, Universitat Autònoma de Barcelona, Barcelona, Spain*

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Linda F. Van Dyk, University of Colorado, Denver School of Medicine, USA

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Sophie Anne Fillon, University of Colorado, Denver, USA

***Correspondence:**
Laura Baldomà
lbaldoma@ub.edu

†These authors have contributed equally to this work.

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The influence of microbiota in human health is well-known. Imbalances in microbiome structure have been linked to several diseases. Modulation of microbiota composition through probiotic therapy is an attempt to harness the beneficial effects of commensal microbiota. Although, there is wide knowledge of the responses induced by gut microbiota, the microbial factors that mediate these effects are not well-known. Gram-negative bacteria release outer membrane vesicles (OMVs) as a secretion mechanism of microbial factors, which have an important role in intercellular communication. Here, we investigated whether OMVs from the probiotic *Escherichia coli* strain Nissle 1917 (EcN) or the commensal *E. coli* strain ECOR12 trigger immune responses in various cellular models: (i) peripheral blood mononuclear cells (PBMCs) as a model of intestinal barrier disruption, (ii) apical stimulation of Caco-2/PMBCs co-culture as a model of intact intestinal mucosa, and (iii) colonic mucosa explants as an *ex vivo* model. Stimulations with bacterial lysates were also performed. Whereas, both OMVs and lysates activated expression and secretion of several cytokines and chemokines in PBMCs, only OMVs induced basolateral secretion and mRNA upregulation of these mediators in the co-culture model. We provide evidence that OMVs are internalized in polarized Caco-2 cells. The activated epithelial cells elicit a response in the underlying immunocompetent cells. The OMVs effects were corroborated in the *ex vivo* model. This experimental study shows that OMVs are an effective strategy used by beneficial gut bacteria to communicate with and modulate host responses, activating signaling events through the intestinal epithelial barrier.

Keywords: Nissle 1917, probiotics, gut microbes, membrane vesicles, microbiota-host crosstalk, intestinal mucosa, Caco-2/PBMC co-culture

INTRODUCTION

Intestinal microbiota has a great impact on human health. These microbial populations provide crucial benefits to the host, such as metabolic activities that promote energy harvest and nutrient absorption from food, development of the host immune system, and prevention of gut colonization and infection by pathogens (O'Hara and Shanahan, 2006; Garrett et al., 2010; Krishnan et al., 2015). Disturbances in microbiota composition can contribute to the development of diverse pathologies (Belkaid and Hand, 2014). Recent metagenomics studies have revealed certain microbial profiles (dysbiosis) associated with inflammatory, metabolic or infectious diseases (Le Chatelier et al., 2013; Robles-Alonso and Guarner, 2013; Sears and Garrett, 2014). Manipulation of gut microbiota through diet, probiotics and other approaches is a promising therapeutic strategy to prevent or alleviate disorders correlated with imbalances in the intestinal microbiota (Shanahan, 2011; Foxx-Orenstein and Chey, 2012).

The intestinal ecosystem is characterized by dynamic and reciprocal interactions between the microbiota, the epithelium and the mucosal immune system. This process requires elaborated regulatory mechanisms to ensure symbiosis and prevent aberrant responses that lead to pathological states. The epithelial layer is the first line of defense against pathogens and is also the surface where the host interacts with the microbiota. Epithelial cells have a critical role in sensing intestinal bacteria. They are equipped with a great variety of receptors that can recognize specific conserved molecular patterns in microbes pattern-recognition receptors (PRRs), such as the membrane-bound toll-like receptors (TLRs). TLRs mainly signal through the adaptor protein MyD88 to activate master transcription factors like NF-kappa B, thus triggering cytokine secretion and activation of host defense mechanisms. Other signaling PRRs are the cytosolic NOD-like receptors, which can modulate apoptosis and inflammatory responses (Jacobs and Braun, 2014; Thaiss et al., 2014; Caballero and Pamer, 2015). However, the gut microbiota does not directly interact with the intestinal epithelium. Both cell types are physically separated by the mucus layer. Commensal microbiota resides in the outer mucus layer, whereas the inner mucin layer is highly compacted and prevents bacteria from accessing the epithelial cells (Johansson et al., 2008; Vaishnava et al., 2011). In this scenario, a key issue is how the crosstalk between microbiota and the host is established. Proteins or factors secreted by microbiota have a relevant role in intestinal communication as they can diffuse through the mucin layer and come into contact with the intestinal mucosa cells.

All bacteria release extracellular vesicles as a means of communication with the environment. The best characterized are the outer membrane vesicles (OMVs) produced by Gram-negative bacteria. These vesicles act as a bacterial secretion pathway for selected proteins and other active compounds in a protected environment. In addition, they allow cell–cell communication, without direct intercellular contact (Kulp and Kuehn, 2010; Kim et al., 2015). Many studies conducted in the last decade with Gram-negative pathogens showed that OMVs are internalized in the host cell and contribute to virulence by delivering cytotoxic factors, as well as mediators that interfere with the immune system (Ellis and Kuehn, 2010; Chatterjee and Chaudhuri, 2013; Schertzer and Whiteley, 2013). At present, microbiota vesicles are seen as key players in signaling processes in the intestinal mucosa (Kaparakis-Liaskos and Ferrero, 2015; Olsen and Amano, 2015). However, studies in this field are still very scarce and mainly focused on *Bacteroides fragilis* (Shen et al., 2012; Stentz et al., 2015), a main Gram-negative group in the gastrointestinal tract of mammals. OMVs from this commensal promote immunomodulatory effects and prevent experimental colitis in mice. These effects are mediated by the capsular polysaccharide A (PSA) through TLR-2. However, a transcriptomic analysis in dendritic cells stimulated with *B. fragilis* ΔPSA-OMVs revealed changes in gene expression that are PSA-independent (Shen et al., 2012). *Akkermansia muciniphila* OMVs are also able to protect the progression of experimental-induced colitis in mice (Kang et al., 2013). Regarding Gram-positive bacteria, studies performed with *Bifidobacterium bifidum* LMG13195 showed that membrane vesicles from this probiotic can activate the maturation of dendritic cells, which trigger the regulatory T cells (Treg) response (López et al., 2012).

Our group reported the first vesicular proteome of a probiotic strain, namely *Escherichia coli* Nissle 1917 (EcN) (Aguilera et al., 2014). This proteomic study identified 192 vesicular proteins that have functions related to adhesion, immune modulation or bacterial survival in host niches, thus indicating that probiotic-derived OMVs contain proteins that can target these vesicles to the host and mediate their beneficial effects on intestinal function (Aguilera et al., 2014). EcN is a Gram-negative probiotic used in the treatment of intestinal disorders, especially in the maintenance of ulcerative colitis (Kruis et al., 2004; Chibbar and Dieleman, 2015). The strain, which was originally isolated from a soldier who survived a severe outbreak of diarrhea during the First World War, is a good colonizer of the human gut and positively affects gastrointestinal homeostasis and microbiota balance. It is known that EcN modulates the expression of antimicrobial peptides and the immune system function in the gut by expressing bacterial factors that specifically interact with host TLRs (Hafez et al., 2010).

The EcN-mediated effects have been evidenced from a great variety of *in vitro* and *in vivo* experiments performed essentially with live probiotic suspensions (Sturm et al., 2005; Schlee et al., 2007; Ukena et al., 2007; Zyrek et al., 2007; Arribas et al., 2009). The aim of this study was to define whether the immune modulation effects promoted by EcN are mediated through released OMVs. We extended the analysis to other commensal *E. coli* strains without probiotic traits, such as ECOR12. We analyzed the modulation of the cytokine/chemokine response by epithelial and immune cells upon stimulation with OMVs isolated from these strains in different *in vitro* and *ex vivo* models. We provide evidence that OMVs are internalized in polarized Caco-2 cells, and that these activated cells trigger an immune response in the underlying immunocompetent cells. *Ex vivo* experiments performed with colonic mucosa explants confirmed the role of OMVs in microbiota-gut signaling processes.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The probiotic strain EcN (serotype O6:K5:H1) was provided by Ardeypharm (GmbH, Herdecke, Germany). ECOR12 is a commensal *E. coli* strain isolated from a healthy human stool sample (Ochman and Selander, 1984). Bacterial cells were grown at 37°C in Luria-Bertani broth (LB). Growth was monitored by measuring the optical density at 600 nm.

Preparation of Bacterial Cell Lysates and OMVs

Bacterial lysates were prepared from cells grown in LB medium. Bacteria were collected by centrifugation (5,000 × *g*, 10 min, 4°C) and resuspended in phosphate buffer saline (PBS) for sonic disruption on ice. Cell debris was removed by centrifugation at 16,000 × *g* for 30 min at 4°C and the supernatant filtered through a 0.22 μm-pore-size filter to remove any residual cell.

Outer membrane vesicles were isolated from culture supernatants as described previously (Aguilera et al., 2014). In brief, bacterial cells grown overnight in LB were pelleted by centrifugation (10,000 × *g*, 20 min, 4°C); the supernatants were filtered through a 0.22 μm-pore-size filter (Millipore), concentrated by centrifugation in a Centricon Plus-70 filter device (Millipore) followed by an additional filtration step. Vesicles were then collected by centrifugation at 150,000 × *g* for 1 h at 4°C, washed and resuspended in PBS. Isolated OMVs were examined by transmission electron microscopy after negative staining as described previously (Egea et al., 2007).

Samples, either lysates or OMVs, were stored at −20°C until use. Sterility of samples was assessed on LB plates. Protein concentration was determined by the method of Lowry et al. (1951).

Immunoblotting of Lipopolysaccharide (LPS)

Protein samples (bacterial lysates and OMVs) were separated on 15% SDS-PAGE and transferred to a Hybond-P polyvinylidene difluoride membrane by using a Bio-Rad MiniTransblot apparatus. The membrane was blocked in PBS-0.05% Tween-20 and 5% skimmed milk (blocking solution) for 1 h at room temperature, and then incubated with specific antibodies against LPS (Abcam; 1:5,000 dilution in blocking solution) for 16 h at 4°C. The secondary antibody was donkey anti-rabbit immunoglobulin horseradish peroxidase-linked, diluted 1:15,000 in blocking solution. The protein-antibody complex was visualized by using the ECL Plus Western blotting detection system (Amersham Pharmacia Biotech).

Cell Lines and Growth Conditions

Caco-2 human colon adenocarcinoma cells (ATCC HTB-37) were obtained from the American Type Culture Collection. Cells (passages 55–65) were routinely grown at 37°C in a 5% CO_2 atmosphere in Dulbecco's modified eagle medium (DMEM) High Glucose containing 25 mM HEPES, 1% non-essential amino acids, 10% heat inactivated fetal calf serum (FCS) (Gibco BRL), penicillin G (100 U/ml) and streptomycin (100 μg/ml; Gibco BRL).

For Transwell cultures, 5×10^5 Caco-2 cells were seeded on the apical compartment of 12 mm polycarbonate inserts (0.4 μm, Transwell Corning) and experiments were performed when confluent monolayers were fully polarized (18–20 days post-confluence). During growth and differentiation the medium was changed every 2 days in both compartments. Monolayer integrity was controlled by measurement of the transepithelial electrical resistance with a Millicell-ERS-2 volt-ohmmeter (Millipore, Madrid, Spain) and by visual assessment of cell layer integrity under the microscope. Prior to apical stimulation with OMVs or bacterial lysates, the medium was changed to DMEM High Glucose containing 25 mM HEPES, 1% non-essential amino acids, 1% heat inactivated FCS and gentamycin (150 μg/ml). For co-culture experiments, human peripheral blood mononuclear cells (PBMCs) were added to the basolateral compartment at a cell density of 2×10^6 cells/ml.

PBMCs Isolation

Peripheral blood mononuclear cells were isolated from fresh buffy coats of six healthy donors, provided by the "Banc de Sang i Teixits" of Barcelona according to the signed agreement with the Institution. Briefly, the buffy coat was centrifuged over a Hystopaque density gradient (Hystopaque-1077, Sigma Aldrich) following the manufacturer's instructions. PBMCs were suspended in DMEM High Glucose containing 25 mM HEPES, non-essential amino acids, 1% heat inactivated FCS and gentamycin (150 μg/ml) and adjusted to 2×10^6 cells/ml. Stimulation with OMVs or bacterial lysates was performed in 24 well plates (PBMCs only) or in 12 mm Transwell inserts (Caco-2/PBMCs co-culture).

Organ Culture of Human Colonic Mucosa

Macroscopically normal colonic tissue was obtained after adenocarcinoma surgery from six patients undergoing right colon resection. Full-thickness colonic wall specimens distant from the tumor and without macroscopic lesions were rinsed under a saline jet and washed twice in sterile saline at 4°C. Mucosal samples weighing 25–35 mg each were separated from underlying tissue and placed with the epithelial surface facing up on culture filter plates (15-mm diameter wells with 500-μm bottom mesh, Netwell culture systems, Costar). Filters were suspended over wells containing 1.5 ml of medium RPMI 1640 (Life Technologies) supplemented with 2 mM glutamine and 150 μg/ml gentamicin. Tissues were incubated during 5 h at 37°C in humidified atmosphere and stimulated by the addition of OMVs or bacterial lysates, as described below. During this time oxygen supply was provided to preserve tissue integrity (95% O_2/5% CO_2). Tissue viability was assessed by measuring lactate dehydrogenase as described elsewhere (Borruel et al., 2003).

Stimulation Conditions

To investigate the interaction between OMVs and cells of the intestinal mucosa, three different experimental approaches were performed: (i) direct stimulation of PBMCs (2×10^6 cells/ml), (ii) apical stimulation of differentiated Caco-2/PBMCs co-cultures

and (iii) apical stimulation of colonic mucosa explants. Each experiment was conducted six times (six individual donors in duplicate). Stimulations were performed with OMVs (50 μg/ml) or bacterial lysates (100 ng/ml) from EcN or ECOR12 strains. The concentration of these bacterial samples was selected according to experimental procedures reported elsewhere (Schaar et al., 2011; Güttsches et al., 2012). Cells were incubated at 37°C in a 5% CO_2 atmosphere for 5 h (for expression analysis by RT-qPCR) or 24 h (for quantification of secreted cytokines). Incubation in growth medium was performed in parallel as a control. For RNA isolation the medium was removed and cells or tissue explants were suspended in appropriate volume of *RNA later*® (Ambion) to preserve RNA integrity and kept at −80°C until RNA extraction. For cytokine quantification, culture supernatants were collected from direct stimulated PBMCs or from the basolateral compartment in co-culture experiments. Supernatants were clarified by centrifugation (300 × *g*, 5 min) and stored in aliquots at −80°C until use. In the *ex vivo* experiments, both culture supernatant and tissue explants were collected and processed after 5 h stimulation.

Cytokine and Chemokine Quantification in Culture Supernatants

Secreted IL-10, TNF-α, and MIP1α were measured using a cytometric bead array system (CBA FlexSet, BD Biosciences) according to manufacturer's instructions and analyzed by flow cytometry (Gallios Beckman Coulter) in the Scientific and Technological Services of the University of Barcelona (CCiT – UB). Measurement of IL-8 and IL-6 was performed using ELISA sets (BD Biosciences). IL-22 was quantified by ELISA using the R&D System Duo Set.

RNA Isolation and Quantitative Reverse Transcription PCR (RT-qPCR)

Total RNA was extracted from PBMCs and Caco-2 cells by using the Illustra RNAspin Mini kit (GE Healthcare) according to the manufacturer's instructions. RNeasy Mini kit (Qiagen) was used to extract total RNA from colonic tissue samples. The concentration and purity of RNA samples were assessed by the ratio of absorbance at 260 and 280 nm in a NanoDrop® spectrophotometer. RNA integrity was verified by visualization of 28S and 18S rRNAs after 1% agarose/formaldehyde gel electrophoresis.

RNA (1 μg for colon explants or 350 ng for PBMCs and Caco-2 cells) was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) in a final volume of 20 μl following manufacturer's recommendations. RT-qPCR reactions were performed in a StepOne Plus PCR cycler (Applied Biosystems) by using the Taqman Gene Expression Master Mix, and the Taqman probes and primers for human IL-10, MIP1α, TNF-α, IL-6, IL-8, IL-12, IL-22, TGF-β, β-defensin-2 (hBD-2), β-defensin-1 (hBD-1) and mucin-1 (MUC1). The standard PCR program used was: one denaturation cycle for 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. A control reaction was performed in the absence of RNA. The housekeeping gene β-actin was used as a normalizing gene. Relative gene expression was calculated as fold-change compared with control and calculated by means of ΔΔCt formula.

OMVs Labeling and Internalization Assay

To monitor OMVs internalization in intestinal epithelial cells, vesicles were fluorescently labeled with rhodamine isothiocyanate B-R18 (Molecular Probes) as described elsewhere (Bomberger et al., 2009). OMVs purified as described above were washed with PBS, resuspended in labeling buffer (50 mM Na_2CO_3, 100 mM NaCl, pH 9.2) in the presence of 1 mg/ml rhodamine isothiocyanate B-R18 and incubated for 1 h at 25°C. Labeled OMVs were pelleted by centrifugation at 100,000 × *g* for 1 h at 4°C, resuspended in PBS (0.2 M NaCl) and washed twice to fully remove the unbound dye. After a final centrifugation step, the rhodamine labeled-OMVs were resuspended in PBS (0.2 M NaCl) containing a protease inhibitor cocktail (Complete Protease Inhibitor Tablet, Roche) and stored at 4°C for up to 6 weeks.

OMVs internalization assays were performed using polarized Caco-2 cells (18–20 days post-confluence) grown in 96-well plate (Corning Incorporated, Costar®). Prior to the assay, the medium was replaced with rhodamine B-R18-labeled OMVs (2 μg/well) suspended in DMEM medium in the absence of phenol red and FCS. Cells were incubated at 37°C and fluorescence was measured over time using a Modulus™ Microplate fluorescence (Turner BioSystems; Ex 570 nm; Em 595 nm). Fluorescence intensity was normalized for fluorescence detected by labeled-OMVs in the absence of epithelial cells.

OMVs internalization was assessed by confocal fluorescence microscopy. Caco-2 cells were grown in 8-well chamber slider (ibidi) and incubated with rhodamine B-R18-labeled OMVs (2 μg) at 37°C for 1 h, and then washed with PBS. Cells were fixed with 3% paraformaldehyde, permeabilized with 0.05% saponin (Sigma Aldrich) and blocked using PBS containing 1% bovine serum albumin. Nuclei were labeled with DAPI. To visualize cell boundaries, the peripheral zonula occludens ZO-1 protein was stained using anti-ZO-1 rabbit IgG antibody (Invitrogen) and Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen). Confocal microscopy was carried out using a Leica TCS SP5 laser scanning confocal spectral microscope with 63x oil immersion objective lens. Images were captured with a Nikon color camera (16 bit). Fluorescence was recorded at 405 nm (blue; DAPI), 488 nm (green; Alexa Fluor 488), and 546 nm (red; rhodamine isothiocynate B-R18). Z-stack images were taken at 0.5 μm. Images were analyzed using Fiji image processing package.

Statistical Analysis

Statistical analysis was performed using SPSS version 20.0 software (SPSS, Inc.) and data were expressed as mean ± SEM ($n = 6$). To establish the distribution of the data, the test Kolmogorov–Smirnov was run. To assess the effect of every experimental condition compared to the other conditions, the *t*-test (normal distribution) or the Mann–Whitney *U*-test (non-normal distribution) were performed. Differences between more than two groups were assessed using one-way ANOVA followed

by Tukey's test. Significant differences were established at $p \leq 0.05$.

Ethical Approval

All the procedures involving human patients were approved by the local ethics committees (Comité Ético de Investigación Clínica, Hospital Vall d'Hebron and Comité Ético del Banc de Sang i Teixits de Barcelona). Written informed consent was obtained from all patients (CEIC: PR(AG)56/2010).

RESULTS

Comparative Protein Profile and LPS Analysis of OMVs Isolated from the Probiotic EcN and the Commensal ECOR12 Strains

Outer membrane vesicles from EcN and ECOR12 were isolated from culture supernatants. Examination of OMVs by transmission electron microscopy revealed that both strains release vesicles ranging from 20 to 60 nm in diameter (**Figure 1A**). To further compare the OMVs isolated from these strains, their protein profile was analyzed by SDS-PAGE and the LPS content was estimated by immunoblotting. Results showed similar protein profiles and LPS amount in both strain vesicles (**Figures 1B,C**). Immunoblotting of the LPS was also carried out in EcN and ECOR12 lysates (**Figure 1C**).

OMVs from EcN and ECOR12 Induce Cytokine Secretion in PBMCs

Human PBMCs, which include several types of immunogenic cells, were used to evaluate the immunomodulatory effects of OMVs secreted by EcN and ECOR12 strains. Direct stimulation of these immune cells can be used as an *in vitro* model of intestinal inflammation and barrier disruption.

PMBCs were stimulated by the addition of OMVs for 24 h in the presence of gentamicin. Stimulations with bacterial lysates were conducted in parallel for comparison. As illustrated in **Figure 2A**, OMVs and lysates from both strains induced the secretion of IL-10, MIP1α, TNF-α, IL-6, and IL-8 by PBMCs ($p \leq 0.05$). Secretion levels of MIP1α, IL-6, and IL-8 were in the ng range. Lysates triggered greater activation than OMVs in this cell model for all the cytokines and chemokines studied. ECOR12 lysates tended to promote increased secretion levels of proinflammatory cytokines than EcN lysates, although only the results for TNF-α were statistically significant (**Figure 2A**).

Expression of these inflammatory and immunomodulatory mediators was also analyzed by RT-qPCR after 5 h-stimulation. The results confirmed that all genes were upregulated in stimulated versus control cells (**Figure 2B**). Although, the changes in gene expression triggered by OMVs or lysates did not exactly match the secretion pattern of the soluble mediators released (**Figure 2A**), the tendency of ECOR12 lysates to produce greater cytokine activation was also apparent.

OMVs from EcN and ECOR12 Activate Cytokine Production by PBMCs in Co-culture with Caco-2 Cells

To evaluate the crosstalk between OMVs, intestinal epithelial cells and immune cells, we used the Caco-2/PBMCs co-culture Transwell model that simulates the interaction of microbiota with the intestinal mucosa (Haller et al., 2000; Fang et al., 2010; Pozo-Rubio et al., 2011). In this model, signaling between epithelial and immune cells occurs through the release of soluble mediators.

Differentiated Caco-2 cells in the apical compartment were challenged with OMVs from EcN or ECOR12, and with bacterial lysates for comparison. After 24 h incubation, the level of released cytokines was measured in the basolateral compartment. Transepithelial electrical resistance was monitored before and after each experiment to ensure the intact barrier function of the monolayer. The results presented in **Figure 3A** show that apical stimulation with OMVs elicited increased secretion of IL-10, MIP1α, TNF-α, IL-6 and IL-8, whereas lysates from the same strains did not produce any activation effect, yielding comparable secreted levels as untreated control cells. Stimulations of Caco-2 monolayers without underlying PBMCs were performed in parallel. Analysis of cytokine secretion in the basolateral compartment showed that polarized Caco-2 cells are almost unresponsive in the absence of crosstalk with immune cells (data not shown). This finding is in accordance with results reported by other groups (Zoumpopoulou et al., 2009; Fang et al., 2010; Pozo-Rubio et al., 2011).

In Caco-2/PBMCs co-cultures the cytokine secretion profile was clearly different from that of PBMCs directly exposed to bacterial samples. As the Caco-2 monolayer constitutes a physical barrier to the bacterial factors, these results showed that OMVs, but not lysates, could stimulate epithelial cells, which in turn may signal to the underlying PBMCs. In this co-culture model, activation of Caco-2 cells by OMVs was corroborated by RT-qPCR analysis of the expression profile of IL-10, MIP1α, TNF-α, IL-6 and IL-8 after 5 h stimulation. OMVs promoted upregulation of all these mediators in Caco-2 cells in the apical compartment (**Figure 3B**). Interestingly, the comparison of data from stimulations performed with vesicles isolated from both strains revealed that OMVs from the probiotic strain EcN promoted a significantly higher increase in the expression of the anti-inflammatory cytokine IL-10 (**Figure 3B**).

Gene expression analysis in PBMCs collected from the basolateral compartment also revealed higher mRNA levels of these mediators than in PBMCs collected from untreated co-cultures (**Figure 3C**). However, due to variability in the data, the results did not reach statistical significance. In this case, the relative increased gene expression values were lower than those of PBMCs directly stimulated by OMVs. These results are consistent with the stimulation of PBMCs by soluble factors released from epithelial cells upon exposure to OMVs.

In this co-culture system, the different cellular responses to OMVs or to bacterial lysates may be attributed to specific OMV-mediated communication and signaling mechanisms in the epithelial barrier. Thus, we sought to prove that OMVs from these *E. coli* strains are internalized in differentiated Caco-2 cells.

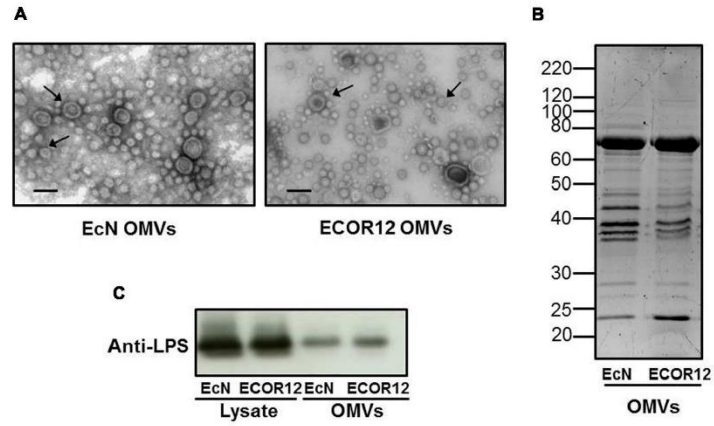

FIGURE 1 | Analysis of EcN and ECOR12 OMVs. (A) Negative staining electron microscopy of isolated vesicles. OMVs are indicated by arrowheads. Bar = 50 nm. **(B)** Comparison of the protein profile of EcN and ECOR12 OMVs. Isolated vesicles (10 μg) were separated in a 10%-SDS-PAGE gel and stained with Sypro® Ruby. Molecular size markers are indicated. **(C)** Western blot analysis of LPS in OMVs (0.1 μg protein) or in bacterial lysates (10 μg protein) obtained from the indicated strains.

We took advantage of the properties of rhodamine isothiocyanate B-R18 fluorescent dye, whose fluorescence is quenched when intercalated into bilayer membranes at a high concentration. However, this dye fluoresces when diluted upon membrane fusion and internalization. Rhodamine B-R18-labeled OMVs applied to the apical side of differentiated Caco-2 cells produced a time-dependent increase in fluorescence. In contrast, no changes in fluorescence emission were observed in non-treated cells or samples containing only labeled-OMVs (**Figure 4A**). These results are compatible with OMVs uptake by intestinal epithelial cells. Internalization of OMVs by Caco-2 cells was assessed by confocal fluorescence microscopy at 1 h incubation with rhodamine B-R18-labeled OMVs. Immunostaining of the peripherally associated membrane protein ZO-1 was performed as an epithelial cell membrane marker. Results presented in **Figure 4B** confirmed the presence of EcN and ECOR12 OMVs in the cytoplasm of Caco-2 cells. Therefore, internalized OMVs can mediate microbiota immunomodulatory effects in the intact intestinal mucosa.

OMVs from EcN and ECOR12 Modulate the Expression of Immuno-modulatory and Defense Mediators in *Ex Vivo* Colon Explants

In this study, the colon organ culture system was used as a closer model to the *in vivo* conditions. Gene expression levels of the selected mediators were analyzed by RT-qPCR (**Figure 5A**). The expression profile of the cytokines IL-10, MIP1α, TNF-α, IL-6, and IL-8 correlated well with the data obtained in the co-culture system. The corresponding genes were upregulated in colonic explants incubated with OMVs, but not in those incubated with bacterial lysates. After 5 h stimulation, statistically significant increases in the cytokine secreted levels were only observed for IL-6 and IL-8 (**Figure 5B**).

In this model, we also examined the expression of other genes related with host immunomodulatory or defense responses, including IL-12, TGF-β, IL-22, hBD-2, hBD-1, and MUC1. OMVs from either EcN or ECOR12 activated transcription of both IL-22 and the antimicrobial peptide hBD-2, while bacterial lysates failed to do so (**Figure 5A**). In contrast, a different expression profile was seen for the other mediators. Expression of both the proinflammatory cytokine IL-12 and TGF-β was downregulated either by OMVs or bacterial lysates. Expression of MUC1 was also significantly reduced (by nearly 40%) upon treatment with all the bacterial samples (**Figure 5A**). Gene encoding the antimicrobial peptide hBD-1 was not differentially expressed in any of the conditions tested (not shown).

Although, samples from both EcN and ECOR12 strains promoted the same expression profile, colonic explants stimulated with OMVs of the probiotic strain EcN showed a tendency toward better anti-inflammatory balance. The values of IL-10/IL-12 and IL-10/TNF-α mRNA ratios were higher in EcN-treated explants than in ECOR12-treated explants. These values were 2.50 ± 0.28 versus 1.86 ± 0.20 for IL-10/IL-12 and 1.08 ± 0.18 versus 0.80 ± 0.12 for IL-10/TNF-α.

DISCUSSION

In the gut, communication between microbiota and intestinal mucosa cells has to be established by soluble mediators that can

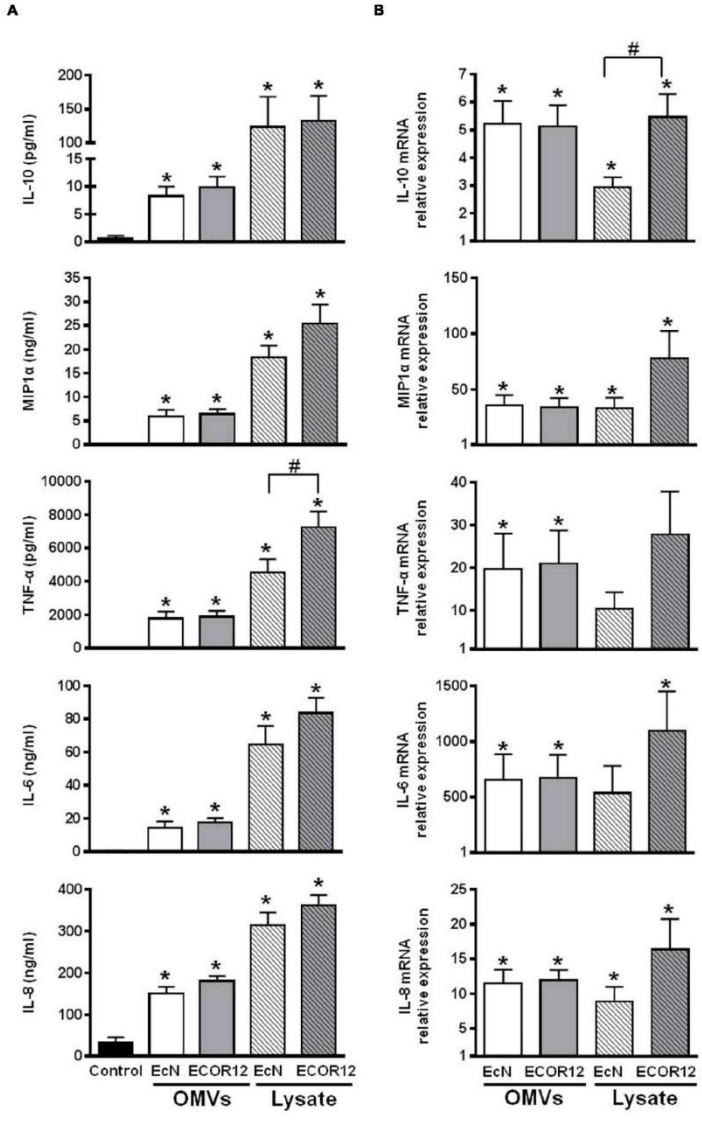

FIGURE 2 | Expression analyses of secreted cytokines and chemokines in PBMCs stimulated with OMVs or bacterial lysates. PBMCs were challenged by addition of OMVs or bacterial lysates from EcN or ECOR12 strains. **(A)** Cytokine concentration in culture supernatants after 24 h stimulation. Data are expressed as mean ± SEM. **(B)** Relative gene expression levels in cells after 5 h stimulation. Data are presented as fold-change compared to untreated control cells. Statistical differences were assessed by one-way ANOVA followed by Tukey's test. $^{*}p \leq 0.05$, versus control cells; $\#p \leq 0.05$, between cells treated with OMVs from EcN or ECOR12.

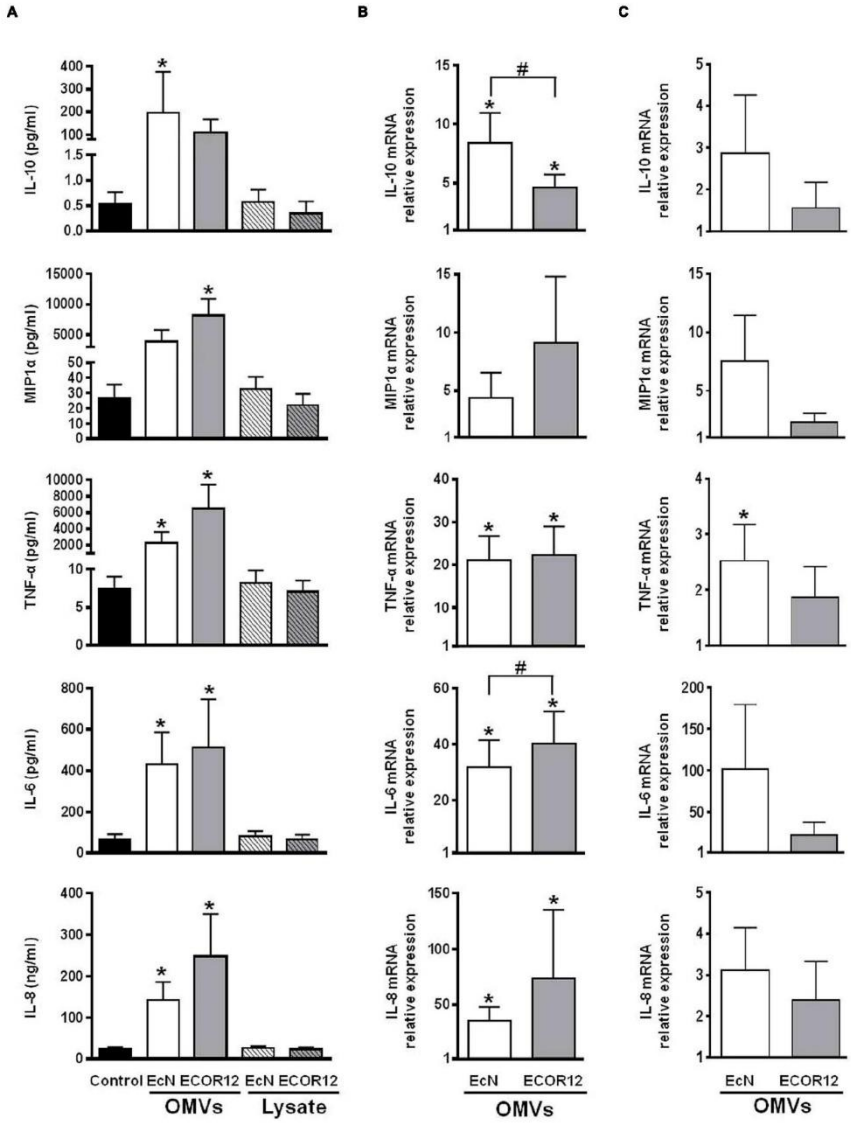

FIGURE 3 | Expression analysis of secreted cytokines and chemokines in Caco2/PBMCs co-cultures apical stimulation. The apical surface of Caco-2 monolayers was challenged by addition of OMVs or bacterial lysates from EcN or ECOR12 strains. **(A)** Cytokine concentration in culture supernatants after 24 h stimulation. Data are expressed as mean ± SEM. Relative gene expression levels in Caco-2 cells **(B)** and in PBMCs **(C)** after 5 h stimulation with OMVs. Data are presented as fold-change compared to untreated control cells. Statistical differences were assessed by the *t*-test or by the non-parametric Mann–Whitney *U*-test. $^{*}p \leq 0.05$, versus control cells; $\#p \leq 0.05$, between cells treated with OMVs from EcN or ECOR12.

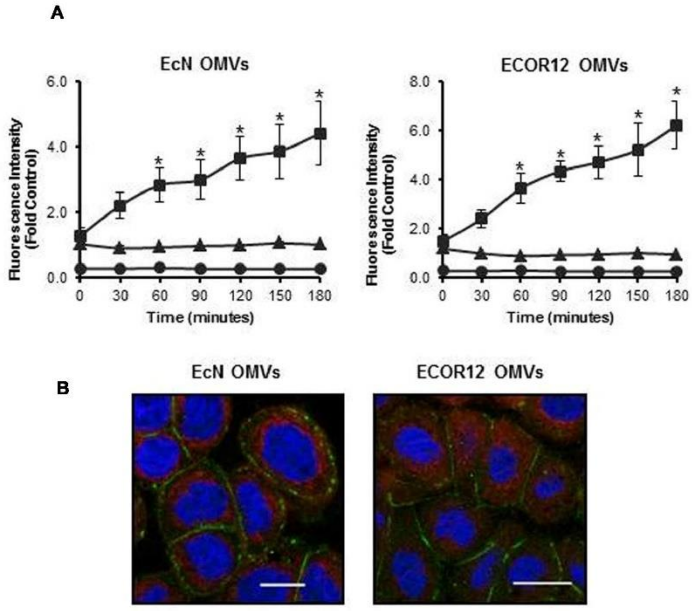

FIGURE 4 | Outer membrane vesicles uptake by differentiated Caco-2 cells. (A) Rhodamine B-R18-labeled OMVs from EcN or ECOR12 were applied to the apical side of polarized Caco-2 cells and fluorescence was measured over time (squares). OMVs (triangles) and cells (circles) alone were used as negative controls for background fluorescence. Statistical differences versus the for background fluorescence emitted by OMVs alone were assessed by one-way ANOVA followed by Tukey's test (*$p < 0.05$). **(B)** Visualization of internalized OMVs by fluorescence microscopy. Caco-2 cells were incubated with rhodamine B-R18 labeled OMVs for 1 h at 37°C. The cell membrane was visualized by immunostaining with antibodies against the zonula occludens ZO-1 protein followed by Alexa Fluor 488-conjugated secondary antibody (green). Nuclei were stained with DAPI (blue). Internalized rhodamine-R18 labeled OMVs are visualized in red. Bar: 20 μm.

diffuse through the mucin layer. Among the bacterial secreted factors, membrane vesicles have a key role in bacteria-host communication, allowing the delivery of effector molecules upon interaction and internalization into the host cells. Many studies performed in the last decade with pathogens evidenced the role of bacterial membrane vesicles as an important mechanism to deliver virulence and immunomodulatory factors to mammalian target cells. However, reports on microbiota vesicles are still scarce.

We focused our study to assess whether OMVs released by probiotic and commensal *E. coli* strains mediate immune modulation in cellular models that mimic damaged or intact intestinal epithelial barrier. To compare the OMVs activity with that of other bacterial fractions, parallel stimulations were performed with bacterial lysates. We analyzed expression of the chemokine MIP1α, the anti-inflammatory cytokine IL-10, and the pro-inflammatory cytokines IL-6, IL-8, and TNF-α. Our results show that direct stimulation with both EcN and ECOR12 OMVs triggered upregulation of all these mediators in PBMCs. In these cells, the genes regulated by EcN lysates had been identified through microarray analysis (Güttsches et al., 2012).The study showed that upregulation of IL-6, IL-8, and TNF-α by the probiotic strain EcN is mediated either by purified LPS or lysates, whereas upregulation of IL-10 is mainly activated by components of the EcN lysate that are not yet identified (Güttsches et al., 2012). Accordingly, the presence of LPS in *E. coli* OMVs may explain the activation of the expression of IL-6, IL-8, and TNF-α, but upregulation of IL-10 by OMVs may be attributed to another vesicle factor. In this context, it should be stressed that EcN OMVs contain some proteins known to modulate the host immune response, such as the flagellar components FlgE and FlgK, and the cytoplasmic moonlighting proteins glyceraldehyde-3-phosphate dehydrogenase and enolase (Aguilera et al., 2014). These moonlighting proteins can switch between different functions, depending on the cell location. Besides their basic metabolic function, when secreted, these proteins fulfill functions that enable bacteria to colonize and modulate the host immune response. As presented above,

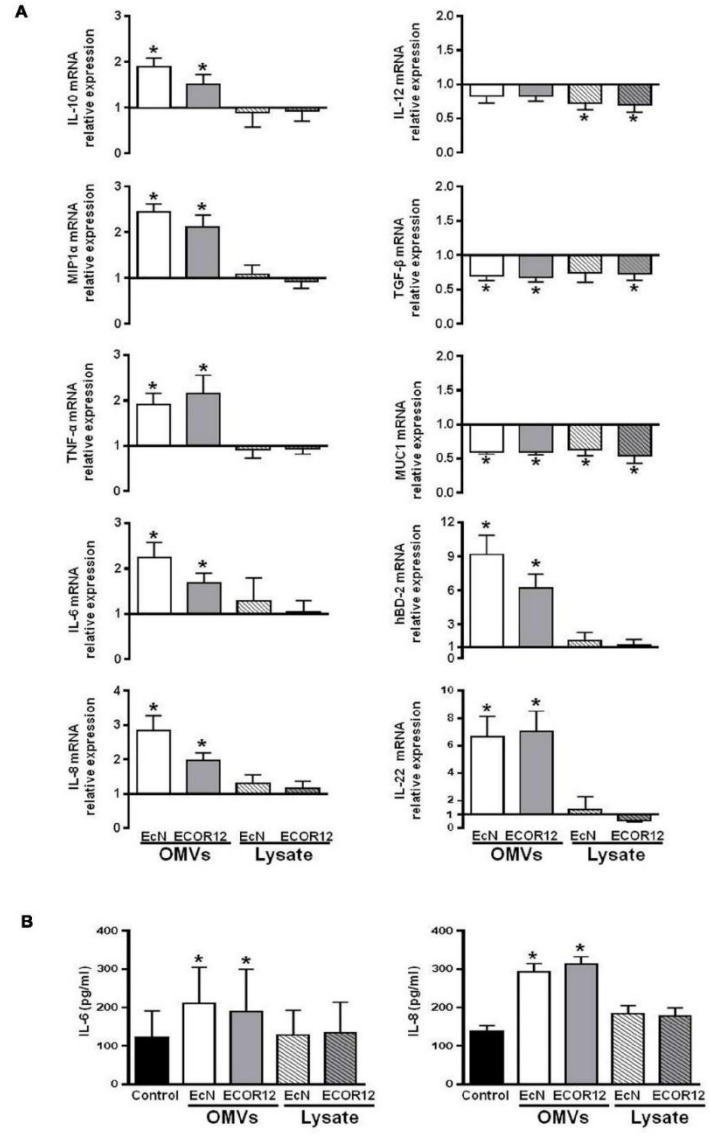

FIGURE 5 | Expression levels of the indicated immunomodulatory and defense mediators in human colonic explants. Colonic tissues were challenged for 5 h with OMVs or bacterial lysates from EcN or ECOR12 strains. **(A)** Relative mRNA levels of the indicated mediators. Data are presented as fold-change compared to untreated colon fragments. **(B)** IL-8 and IL-6 protein levels in culture supernatants. Data are presented as mean ± SEM. Statistical differences were assessed by the *t*-test. $^*p \leq 0.05$, versus control.

cytokine secretion levels were lower in PBMCs exposed to OMVs than in cells exposed to bacterial lysates. The greatest difference was observed for IL-10. For this anti-inflammatory cytokine, the secretion levels were more than 10-fold higher in cells challenged with bacterial lysates, whereas the relative mRNA levels were similar for both OMV and lysate-stimulated cells. According to the molecular composition, we could speculate that bacterial lysates and OMVs activate different signaling pathways or post-transcriptional regulatory mechanisms that would account for the different cytokine secreted levels after 24 h incubation.

The profile of cytokine production by PBMCs incubated with microbiota lysates was clearly different from the profile of cytokine secretion in the co-culture model, in which the epithelial barrier formed by differentiated Caco-2 cells prevent direct access of the effector molecules to underlying PBMCs. In this model, secretion of the analyzed cytokines did not increase over the control levels when the co-culture was apically stimulated with bacterial lysates. Thus, intestinal epithelial cells were poorly responsive to lysate components, which are mainly enriched with cytoplasmic proteins and factors. In contrast, OMVs elicited an immune response in the epithelial cell monolayer as shown by the increased gene expression levels of all the cytokines and chemokines analyzed. This is compatible with the presence in *E. coli* OMVs of PPR-ligands, such as LPS (TLR-4 ligand) or peptidoglycan (NOD-1/NOD-2 ligand) that can trigger NF-kappa B activation. Here, we have shown that microbiota *E. coli* OMVs are internalized in differentiated Caco-2 cells. Therefore, vesicle uptake can assist the delivery of specific bacterial ligands to cytosolic NOD-like receptors. In this co-culture model, activation of the underlying PBMCs upon apical stimulation by microbiota OMVs was assessed by RT-qPCR. Although, the relative gene expression values were not statistically significant after 5 h incubation, the mRNA levels were manifestly higher in co-cultures challenged with OMVs than in non-treated cultures. Upregulation and increased secretion of IL-10, TNF-α, and IL-6 was also described in a co-culture model of intestinal epithelial cells and dendritic cells after apical stimulation with EcN bacterial cells (Zoumpopoulou et al., 2009). Our results prove that PBMCs are stimulated by soluble factors released from epithelial cells upon exposure to OMVs, and show for the first time the ability of microbiota vesicles to mediate signaling events through the intestinal epithelial barrier.

The role of commensal and probiotic *E. coli* OMVs as modulators of the intestinal homeostasis and immunity was corroborated in the *ex vivo* model of colon organ culture, which is closer to the *in vivo* conditions. As in the co-culture model, upregulation of MIP1α, IL-10, TNF-α, IL-6, and IL-8 in colonic mucosa explants was only observed in samples exposed to OMVs. These vesicles also promoted upregulation of the antimicrobial peptide hBD-2. Epithelial hBD-2 plays an important role in intestinal barrier function and many probiotics can induce its secretion (Wehkamp et al., 2004; Möndel et al., 2009). Induction of the expression of hBD-2 by EcN depends on flagella synthesis, as EcN mutants with deletions in genes *fliC* or *flgE* fail to induce this antimicrobial peptide (Schlee et al., 2007). Interestingly, not all *E. coli* strains activate hBD-2 expression (Wehkamp et al., 2004; Möndel et al., 2009). Our results show that OMVs from both EcN and ECOR12 promote hBD-2 upregulation, with higher relative expression levels in colonic fragments challenged with the probiotic OMVs. Induction may be mediated by flagella-associated proteins present in EcN OMVs, such as FliC or FlgE (Aguilera et al., 2014). These results provide evidence that OMVs released by commensal bacteria may also have positive impact on inducible antimicrobial defense mechanisms. In contrast, OMVs from these *E. coli* strains do not significantly modify expression of hBD-1. This antimicrobial peptide is constitutively expressed at high levels by colonic epithelial cells, and functions as a component of the host innate defenses at the mucosal surface. Our results are in accordance with the expression pattern reported for this β-defensin. In contrast to hBD-2, expression of hBD-1 is not inducible by bacterial components or inflammatory stimuli. However, certain enteric pathogens promote hBD-1 downregulation as a mechanism to overcome natural host defenses (reviewed by Cobo and Chadee, 2013).

Another cytokine upregulated in colonic explants by EcN and ECOR12 OMVs is IL-22. This cytokine is mainly expressed by immune cells and displays both pro-inflammatory and anti-inflammatory functions, depending on the tissue and the stimuli that direct its secretion. The pro-inflammatory properties are linked to its production by activated T helper 17 (Th17) cells. The IL-22 targets are typically non-hematopoietic cells, such as epithelial cells. In the intestine, the innate lymphoid cells resident in the lamina propria are a main source of IL-22. This population of cells is essential for the integration of microbiota-derived signals and the control of the adaptive immune response. IL-22 released by gut innate lymphoid cells helps to maintain the integrity of the epithelial barrier by inducing the expression of β-defensins and the production of mucin by globet cells (Nikoopour et al., 2015). Therefore, IL-22 has a relevant role in host-microbiota homeostasis. Expression of this cytokine by innate lymphoid cells and T cells is activated by certain microbiota groups known to protect against food allergen sensitization, such as Clostridia. As a result, increased IL-22 levels reinforce the intestinal epithelial barrier, and therefore limit the access of allergens to the systemic circulation (Stefka et al., 2014). Upregulation of IL-22 by OMVs from EcN and ECOR12 strongly suggest that certain commensal *E. coli* strains can confer intestinal barrier protection, and prevent aberrant responses to food components. In addition, this finding supports the role of microbiota vesicles as a mechanism to deliver regulatory signals to the immune cells resident in the intestinal mucosa.

The expression of genes encoding IL-12, TGF-β and MUC1 was reduced in colonic explants challenged with bacterial samples. Microbiota OMVs and bacterial lysates promoted significant downregulation of TGF-β and MUC1. Relative expression levels were reduced by 25 and 40%, respectively. Expression of the pro-inflammatory cytokine IL-12 was also downregulated, although to a lesser extent.

TGF-β is a pleiotropic cytokine with potent regulatory and inflammatory activities. Its effects depend on cellular and

environmental factors. This mediator promotes differentiation of induced Treg cells, which secrete anti-inflammatory cytokines such as IL-10, and help to control inflammation. However, in the presence of IL-6, TGF-β can trigger differentiation of Th17 cells through induction of the transcriptional factor Runx1, promoting further inflammation (Sanjabi et al., 2009; Liu et al., 2015). Th17 cells are especially abundant in the intestinal mucosa surfaces, where, in cooperation with Treg cells, they contribute to preserving intestinal homeostasis. Alteration in the Th17 (pro-inflammatory)/Treg (anti-inflammatory) balance toward excessive production of Th17 cells leads to the pathogenesis of inflammatory bowel diseases (Llopis et al., 2009). Several probiotics favor the Treg response leading to the induction and secretion of anti-inflammatory cytokines such as IL-10 and TGF-β. Our results show that EcN and ECOR12 trigger downregulation of TGF-β expression. As the pro-inflammatory effects of TGF-β are related with the differentiation of Th17 cells, the reduction in TGF-β levels promoted by these microbiota strains may contribute to restoring the Th17/Treg balance under inflammatory conditions. This mechanism may explain the effectiveness of the probiotic EcN in the remission of ulcerative colitis (Kruis et al., 2004).

MUC1 is a membrane-anchored mucin, located in the apical surface of mucosal epithelial cells. Production of this mucin by colonic epithelial cells is stimulated by IL-17 released by Th17 cells, and MUC1 in turn negatively regulates the Th17 cell responses in inflamed gut (Nishida et al., 2012). In fact the MUC1 gene has been linked with susceptibility to inflammatory bowel disease (Apostolopoulos et al., 2015). This mucin has been associated with barrier functions. However, different models have shown contradictory results. Deficiency of MUC1 results in increased colonic permeability and IL-17 responses in knockout mice lacking the T cell receptor (Nishida et al., 2012), whereas knockdown of MUC1 in corneal epithelial cells did not modify the barrier function (Gipson et al., 2014). *In vitro* studies performed in the colon adenocarcinoma cell line LS174T showed upregulation of MUC1 by probiotic strains, including EcN. However, this effect was not observed in an *in vivo* murine model (Becker et al., 2013). Similarly, analysis of MUC1 expression in mice colonized with EcN during the first week of life did not reveal any increase in the MUC1 mRNA levels when compared to the control group (Bergström et al., 2012).

Interestingly, both MUC1 and TGF-β are overexpressed in several cancer types (Khanh do et al., 2013; Apostolopoulos et al., 2015). As EcN and commensal *E. coli* strains promote downregulation of both mediators in intestinal mucosa explants, we may hypothesize that these microbiota strains could help to reduce cancer progression or to increase treatment effectiveness (Viaud et al., 2015). This is especially interesting in the context of immunotherapy strategies in which the individual response to these therapies has been shown to be dependent on gut microbiota (Sivan et al., 2015; Vétizou et al., 2015).

CONCLUSION

Recent knowledge supports that microbiota is a source of regulatory signals that influence the development and maturation of the digestive and immune systems. Nowadays, potential clinical applications of gut microbes are foreseen. However, translation of microbiota-based drugs to human health care requires deep knowledge of the molecular mechanisms involved in microbiota-host interaction (Jia et al., 2008; Shanahan, 2011). Our study proves the ability of microbiota vesicles to mediate signaling events to the immune system through the intestinal epithelial barrier. Therefore, OMVs are an effective strategy used by beneficial bacteria of the human microbiota to communicate with intestinal mucosa cells, promoting the delivery of mediators that trigger host immune and defense responses. The *in vivo* beneficial effects of the probiotic EcN on gut homeostasis, especially modulation of the immune response and barrier function, can be mediated by released OMVs.

AUTHOR CONTRIBUTIONS

LB, JB, MA, conceived of the study with the participation of EV and RG in experimental design. LB and JB wrote the manuscript. MA, EV, RG, LA, and MJF carried out data interpretation and statistical analysis as well as participated in revising the manuscript. MJF and LA prepared OMVs, Caco-2 cultures, PBMC isolation, Caco-2/PBMC cocultures, cytokine analysis and related work. MJF, LA with the help of EV and MA participated in the *ex vivo* experiments performed with colonic mucosa explants. MAC performed OMVs labeling and internalization studies in Caco-2 cells. All authors read and approved the final manuscript.

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4. CHAPTER 2

Intestinal anti-inflammatory effects of outer membrane vesicles from *Escherichia coli* Nissle 1917 in DSS-experimental colitis in mice.

María José Fábrega, Alba Rodríguez Nogales, José Garrido Mesa, Francesca Algieri, Josefa Badía, Rosa Giménez, Julio Gálvez and Laura Baldomà.

RESUMEN

Escherichia coli Nissle 1917 (EcN) es una cepa probiótica con eficacia demostrada en inducir y mantener la remisión de la colitis ulcerosa. Sin embargo, los factores microbianos que median estos efectos beneficiosos no son del todo conocidos. Las bacterias Gram negativas secretan vesículas de membrana externa (OMVs) como vía de liberación directa de determinadas proteínas bacterianas y compuestos activos hacia el huésped. De hecho, las vesículas liberadas por la microbiota intestinal están emergiendo como piezas clave en procesos de señalización de la mucosa intestinal. En este estudio, se usó un modelo en ratón, de colitis inducida por sulfato sódico de dextrano (DSS) en ratón, para investigar el potencial de las OMVs de EcN de mejorar el daño en la mucosa y la inflamación del intestino. El protocolo experimental desarrollado incluyó un pretratamiento con OMVs durante 10 días, antes de la ingesta del DSS, y un período de recuperación de 5 días. La administración oral de OMVs de EcN purificadas (5µg/día) disminuyó significativamente la pérdida de peso asociada al DSS y mejoró los síntomas clínicos y valores histológicos. El tratamiento con OMVs contrarrestó la expresión alterada de citoquinas y marcadores de barrera intestinal. Este estudio demuestra por primera vez, que las OMVs de EcN pueden mediar los efectos antiinflamatorios y de protección de barrera, previamente reportados para esta cepa probiótica en modelos de colitis experimental. Remarcablemente, el uso de probióticos en la salud humana requiere el conocimiento de los mecanismos moleculares implicados en las interacciones probiótico-huésped. Además, las OMVs, son una forma bacteriana no replicativa, que podrían ser exploradas como una nueva aproximación terapéutica derivada de los probióticos, con un menor riesgo de efectos adversos, comparado con la administración del probiótico.

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Intestinal Anti-inflammatory Effects of Outer Membrane Vesicles from *Escherichia coli* Nissle 1917 in DSS-Experimental Colitis in Mice

María-José Fábrega[1,2†], Alba Rodríguez-Nogales[3†], José Garrido-Mesa[3], Francesca Algieri[3], Josefa Badía[1,2], Rosa Giménez[1,2], Julio Gálvez[3‡] and Laura Baldomà[1,2‡]*

[1] Departament de Bioquímica i Fisiologia, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Institut de Biomedicina de la, Universitat de Barcelona, Barcelona, Spain, [2] Microbiota Intestinal, Institut de Recerca Sant Joan de Déu, Esplugues de Llobregat, Spain, [3] Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas, Department of Pharmacology, ibs.GRANADA, Center for Biomedical Research, University of Granada, Granada, Spain

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Hani El-Nezami, University of Hong Kong, Hong Kong
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***Correspondence:**
Laura Baldomà
lbaldoma@ub.edu

†These authors have contributed equally to this work.

‡These authors have contributed equally to the supervision of the study.

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Escherichia coli Nissle 1917 (EcN) is a probiotic strain with proven efficacy in inducing and maintaining remission of ulcerative colitis. However, the microbial factors that mediate these beneficial effects are not fully known. Gram-negative bacteria release outer membrane vesicles (OMVs) as a direct pathway for delivering selected bacterial proteins and active compounds to the host. In fact, vesicles released by gut microbiota are emerging as key players in signaling processes in the intestinal mucosa. In the present study, the dextran sodium sulfate (DSS)-induced colitis mouse model was used to investigate the potential of EcN OMVs to ameliorate mucosal injury and inflammation in the gut. The experimental protocol involved pre-treatment with OMVs for 10 days before DSS intake, and a 5-day recovery period. Oral administration of purified EcN OMVs (5 μg/day) significantly reduced DSS-induced weight loss and ameliorated clinical symptoms and histological scores. OMVs treatment counteracted altered expression of cytokines and markers of intestinal barrier function. This study shows for the first time that EcN OMVs can mediate the anti-inflammatory and barrier protection effects previously reported for this probiotic in experimental colitis. Remarkably, translation of probiotics to human healthcare requires knowledge of the molecular mechanisms involved in probiotic–host interactions. Thus, OMVs, as a non-replicative bacterial form, could be explored as a new probiotic-derived therapeutic approach, with even lower risk of adverse events than probiotic administration.

Keywords: probiotic, *Escherichia coli* Nissle 1917, outer membrane vesicles, DSS-experimental colitis, mouse model, immune modulation

Abbreviations: Cox-2, cyclooxygenase 2; DAI, disease activity index; DSS, dextran sodium sulfate; EcN, *Escherichia coli* Nissle 1917; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hBD-2, human β-defensin; IBD, inflammatory bowel diseases; iNOS, inducible nitric oxide synthase; LB, Luria-Bertani broth; LPS, lipopolysaccharide; MIP-2, macrophage inflammatory protein 2; MMPs, matrix metalloproteinases; NO, nitric oxide; OMVs, outer membrane vesicles; PAGE, polyacrylamide gel electrophoresis; PBMCs, peripheral blood mononuclear cells; PBS, phosphate buffered saline; PRRs, pattern-recognition receptors; SDS, sodium dodecyl sulfate; TEM, transmission electron microscopy; TFF-3, trefoil factor 3; TJ, tight junction; TLRs, toll-like receptors; TNF-α, tumor necrosis factor-α; Treg, regulatory T cells; UC, ulcerative colitis; ZO-1, zonula occludens-1.

INTRODUCTION

The term IBD mainly refers to UC and Crohn's disease. These are chronic inflammatory disorders of the intestinal tract that may cause life-threatening complications and have an increasing incidence worldwide. They are multifactorial diseases, and although their etiology remains poorly understood, they involve a dysregulated immune response against commensal gut microbes in genetically susceptible individuals with impaired intestinal epithelial integrity (Zhang and Li, 2014). Moreover, several studies have reported that IBD patients have lower microbiota diversity than healthy subjects, and an altered intestinal microbiota balance, which is known as dysbiosis (Ott et al., 2004; Sokol et al., 2008; Manichanh et al., 2012).

Conventional IBD therapies are complex, and not all patients show complete beneficial effects when treated with any of the drugs currently available to manage these intestinal conditions, including salicylates, corticoids, immunosuppressants and biological agents (Ko and Auyeung, 2014). Since dysbiosis is common in IBD, various therapeutic approaches targeting the modulation of the gut microbiota have been explored (Qiao et al., 2016). In this context, many studies have evaluated the therapeutic potential of certain bacteria, including commensal and probiotic strains, to ameliorate IBD in clinical trials (Fedorak, 2010; Wasilewski et al., 2015) or in animal models of colitis (Ewaschuk et al., 2008; Garrido-Mesa et al., 2011; Shen et al., 2012; Kang et al., 2013; Martín et al., 2015; Souza et al., 2016). Overall, these studies have shown the ability of these bacteria to exert beneficial effects on parameters related with gut function, including improvement of gut permeability, reduction of inflammatory cytokine production and/or release, and amelioration of the histological alterations observed in these inflammatory conditions. In some cases, these effects may be mediated, at least in part, by bacterial secreted factors (Ewaschuk et al., 2008; Martín et al., 2015) or by released membrane vesicles (Shen et al., 2012; Kang et al., 2013).

The probiotic EcN positively affects gastrointestinal homeostasis and microbiota balance. In fact, various clinical trials have reported its therapeutic benefits in inducing and maintaining remission of UC (Scaldaferri et al., 2016). EcN shows similar efficacy to the aminosalicylate mesalazine (Losurdo et al., 2015), which supports its use in the treatment of human UC (Floch et al., 2011). Similarly, the intestinal anti-inflammatory effects of this probiotic have been reported in experimental models of colitis in mice and rats (Grabig et al., 2006; Ukena et al., 2007; Arribas et al., 2009; Garrido-Mesa et al., 2011; Souza et al., 2016). Several mechanisms have been reported to be involved in the beneficial effects of this probiotic in IBD, such as its ability to modulate the host immune response toward an anti-inflammatory balance (Trebichavsky et al., 2010). In addition, EcN reinforces the intestinal epithelial barrier by strengthening TJs between intestinal epithelial cells (Ukena et al., 2007; Zyrek et al., 2007; Hering et al., 2014) and increasing the expression of antimicrobial factors such as microcins (Sassone-Corsi et al., 2016) and β-defensin-2 (Schlee et al., 2007). It is important to note that these effects were mainly established when live probiotic suspensions were administered. There is little reported information describing the bacterial factors that could mediate these effects. In this context, membrane vesicles released by commensal bacteria are currently receiving great attention as key players in signaling processes in the intestinal mucosa (Kaparakis-Liaskos and Ferrero, 2015; Olsen and Amano, 2015). The release of bacterial vesicles provides a mechanism for the delivery of microbial proteins and active compounds directly to the host, thus avoiding direct intercellular contact. Recently, it has been reported that OMVs released by the probiotic EcN enter intestinal epithelial cells via clathrin-mediated endocytosis, and thereby can trigger host immune and defense responses (Cañas et al., 2016). In fact, *in vitro* and *ex vivo* studies have shown that EcN OMVs induce the expression of antimicrobial peptides and modulate the cytokine/chemokine response of gut epithelial and immune cells (Fábrega et al., 2016). In addition, in intestinal epithelial cell lines, these vesicles promote upregulation of the TJ-proteins ZO-1 and claudin-14, but down-regulation of claudin-2, thus reinforcing intestinal barrier functions and reducing gut permeability (Alvarez et al., 2016).

Probiotics are Generally Regarded As Safe (GRAS), yet some concerns about the potential risk associated with their use should be considered, especially in immunocompromised individuals and neonates. In conditions of altered intestinal barrier, the probiotic may cause invasive infections leading to sepsis (Ledoux et al., 2006; Bleich et al., 2008; Gronbach et al., 2010). In this context, the use of probiotic- derived bioactive factors, such as OMVs, could potentially have healing benefits, while avoiding the risks associated with the administration of live bacteria.

Based on previous *in vitro* studies (Alvarez et al., 2016; Fábrega et al., 2016) we hypothesized that EcN OMVs may contribute to the immunomodulatory and barrier strengthening effects of EcN in the gut. The aim of the present study was to evaluate the effects of OMVs isolated from EcN in the dextran sodium sulfate (DSS) model of mouse colitis, an experimental model that is widely used in preclinical assays to study new potential treatments for human IBD (Claes et al., 2011).

MATERIALS AND METHODS

EcN Culture and OMVs Preparation

The probiotic strain EcN (serotype O6:K5:H1) was provided by Ardeypharm (GmbH, Herdecke, Germany). OMVs were isolated from culture supernatants of EcN grown overnight at 37°C in Luria-Bertani broth (LB) as previously described (Aguilera et al., 2014). Briefly, bacterial cells were pelleted by centrifugation at 10,000 × *g* for 20 min at 4°C. The supernatant was filtered through a 0.22 μm-pore-size filter (Millipore) to remove residual bacteria, concentrated by centrifugation in a Centricon Plus-70 filter device (Millipore) followed by an additional filtration step. OMVs were then recovered by centrifugation at 150,000 × *g* for 1 h at 4°C, washed and resuspended in sterile PBS. Aliquots were stored at −20°C. The sterility of samples was assessed on LB-agar plates. Protein concentration was measured using the method of (Lowry et al., 1951).

Negative Staining and Transmission Electron Microscopy

Isolated OMVs were examined by transmission electron microscopy (TEM) after negative staining as described previously (Aguilera et al., 2014). A drop of OMV suspension was adsorbed for 2 min on Formvar/carbon coated-grids that were previously activated by UV light. Grids were washed with distilled water, stained with 2% uranyl acetate for 1 min, air dried and examined by TEM (Jeol, JEM 1010, Japan).

Dextran Sodium Sulfate (DSS) Model of Mouse Colitis

All studies were carried out in accordance with the "Guide for the care and the Use of Laboratory Animals" as promulgated by the National Institute of Health. The protocol was approved by the Ethics Committee of the University of Granada (Ref. No. CEEA-2010-286). Male C57BL/6J mice (7- to 9-weeks-old; approximately 20 g) were obtained from Janvier (St Berthevin Cedex, France). Animals were randomly assigned to three groups: non-colitic ($n = 6$), colitic ($n = 9$) and OMVs-treated ($n = 9$). Mice were fed *ad libitum* during the entire experimental period (20 days) on AIN-93G growth purified diet, and animal body weight, as well as food and water intake, were evaluated daily. The experimental design is shown in **Figure 1**. Every day throughout the experimental period (20 days), the two reference groups (non-colitic and colitic) received PBS solution (200 μl), and the treated group received EcN OMVs (5 μg in 200 μl of PBS) by means of an oesophageal catheter. Ten days after starting the experiment, colitis was induced by adding DSS (36–50 kDa, MP Biomedical, Scarborough, ON, United States) to the drinking water at a final concentration of 3% (Mähler et al., 1998). This treatment was applied to all groups for 5 days, except for mice from the reference non-colitic group, which drank tap water throughout the experiment. During this DSS-induced colitis period, the OMVs-treated group was daily administered with EcN OMVs (5 μg/mouse in 200 μl PBS). After colitis damage, 200 μl of PBS were administered daily to all groups except for the OMVs-treated model, which continued with the OMVs treatment for five additional days. All mice were sacrificed 20 days after beginning of the experiment. Animals were previously anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). After starting the DSS intake, animal body weight, the presence of gross blood in the feces and stool consistency were individually evaluated daily (by an observer who was unaware of the treatment). Each parameter was assigned a score according to the criteria proposed previously by (Cooper et al., 1993) and used to calculate an average daily DAI (**Table 1**).

Once mice were sacrificed, the colon was excised from anus to caecum, emptied and washed with PBS before being measured and weighed. Representative specimens (0.5 cm length containing all wall layers) were taken from the distal inflamed region and fixed in 4% buffered formaldehyde for histological analysis. Other fragments were placed in RNA later stabilization reagent (Qiagen GmbH, Hilden, Germany) and stored at −80°C until RNA extraction. The remaining colonic tissue was subsequently sectioned in different longitudinal fragments to be used for biochemical determinations.

Colonic Organ Culture and ELISA Assays

Small fragments of colonic tissue were weighted, cut and then cultured in DMEM (Gibco, Grand Island, NY, United States) supplemented with 10% (v/v) of heat inactivated fetal bovine serum, 1% penicillin/streptomycin, 1% amphotericin, 2% glutamine and 0.45% glucose and incubated at 37°C in a 5% CO_2 atmosphere. After 24-h culture, the supernatants were collected, centrifuged and stored at −80°C until assay. IL-1β, IL-6 and IL-10 levels were measured using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, United States) according to the manufacturer's instructions. The results were expressed as pg/mg protein.

Histological Studies

The colonic samples that were fixed in formaldehyde were paraffin-embedded, sectioned (5 μm) at different levels, and stained with haematoxylin and eosin. Tissues were reviewed in a blinded fashion and the histological damage was assessed according to a previously validated intestinal histologic inflammatory score (Camuesco et al., 2012). This score takes into account the presence of ulceration, infiltration, oedema and the condition of crypts. After evaluation of these features a score ranging from 0 (healthy tissue) to 3 or 4 (severe damage), depending on the item, was assigned to each one. For each sample, the total score was calculated from the sum of each item's score.

Gene Expression Analysis in Colonic Samples by RT-qPCR

Tissue was homogenized in 1 ml of QIAzol using a Precellys 24 homogenizer (Bertin Technologies, Montigny-Le bretonneux, France). Total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Purity and RNA concentration were measured by the absorbance ratio at 260 and 280 in a Thermo Scientific Nano Drop TM 2000 Spectrophotometer. RNA integrity was verified by visualization of 28S and 18S rRNAs after 1% agarose/formaldehyde gel electrophoresis. RNA (2 μg) was reverse transcribed using oligo (dT) primers (Promega, Southampton, United Kingdom). The resulting cDNA (20 ng) was amplified on optical grade 48-well plates in an Eco^{TM} Real Time PCR System (Illumina, San Diego, CA, United States), using the KAPA Sybr Fast qPCR Master Mix (Kapa Biosystems, Inc., Wilmington, MA, United States) and specific primers for each gene (**Table 2**). The $2^{-\Delta\Delta Ct}$ method was used to normalize expression results. The values of the housekeeping glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were used to normalize the values obtained for each of the genes under study. Relative gene expression was calculated by means of the ΔΔCt formula and expressed as fold-change compared with the non-colitic control.

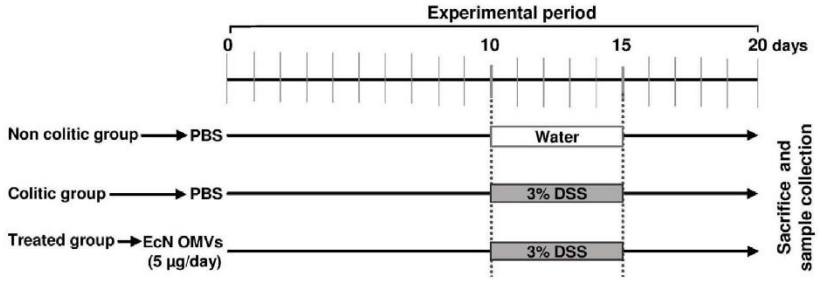

FIGURE 1 | Experimental design to evaluate the potential of EcN OMVs to alleviate DSS-induced colitis in mice. The OMV-treated group daily received intragastrically 200 µl of PBS containing 5 µg of EcN OMVs over the 20-day experimental period, whereas the colitic group received 200 µl of PBS. The non-colitic control group was not challenged with DSS and received 200 µl of PBS over the entire experimental period.

SDS–PAGE and Western Blot Analysis

Protein concentration of colonic samples was measured using a BCA Protein Assay Kit. The intestinal inducible isoform of nitric oxide synthase (iNOS) was analyzed by Western blot. Samples (50 µg protein) were mixed with SDS–PAGE sample buffer, boiled for 5 min at 95°C and electrophoresed on 6% SDS–PAGE gel. Proteins were transferred to a HyBond-P polyvinylidene difluoride membrane using a Bio-Rad Mini Transblot apparatus. The membrane was blocked in PBS-0.05% Tween-20 and 5% skimmed milk (blocking solution) for 1 h at room temperature, and then incubated with specific antibodies against iNOS (Transduction Laboratories, Becton Dickinson Biosciences, Madrid, Spain), 1:2,000 dilution in blocking solution, for 16 h at 4°C, followed by incubation with peroxidase-conjugated anti-rabbit IgG antibody (1:3,000) for 1 h. The protein-antibody complex was visualized using the ECL Plus Western blotting detection system (Amersham Pharmacia Biotech). Control of protein loading and transfer was conducted by detection of the β-actin levels.

Statistics

Statistical analysis was performed using SPSS version 20.0 software (SPSS, Inc.) and GraphPad Prism version 6.0 (GraphPad Software Inc., La Jolla, CA, United States), and data were expressed as mean ± SEM. Differences between more than two groups were assessed using one-way ANOVA followed by Tukey's test. Significant differences were established at $P \leq 0.05$.

TABLE 1 | Scoring index for disease activity.

Score	Weight loss (%)	Stool consistency	Rectal bleeding
0	None	Normal	Normal
1	1–5		
2	5–10	Loose stools	
3	10–20		
4	>20	Diarrhea	Gross bleeding

Disease Activity Index was calculated by adding combined scores for weight loss, stool consistency and rectal bleeding, and dividing the resulting global score by three. Adapted from Cooper et al. (1993).

RESULTS

EcN OMVs Improve Clinical Signs and Colonic Histology in DSS-Induced Mouse Colitis

OMVs were isolated from cell-free culture supernatants and evaluated by negative stain-TEM. Images showed spherical vesicles ranging in size from approximately 20–60 nm in diameter (**Figure 2**).

The *in vivo* protective effects of EcN OMVs were evaluated in male C57BL/6J mice treated with 3% DSS following the protocol outlined in **Figure 1**, which involves pre-treatment with OMVs for 10 days before DSS intake, and a 5-day recovery period. The administration of EcN OMVs for 10 days before DSS intake did not result in significant modifications in body weight in comparison with non-treated mice. Body weight increase was around 15–17% in all groups. When DSS was orally administered to mice, the intestinal inflammatory status was induced, and characterized in the control colitic group (DSS control) by marked body weight loss (**Figure 3A**) and diarrhea with bleeding feces, which resulted in an increased DAI score in this group from day 5 (**Figure 3B**). The administration of EcN OMVs to colitic mice (DSS-OMVs) ameliorated body weight loss (**Figure 3A**) and notably reduced the DAI score from day 6 after DSS intake in comparison with the control colitic group. Statistically significant differences were obtained on the last day of the study (**Figure 3B**). Once mice had been sacrificed, macroscopic evaluation of colonic segments confirmed the beneficial impact of EcN OMVs on the inflammatory process. In fact, colon length is inversely associated with the severity of DSS-induced colitis (Pandurangan et al., 2015), and the colonic weight/length ratio is widely used as an indicator of the colonic oedema that typically occurs in experimental colitis (Hou et al., 2014). The colonic weight/length ratio values were significantly higher in the colitic group than in

TABLE 2 | Primer sequences used for quantitative RT-PCR.

Gene	Sequence (5′-3′)	Annealing T (°C)	Gene accession number
GAPDH	FW: CCATCACCATCTTCCAGGAG RV: CCTGCTTCACCACCTTCTTG	60	NM_008084
IL-1β	FW: TGATGAGAATGACCTCTTCT RV: CTTCTTCAAAGATGAAGGAAA	60	NM_008361.4
IL-6	FW: TAGTCCTTCCTACCCCAATTTCC RV: TTGGTCCTTAGCCACTCCTTCC	60	NM_031168.2
IL-10	FW: TCCTTAATGCAGGACTTTAAGGG RV: GGTCTTGGAGCTTATTAAAAT	56	NM_010548.2
IL-12	FW: CCTGGGTGAGCCGACAGAAGC RW: CCACTCCTGGAACCTAAGCAC	60	NM_001159424
IL-17	FW: GCTCCAGAAGGCCCTCAGACTACC RV: CTTCCCTCCGCATTGACACAGC	60	NM_010552.3
TNF-α	FW: AACTAGTGGTGCCAGCCGAT RV: CTTCACAGAGCAATGACTCC	60	NM_013693.3
INF-γ	FW: GAACTGGCAAAAGGATGGTGA RV: TGTGGGTTGTTGACCTCAAAC	60	NM_008337.4
MIP-2	FW: AGTTAGCCTTGCCTTTGTTCAG RV: CAGTGAGCTGCGCTGTCCAATG	57	NM_009140.2
MMP-2	FW: TGCCGGCACCACTGAGGACTAC RV: GGGCTGCCACGAGGAACA	56	XM_006530751.2
MMP-9	FW: TGGGGGCAACTCGGC RV: GGAATGATCTAAGCCCAG	60	NM_013599.4
iNOS	FW: GTTGAAGACTGAGACTCTGG RV: ACTAGGCTACTCCGTGGA	67	NM_010927.4
COX-2	FW: GGGTTGCTGGGGGAAGAAATG RV: GGTGGCTGTTTTGGTAGGCTG	60	NM_011198.4
TFF-3	FW: CCTGGTTGCTGGGTCCTCTG RV: GCCACGGTTGTTACACTGCTC	60	NM_011575.2
Occludin	FW: ACGGACCCTGACCACTATGA RV: TCAGCAGCAGCCATGTACTC	56	NM_008756.2
ZO-1	FW: GGGGCCTACACTGATCAAGA RV: TGGAGATGAGGCTTCTGCTT	56	XM_006540782

the OMVs-treated group (**Figure 3C**), thus revealing that EcN OMVs intake attenuated the colonic oedema associated with DSS-induced intestinal inflammation in mice.

Microscopic evaluation of the colonic samples also evidenced the intestinal anti-inflammatory effects of EcN OMVs on DSS-induced colitis. Intestinal tissue from the control colitic group revealed common characteristics that were previously reported for this experimental model of colitis (Algieri et al., 2014), including mucosal ulceration, with loss of the normal crypt structure and intense goblet cell depletion, together with inflammatory cell infiltration and oedema (**Figure 4A**). Evaluation of colonic damage by the scoring method described above yielded high histological scores in colitic mice (DSS control group). In contrast, the colitic group treated with EcN OMVs showed recovery from the inflammatory process with an improvement in mucosal barrier integrity, as evidenced by a smaller ulceration surface and the presence of goblet cells replenished with their mucin content. In addition, samples from mice treated with OMVs showed lower inflammatory cell infiltration and tissue oedema than samples from the control colitic group. Consistently, microscopic evaluation of colonic damage in the OMV-treated colitic mice resulted in significant improvement in the histologic scores ($P < 0.05$ vs. control colitic group) (**Figure 4B**).

Intestinal Anti-inflammatory Effects of EcN OMVs Are Associated with an Improvement in the Altered Immune Response in DSS-Induced Mouse Colitis

It is well-known that DSS-induced colonic inflammation is associated with an altered immune response (Xu et al., 2014). Consistently, results from the RT-qPCR analysis performed in the present study revealed that mice from the colitic group showed significant higher mRNA expression of the pro-inflammatory cytokines IL-1β, TNF-α, IL-6, MIP-2 and INFγ than the non-colitic group. A trend of higher expression of cytokines IL-12 and IL-17 was also observed in the colitic group but the values did not reach statistical significance when compared with non-colitic mice. The treatment of colitic mice with EcN OMVs decreased the expression of all pro-inflammatory cytokines assayed. The results were statistically significant for IL-1β, TNF-α and IL-17 in comparison with control colitic mice (**Figure 5A**). Moreover, the colonic inflammatory process induced by DSS was associated with

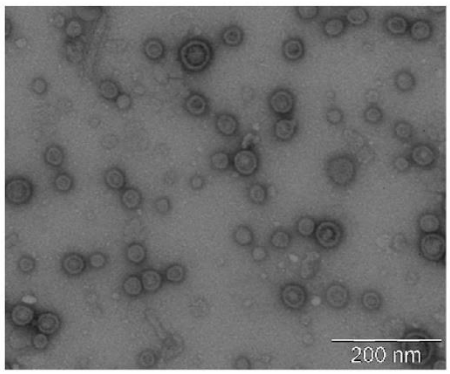

FIGURE 2 | Negative staining electron microscopy of isolated EcN OMVs. Vesicles were isolated from 1-liter LB culture of EcN and resuspended in a final volume of 0.2 ml PBS. A representative image of OMV samples (1:20 dilution) is shown. Scale bar: 200 nm.

lower expression of the anti-inflammatory cytokine IL-10, which was significantly counteracted by administration of EcN OMVs (**Figure 5A**).

The observed changes in colonic mRNA expression correlated well with the protein levels, as evidenced from the quantitative ELISA results of three representative cytokines (IL-10, IL-6 and IL-1β) (**Figure 5B**). Levels of the pro-inflammatory cytokines IL-6 and IL-1β were significantly lower in the colitic group treated with EcN OMVs than in the corresponding control group (DSS control). Administration of EcN OMVs counterbalanced the DSS-mediated reduction in the colonic levels of the anti-inflammatory cytokine IL-10, although these results did not reach statistical significance, probably due to variability in the data (**Figure 5B**).

The beneficial effects of EcN OMVs were also associated with the recovery of a marker of intestinal barrier function, the intestinal TFF-3, whose expression is downregulated in inflamed colonic tissue after DSS administration to mice. It is interesting to note that EcN OMVs restored the mRNA levels of TFF-3 to values similar to those found in the non-colitic control group (**Figure 6**). Treatment with EcN OMVs did not counteract DSS-induced downregulation of ZO-1, but tended to increase occludin mRNA levels in DSS-treated mice, although data did not reach statistical significance (**Figure 6**). In the context of tissue remodeling, we also measured the mRNA expression of two relevant MMPs, MMP-9 and MMP-2, which have opposing roles. MMP-9 promotes tissue injury in colitic mice, whereas MMP-2 has a protective role and contributes to the maintenance of gut barrier function (Garg et al., 2009). Accordingly, mice in the colitic group showed greater expression of MMP-9 and lower expression of MMP-2 than non-colitic mice. Both DSS-mediated effects were countered by treatment with EcN OMVs, but only the reduction in colonic MMP-9 mRNA levels was statistically significant (**Figure 6**). Consequently, the effects exerted by EcN OMVs on the altered expression of MMPs during colitis can contribute to their ability to protect against tissue damage induced by DSS.

In addition, RT-qPCR analysis was performed for the inflammatory enzymes COX-2 and inducible iNOS, known to be upregulated in DSS-induced colitis. Administration of EcN OMVs to colitic mice significantly reduced the expression of both enzymes, which were highly expressed in the colonic tissue of untreated colitic mice (**Figure 7A**). Western blotting of iNOS confirmed the compensatory effect mediated by EcN OMVs at protein level (**Figure 7B**).

DISCUSSION

The etiology of IBD has not been completely elucidated to date. However, it has been proposed that intestinal dysbiosis is crucial, since it can promote an inappropriate immune response in

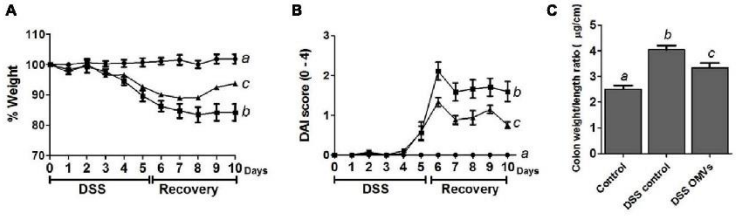

FIGURE 3 | EcN OMVs treatment improves clinical signs of DSS-induced colitis in mice. After the 10-day pre-treatment period, mice received 3% DSS in drinking water for 5 days. Mice were sacrificed 5 days later. Mice from the OMVs-treated group (triangles; $n = 9$) were administered with EcN OMVs (5 µg/day) throughout the experiment, whereas the two other experimental groups, colitic DSS control (squares; $n = 9$) and non-colitic control (circles; $n = 6$), received PBS instead. **(A)** Weight evolution in animals over the DSS-treatment and the recovery period. Values are presented as percentage of the body weight at the beginning of DSS intake (day 0). **(B)** DAI score of each experimental group from the beginning of the induction of colitis by DSS treatment (day 0) until sacrifice (day 10), calculated as described in the Section "Materials and Methods." **(C)** Colon weight/length ratio calculated following resection. Data are expressed as means ± SEM. Different letters indicate significant differences between groups ($P < 0.05$).

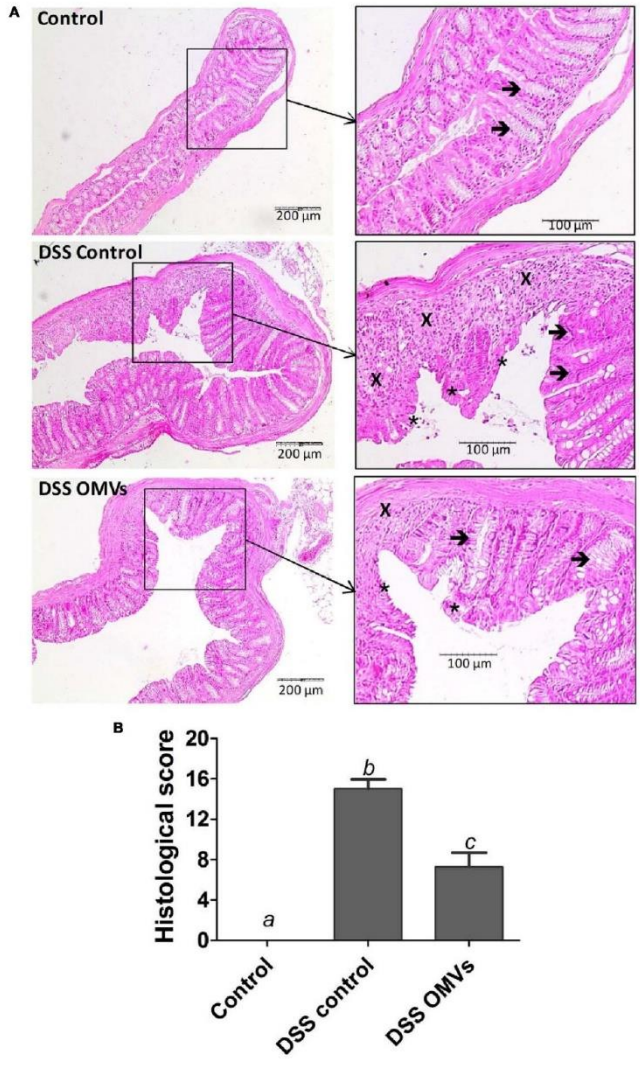

FIGURE 4 | EcN OMVs treatment promotes recovery of DSS-induced intestinal injury and inflammation in mice. On day 20, colons were excised and processed for microscopic analysis. **(A)** Histological images of colonic tissue stained with haematoxylin and eosin showing the effect of EcN OMVs on DSS-induced colitis. Representative images of each experimental group are shown: Control, DSS control and DSS OMVs. In Control, images show the normal appearance of the intact mucosa containing the crypts with goblet cells plenty of their mucin content (arrows). In DSS control, images show changes in the mucosa with areas of ulceration on the epithelial layer (asterisks), reduction of goblet cells with depletion of their mucin content (arrows) and intense inflammatory cell infiltrate (cross). In DSS OMVs, an improvement of the colonic histology is observed, with reduced area of ulceration, mostly in process of recovery (asterisks), presence of goblet cells replenished with their mucin content (arrow) and reduced inflammatory cell infiltrate (cross). **(B)** Histological scores calculated after microscopic analyses of longitudinal colon sections as described in the Section "Materials and Methods." Results are expressed as mean ± SEM. Control group ($n = 6$), DSS control group ($n = 9$), DSS OMVs group ($n = 9$). Different letters indicate significant differences between groups ($P < 0.05$).

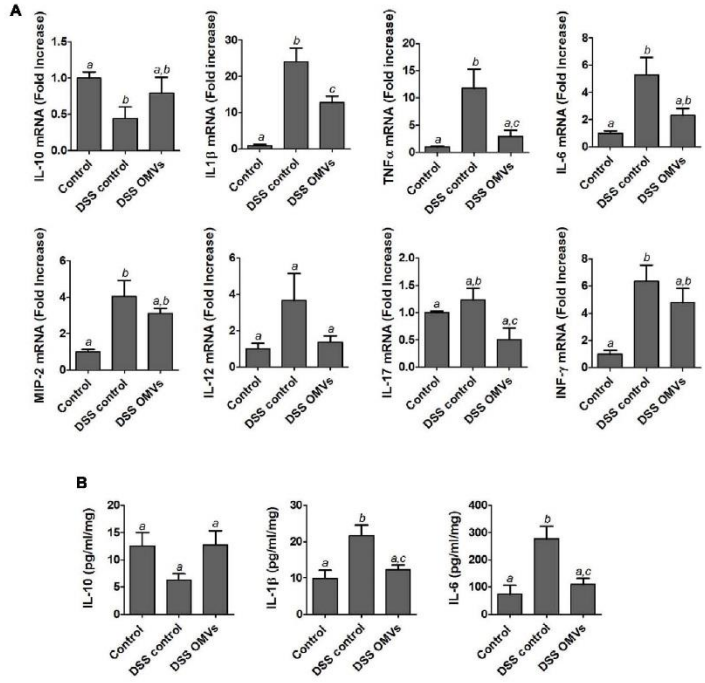

FIGURE 5 | Effects of EcN OMVs treatment on colonic cytokine expression in DSS colitic mice. **(A)** Relative mRNA levels of the indicated cytokines measured by RT-qPCR in colonic tissue. Data are presented as fold-change compared to the non-colitic control group (expression value set to 1). **(B)** IL-10, IL-1β and IL-6 protein levels measured by ELISA in culture supernatants of colonic fragments from mice of each experimental group incubated for 24 h in complete DMEM medium as described in the Section "Materials and Methods." Data are expressed as mean ± SEM. Control group (n = 6), DSS control group (n = 9), DSS OMVs group (n = 9). Different letters indicate significant differences between groups ($P < 0.05$).

genetically susceptible individuals, leading to the inflammatory status that characterizes these intestinal conditions (Becker et al., 2015). Accordingly, restoration of intestinal microbiota composition through the administration of probiotics could have beneficial effects against the inflammatory response, as has been shown both in experimental models and in human IBD (Claes et al., 2011; Bellaguarda and Chang, 2015). EcN is one of the probiotics reported to have a beneficial effect in IBD, and plays a prominent role in therapy for this group of intestinal conditions in humans. In fact, the 2011 update of the American Recommendations for Probiotic Use gave very strong "A" recommendations for its use to maintain remission in human UC (Floch et al., 2011). The therapeutic efficacy of EcN has been more recently supported in a systematic review and meta-analysis (Losurdo et al., 2015). Among the mechanisms that might be involved in the beneficial effects exerted by EcN, its immunomodulatory properties could play a key role. In the gut, the probiotic can counteract altered cytokine production by immune cells (Sturm et al., 2005; Guzy et al., 2008) and restore damaged epithelium through the modulation of TJ and zonula occludens proteins (Ukena et al., 2007; Zyrek et al., 2007). Although direct interaction between the bacteria EcN and the immune or epithelial cells could be responsible for the observed effects, other mechanisms involving the production and release of secreted bacterial factors cannot be ruled out. Among these secreted factors, membrane vesicles play a key role in bacteria–host communication, allowing the delivery of effector molecules upon interaction and internalization into the host cells. In fact, OMVs enclose many of the biological components that can be found in the bacterium and, specifically, ligands of pattern recognition receptors such as LPS (TLR-4 ligand), peptidoglycan (NOD-1/NOD-2 ligand) or DNA (TLR-9 ligand), among others. These receptors, which are expressed in epithelial and immune cells, are key components of innate immunity as they sense gut microbes and trigger appropriate immune responses. Therefore, bacteria-released OMVs can mediate most of the effects of the

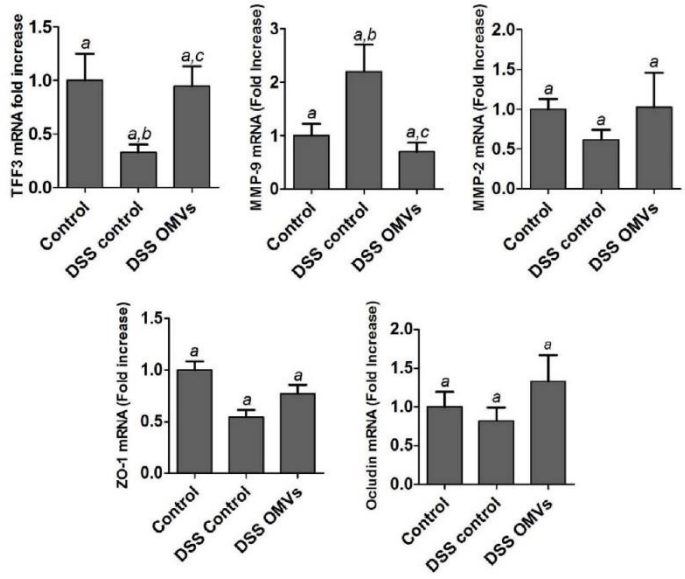

FIGURE 6 | Effects of EcN OMVs treatment on colonic expression of markers of intestinal barrier function in DSS colitic mice. Relative mRNA levels of TFF-3, MMP-9, MMP-2, ZO-1 and occludin were measured by RT-qPCR in colonic tissue. Data are presented as fold-change compared to the non-colitic control group. Control group (n = 6), DSS control group (n = 9), DSS OMVs group (n = 9). Data are presented as mean ± SEM. Different letters indicate significant differences between groups ($P < 0.05$).

bacterium. Remarkably, OMVs as non-replicative bacterial forms further reduce the low risk of adverse events associated with the administration of the probiotic (Bleich et al., 2008; Gronbach et al., 2010; Olier et al., 2012).

Previous studies performed in human cell lines and human colonic explants have shown the immunomodulatory and barrier strengthening potential of EcN OMVs (Alvarez et al., 2016; Fábrega et al., 2016). Since orally administered live EcN suspensions ameliorate DSS-induced colitis in mice (Grabig et al., 2006; Garrido-Mesa et al., 2011; Olier et al., 2012), we sought to explore whether EcN OMVs could prevent colonic damage in the same experimental model of intestinal inflammation. This is a well-established animal model that resembles human UC, and it has been validated for the translation of mouse data to humans (Jiminez et al., 2015).

The present study demonstrates that oral administration of OMVs isolated from EcN has intestinal anti-inflammatory properties in the DSS experimental model of colitis, as previously reported when a suspension of the viable probiotic was administered (Garrido-Mesa et al., 2011). The beneficial effects of EcN OMVs were already observed during the course of the experiment. In fact, reduction in weight loss and DAI evolution in mice treated with OMVs, compared to the control colitic group, clearly evidenced amelioration of the impact of the colitic process and improvement in the health status of mice.

Likewise, OMVs administration to colitic mice significantly reduced colonic damage. This was shown macroscopically by a significant reduction in the colonic weight/length ratio, which indicated amelioration of the tissue oedema that characterizes this colonic inflammatory process, and microscopically with recovery of the altered tissue histology associated with DSS colitis. The OMV-mediated anti-inflammatory effect was clearly associated with an improvement in the altered immune response accompanying DSS-induced colonic damage, thus mimicking the immunomodulatory properties described for this probiotic both *in vivo* and *in vitro* (Kruis et al., 2004; Grabig et al., 2006; Arribas et al., 2009; Garrido-Mesa et al., 2011; Scaldaferri et al., 2016). It is known that the acute mucosal inflammation induced by DSS results in activation of several cells involved in the intestinal innate immune response, including epithelial cells, macrophages and dendritic cells, leading to upregulated expression and/or production of pro-inflammatory cytokines, such as IL-1β, TNF-α and IL-6. This subsequently facilitates the sustained inflammatory response associated with an imbalance in Th1/Th17 and Treg cell responses, which results in increased expression and/or production of other cytokines such as IFN-γ, IL-12 (Th1) and IL-17 (Th17), together with a reduction in the

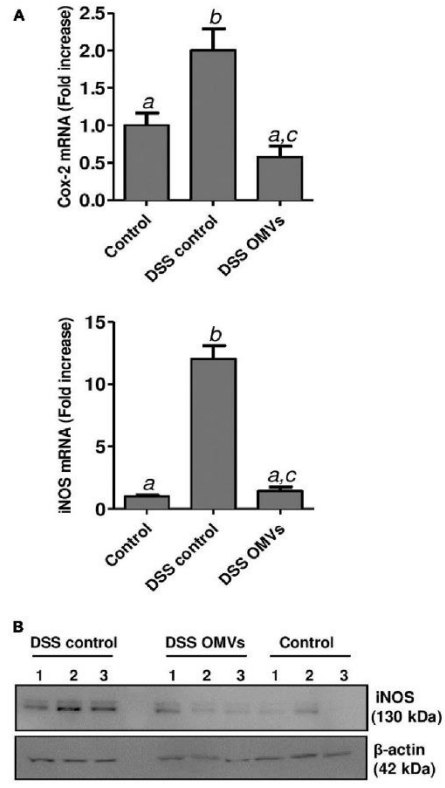

FIGURE 7 | Effects of EcN OMVs treatment on colonic COX-2 and iNOS expression. **(A)** Relative mRNA levels of COX-2 and iNOS measured by RT-qPCR in colonic tissue. Data are presented as fold-change compared to the non-colitic control group. Different letters indicate significant differences between groups ($P < 0.05$). **(B)** Western blot analysis of iNOS levels in colonic fragments collected from three mice (lines 1, 2, 3) of each group. Anti-β-actin antibody was used as internal control. Representative images are shown.

anti-inflammatory cytokine IL-10 (Treg) (Neurath, 2014). Our results show that administration of EcN OMVs ameliorates the altered cytokine profile observed in colitic mice treated with DSS.

Different mechanisms could be involved in these immunomodulatory properties. Of particular significance is the ability to restore intestinal epithelial integrity. In this context, it has been reported that the intestinal anti-inflammatory properties of EcN are related to its ability to reinforce TJs of intestinal epithelial cells, thus counteracting the altered permeability status in the intestinal epithelium that characterizes intestinal inflammation (Ukena et al., 2007). Notably, impairment of the epithelial barrier function has been proposed as one of the first events to occur in intestinal inflammation. This facilitates the access of antigens from the intestinal lumen and triggers the exacerbated immune response. Consequently, rapid promotion of mucosal healing has been considered crucial in the management of intestinal inflammation (Dave and Loftus, 2012). Under conditions of intact epithelial barrier EcN OMVs (100 μg/ml) upregulate expression of claudin-14 and ZO-1 in T-84 and Caco-2 monolayers (Alvarez et al., 2016). However, oral administration of EcN OMVs does not prevent DSS-induced downregulation of ZO-1 mRNA. Thus, the effects previously observed in *in vitro* models of intact epithelial barrier are not appreciated in the *in vivo* model of experimental colitis. This suggests that different regulatory mechanisms could be activated by EcN OMVs in the presence of highly expressed inflammatory mediators. Notably, ZO-1 is a target of post-translational modifications that regulate the intracellular fate of this protein. Reinforcement of the epithelial barrier can also be triggered by stimuli that promote ZO-1 location at the cell boundaries, and hence its association with TJ structures. In this context, it has been described that TFF-3 promotes redistribution of ZO-1 from the cytoplasmic compartment to the intercellular junctions in Caco-2 cell monolayers, which results in an increased level of co-localisation with occludin. These regulatory effects were not accompanied by changes in ZO-1 or occludin protein levels (Buda et al., 2012). Expression of TFF-3 is downregulated in intestinal inflammation (Lozano-Pérez et al., 2014), as observed in the present study. In connection with this, here we show that EcN OMVs increase colonic expression of this bioactive peptide involved in epithelial protection and repair (Podolsky et al., 2009), and preserve the damaged colonic cytoarchitecture of the mucosa in colitic mice. In addition to beneficial effects on epithelial integrity restoration, increased expression of TFF-3 can help to ameliorate the altered immune response exerted by EcN OMVs in experimental colitis, since overexpression of this peptide has been reported to abolish the IL-1β-induced upregulation of the pro-inflammatory cytokines IL-6, IL-8 and TNFα (Lin et al., 2013). Another plausible mechanism exploited by EcN OMVs to protect intestinal barrier function in colitic mice is downregulation of MMP-9 expression. Upregulation of this gelatinase contributes to the pathogenesis of IBD by disrupting TJs between intestinal epithelial cells, leading to increased intestinal permeability and the subsequent pro-inflammatory effects (Nighot et al., 2015). Moreover, treatment of colitic mice with EcN OMVs tends to increase, or at least preserve, MMP-2 expression, thus protecting against DSS-associated intestinal barrier loss (Garg et al., 2009).

Other markers of inflammation and tissue damage that are over-expressed in DSS-induced colitis are COX-2 and iNOS (Kolios et al., 2004). These enzymes are in fact defense mechanisms against the injury, inflammation and infection that occur in pathological conditions, and are highly expressed at damaged sites. Increases in COX-2 levels are seen in chronic intestinal inflammation, which is one of the risk factors for colorectal cancer (Wang and DuBois, 2010). Expression of iNOS is induced by certain pro-inflammatory cytokines such as TNF-α or IFN-γ, as well as by bacterial products. Although increased NO production through upregulation of iNOS is part of the

intestinal antibacterial response, excess NO has been associated with intestinal inflammation in IBD patients and attributed to the immune dysregulation that occurs in these intestinal disorders (Kolios et al., 2004). In fact, elevated iNOS expression has been demonstrated in clinical IBD, caused mainly by infiltrating macrophages in intestinal mucosa (Rachmilewitz et al., 1995; Palatka et al., 2005). In addition, excessive NO production mainly through iNOS has been correlated with tissue injury and mucosal lesions observed in both human IBD and DSS-induced colitic mice. Consequently, probiotics or natural compounds with proven efficacy in attenuating colitis in DSS-treated mice reduce iNOS expression (Camuesco et al., 2004; Garrido-Mesa et al., 2015; Ren et al., 2015; Senol et al., 2015; Utrilla et al., 2015). EcN is among these probiotics (Garrido-Mesa et al., 2011). Here we show for the first time the ability of OMVs from a probiotic strain, specifically EcN, to counteract DSS-induced expression of both iNOS and COX-2. This correlates with reduced expression of the pro-inflammatory cytokines TNF-α and IFN-γ, and lower inflammatory cell infiltration in OMVs-treated mice.

At present, the molecules in EcN OMVs responsible for the anti-inflammatory and/or barrier protective effects remain unknown. Olier et al. (2012) reported that the genotoxic polyketide colibactin is required for the *in vivo* anti-inflammatory effects of EcN. Moreover, deficiency in colibactin biosynthesis leads to exacerbation of colitis severity in DSS-treated mice. These facts lead authors to considered colibactin as an immunomodulin. Whether this bacterial effector is exported and delivered into the host cells remains elusive. As EcN OMVs exert anti-inflammatory effects in experimental colitis, we may speculate that colibactin could be secreted through OMVs, at least in part. However, the contribution of other specific probiotic OMV-associated factors cannot be ruled out. Regarding mediators of intestinal barrier protection, it has been shown that the secreted protein TcpC contributes to the *in vitro* strengthening ability of EcN when the epithelial barrier is intact (Hering et al., 2014). However, the effect of TcpC is not associated with OMVs (Alvarez et al., 2016). Thus, OMV-linked factors other than TcpC should contribute to the modulation of mucosal healing markers like TFF-3 and MMP-9, observed in this experimental murine colitis model.

CONCLUSION

Recently acquired knowledge shows that microbiota plays an important role in the pathogenesis of IBD; dysbiosis is a common feature of these inflammatory conditions. Nowadays, potential clinical applications of probiotics are being explored to restore microbiota imbalances and take advantage of their health-promoting capacities. However, the translation of probiotics or microbiota-based drugs to human health-care requires deep knowledge of the molecular mechanisms involved in microbiota–host interactions (Jia et al., 2008; Shanahan, 2011). In this regard, bacterial vesicles are key players in signaling processes in the intestinal mucosa, as they act as a secretion and delivery pathway for selected bacterial proteins and active compounds directly to the host cells. Among probiotics with promising results in IBD clinical assays and experimental colitis models, EcN has beneficial effects on the remission of UC. Our study proves that oral administration of OMVs isolated from this probiotic has intestinal anti-inflammatory effects in the DSS experimental model of colitis, similarly as previously reported for the administration of viable probiotic suspensions. Therefore, OMVs could represent a safe probiotic-derived strategy targeting intestinal inflammatory processes as they are free from bacteria and can elicit the same effect on gastrointestinal health as the probiotic itself.

AUTHOR CONTRIBUTIONS

LB, JG, JB and RG contributed to the conception and design of the study. M-JF and AR-N were the responsible to perform all the experiments and analysis of data. JG, JG-M, and FA contributed to the histological studies. JG, LB, JB, and RG supervised the work, interpreted the data and wrote the drafted manuscript. All the authors revised the manuscript and approved the final version.

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5. CHAPTER 3

Outer membrane vesicles from probiotic and commensal *Escherichia coli* activate NOD-mediated immune responses in intestinal epithelial cells

María Alexandra Cañas, María José Fábrega, Rosa Giménez, Josefa Badía and Laura Baldomà.

RESUMEN

La microbiota intestinal es fundamental en el mantenimiento de la homeostasis intestinal y la salud humana. Las vesículas bacterianas extracelulares tienen un papel clave en la comunicación bacteria-huésped, ya que éstas permiten la liberación de moléculas efectoras dentro de la célula. Las vesículas de membrana externa (OMVs) secretadas por bacterias Gram-negativas transportan ligandos de PRRs, los cuales son componentes clave de la inmunidad innata, capaces de reconocer microorganismos intestinales y desencadenar una correcta respuesta inmunitaria. Los receptores citosólicos NOD1 y NOD2 reconocen específicamente el peptidoglicano presente en la pared celular bacteriana. Estos receptores inmunitarios son esenciales en la defensa del huésped frente a las infecciones bacterianas y en la regulación de la respuesta inflamatoria. Aportaciones recientes muestran que los NODs son también fundamentales para el mantenimiento de la homeostasis y el balance microbiano. El peptidoglicano de patógenos no invasivos es reconocido por estos receptores citosólicos a través de las OMVs, las cuales son internalizadas vía endocitosis. Sin embargo, aún se desconoce si esta vía puede ser usada por la microbiota para activar la señalización a través de receptores tipo NOD. En este trabajo, nosotros mostramos que las OMVs aisladas del probiótico *Escherichia coli* Nissle 1917 y de la cepa comensal ECOR12 activan la señalización de la vía de NOD1 en células de epitelio intestinal. La señalización de NOD1 y la inhibición de RIP2 anuló significativamente la activación de NF-κB mediada por OMVs y la subsecuente respuesta de IL-6 e IL-8. Análisis de microscopía confocal de fluorescencia confirmaron que las OMVs endocitadas colocalizan con NOD1, desencadenan la formación de agregados de NOD1, y promueven la asociación de NOD1 con endosomas tempranos. Este estudio muestra por primera vez la activación de la vía de señalización NOD1 por vesículas extracelulares liberadas por la microbiota intestinal.

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Outer membrane vesicles from probiotic and commensal Escherichia coli activate NOD1-mediated immune responses in intestinal epithelial cells

María-Alexandra Cañas[1], MARIA JOSE FABREGA[1], Rosa Giménez[1], Josefa Badia[1], Laura Baldomà[1*]

[1]University of Barcelona, Spain

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Outer membrane vesicles from probiotic and commensal *Escherichia coli* activate NOD-mediated immune responses in intestinal epithelial cells

María-Alexandra Cañas[1,2,a], María-José Fábrega[1,2,a], Rosa Giménez[1,2], Josefa Badia[1,2,b] Laura Baldomà[1,2b].

[1] Secció de Bioquímica i Biología Molecular, Departament de Bioquímica i Fisiologia, Facultat de Farmàcia, Universitat de Barcelona, Barcelona, Spain. [2] Institut de Biomedicina de la Universitat de Barcelona, Institut de Recerca Sant Joan De Déu (IR-SJD), Barcelona, Spain.

[+a]Both authors contributed equally to this work
[b]Both authors contributed equally to the supervision of the study
*Correspondence:
Laura Baldomà
lbaldoma@ub.edu

ABSTRACT

Gut microbiota plays a critical role in maintaining human intestinal homeostasis and host health. Bacterial extracellular vesicles are key players in bacteria-host communication, as they allow delivery of effector molecules into the host cells. Outer membrane vesicles (OMVs) released by Gram-negative bacteria carry many ligands of pattern recognition receptors that are key components of innate immunity. NOD1 and NOD2 cytosolic receptors specifically recognize peptidoglycans present within the bacterial cell wall. These immune receptors are essential in host defence against bacterial infections and in the regulation of inflammatory responses. Recent contributions show that NODs are also fundamental to maintain intestinal homeostasis and microbiota balance. Peptidoglycan from non-invasive pathogens is delivered to cytosolic NODs through OMVs, which are internalized via endocytosis. Whether this pathway could be used by microbiota to activate NOD receptors remains unexplored. Here, we report that OMVs isolated from the probiotic *Escherichia coli* Nissle 1917 and the commensal ECOR12 activate NOD1 signalling pathways in intestinal epithelial cells. NOD1 silencing and RIP2 inhibition significantly abolished OMV-mediated activation of NF-κB and subsequent IL-6 and IL-8 expression. Confocal fluorescence microscopy analysis confirmed that endocytosed OMVs colocalize with NOD1, trigger the formation of NOD1 aggregates, and promote NOD1 association with early endosomes. This study shows for the first time the activation of NOD1-signalling pathways by extracellular vesicles released by gut microbiota.

Key words: *gut microbiota, Escherichia coli Nissle 1917, NF-kB activation, bacterial extracellular vesicles, NOD1*

INTRODUCTION

There is strong scientific evidence that the gut microbiota has a fundamental role in the well-being of the host. Indeed, this microbial community is a key orchestrator of the immune system, contributing to maintenance of tolerogenic immune responses (Maynard et al., 2012). Communication between microbiota and intestinal epithelial cells is essential to preserve proper microbiota balance and intestinal homeostasis. Under healthy conditions, this inter-kingdom communication is mediated through secreted bacterial compounds that, unlike whole bacteria, can diffuse through the intestinal mucus layer and interact with epithelial cells (Sánchez et al., 2010; Hevia et al., 2015). Besides soluble proteins, Gram-negative bacteria also release active mediators into the intestinal lumen through outer membrane vesicles (OMVs) (Ahmadi- Badi et al. 2017). Most studies have been focused on pathogen released OMVs and have proven their role in virulence (Yoon et al., 2011; Pollak et al., 2012; Bielaszewska et al., 2013; Schwechheimer and Kuehn, 2015; Jäger et al., 2015, Jung et al., 2016).

In contrast, vesicles released by commensal and probiotic bacteria have been associated with beneficial effects for the host. Therefore, it has been suggested that microbiota vesicles may act as key players to maintain intestinal homeostasis (Kaparakis-Liaskos and Ferrero, 2015; Patten et al., 2017). However, there are relatively few reports on this field. Studies performed with prevalent Gram-negative bacteria that reside in the human gut showed that OMVs released by *Bacteroides fragilis* and *Akkermansia muciniphila* promote immunomodulatory effects and prevent gut inflammation in mice models of experimental colitis (Shen et al., 2012; Kang et al., 2013). In this context, we have proved that OMVs from the probiotic *Escherichia coli* Nissle 1917 (EcN) and other commensal *E. coli* strains deliver mediators that trigger host immune and defence responses. These vesicles are internalized by intestinal epithelial cells via clathrin-mediated endocytosis and sorted to lysosomes through endocytic compartments (Cañas et al., 2016). OMVs from microbiota *E. coli* strains exhibit immunomodulatory activity on different *in vitro* models of intestinal barrier and in human colonic explants, regulating expression of antimicrobial peptides and inflammatory biomarkers towards an anti-inflammatory profile (Fábrega et al., 2016). Oral administration of OMVs

isolated from the probiotic EcN ameliorate inflammation and colitis progression in DSS-treated mice, similarly to previously reported for the administration of viable probiotic suspensions (Fábrega et al., 2017). In addition to immune modulation, EcN OMVs reinforce the intestinal barrier and reduce gut permeability by promoting upregulation of tight junction proteins ZO-1 and claudin-14, and downregulation of claudin-2 (Alvarez et al., 2016).

Despite these early findings that indicate a key role of bacterial vesicles in signalling processes at the intestinal mucosa, the specific molecular mechanisms and pathways involved in microbiota OMVs-host crosstalk remains largely unexplored. In this regard, vesicles are loaded with microbial-associated molecular patterns (MAMPs) including DNA, RNA, lipoproteins, LPS or peptidoglycan, which allow OMVs to interact directly with host cells via pattern recognition receptors (PRRs) and activate signalling pathways that result in cytokine/chemokine modulation (Kaparakis-Liaskos and Ferrero, 2015; Patten et al., 2017). Besides recognition of extracellular Toll-like receptors (TLRs), commensal bacteria can also signal through cytosolic NOD-like receptors (NLRs), which include two members, NOD1 and NOD2 (Natividad et al., 2012).

NOD1 is constitutively expressed in a wide variety of cell types and especially by epithelial cells in the intestinal tract, whereas NOD2 is predominantly expressed in immune cells of myeloid origin. However, low expression of NOD2 has also been reported in intestinal epithelial cells, where certain stimuli such as pro-inflammatory cytokines or infection with enteroinvasive pathogens can up-regulate its expression (Rosenstiel et al., 2003; Kim et al., 2004). NOD receptors consist of three domains: (i) an N-terminal caspase-activated recruitment domain (CARD) that allows protein-protein interactions, (ii) a central NACHT domain involved in receptor dimerization, and (iii) a C-terminal leucine-rich repeat (LRR) domain that recognizes specific ligands. Both NOD1 and NOD2 are activated by intracellular fragments of bacterial peptidoglycan (PG), thereby acting as intracellular sensors of bacterial infection. NOD1 detects D-glutamyl-meso-diaminopimelic acid (iE-DAP) present in Gram-negative PG, but also in specific groups of Gram-positive bacteria, while NOD2 detects muramyl dipeptide (MDP), which is present in all groups of bacteria. PG interaction elicits NOD oligomerization, which in turn promotes the recruitment of the downstream receptor-interacting protein 2 (RIP2), a specific kinase that triggers activation of NF-

κB and mitogen-activated protein kinase (MAPK) pathways leading to the expression of inflammatory genes. NOD receptors have essential roles not only in host responses against bacterial infection, but also in the regulation of intestinal inflammatory response to microbiota, and thus in maintaining intestinal homeostasis (reviewed in Philpott et al., 2014; Caruso et al., 2014; Kaparakis-Liaskos, 2015).

In addition to PG entry through cell infection by invasive bacteria, other intracellular PG delivery pathways have been described in non-invasive bacteria. These alternative routes include PG translocation through bacterial secretion systems, uptake by endocytosis or by specific membrane transport systems (PEPT), and delivery via OMVs (reviewed in Philpott et al., 2014; Kaparakis-Liaskos, 2015). Delivery of PG inside the host cell and further activation of NOD signalling cascades by bacterial OMVs have been studied in pathogens, specifically *Helicobacter pylori* (Kaparakis-Liaskos et al., 2010), *Vibrio cholerae* (Chatterjee and Chaudhuri 2013) and *Aggregatibacter actinomycetemcomitans* (Thay et al., 2014). OMVs from these pathogens induce an inflammatory response that is NOD1-dependent. Studies performed with *H. pylori* OMVs revealed that delivery of endocytosed PG-containing OMVs to cytosolic NOD1 is triggered by recruitment of the cytosolic receptor to early endosomes. This intracellular trafficking was shown to be essential for NOD1-PG interaction and activation of the signalling cascade that leads to nuclear NF-kB translocation (Irving et al., 2014).

Intracellular PG delivery by means of bacterial extracellular vesicles points to a plausible mechanism used by microbiota to activate NOD-mediated host immune responses. The aim of the present study was to evaluate whether OMVs from probiotic and commensal *E. coli* could activate NOD signalling pathways in intestinal epithelial cells. Here we show that in Caco-2 cells NOD1, but not NOD2, is essential for the immune responses mediated by EcN or ECOR12 OMVs. OMV-mediated activation of NF-κB and the subsequent IL-6 and IL-8 responses are significantly reduced by NOD1 silencing or RIP2 inhibition. Colocalization of endocytosed OMVs with NOD1 was confirmed by confocal fluorescence microscopy. This analysis also provides evidence of NOD aggregation and association with early endosomes in cells stimulated with EcN or ECOR12 OMVs.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The probiotic strain *Escherichia coli* Nissle 1917 (EcN) (serotype O6:K5:H1) was provided by Ardeypharm GmbH, Herdecke, Germany. ECOR12 is a commensal strain isolated from the stool of a healthy human (Ochmann and Selander, 1984). Bacterial cells were routinely grown at 37°C in Luria–Bertani broth (LB) with constant rotation (150 rpm). Growth was monitored by measuring the optical density at 600 nm.

Isolation and labelling of OMVs

OMVs were isolated from culture supernatants as described previously (Aguilera et al., 2014). Briefly, bacterial cells were grown aerobically overnight in LB and pelleted by centrifugation at 10,000×*g* for 20 min at 4°C. The supernatants were filtered through a 0.22 µm-pore-size filter (Merck Millipore) to remove residual bacteria and concentrated using a 10k Centricon® Plus-70 filter device (Merck Millipore). Vesicles were collected by centrifugation at 150,000×*g* for 1 h at 4°C in an Optima ™ L-90K ultracentrifuge (Beckman Coulter), washed twice and resuspended in an adequate volume of phosphate buffered saline (PBS) and stored at -20°C. Sterility of samples was assessed on LB plates. Protein concentration was determined by the method of (Lowry et al., 1970). The reproducibility of OMV preparations was assessed by transmission electron microscopy after negative staining and SDS-PAGE as described previously (Aguilera et al., 2014).

OMVs were fluorescently labelled with BODIPY®FL vancomycin (ThermoFisher Scientific), which selectively reacts with PG. To this end, EcN and ECOR12 OMVs (2 mg/ml) were incubated for one hour at room temperature in the presence of 4 ng/ml of BODIPY®FL vancomycin. Unbound dye was removed by washing two times in PBS. Labelled OMVs were then collected by centrifugation at 100,000*xg* for 1 hour at 4°C.

Cell culture and stimulation conditions

The human colonic cells lines Caco-2 (ATCC HTB37) and HT-29 (ATCC HTB-38) were obtained from the American Type Culture Collection. Cells were cultured in

DMEM High Glucose (Dulbecco's Modified Eagle Medium) (Life Technologies) supplemented with 10% (v/v) fetal bovine serum (FBS), 25 mM HEPES, 1% non-essential amino acids, penicillin (100 U/ml) and streptomycin (100 µg/ml) (Gibco BRL, MD, USA). Cultures were incubated at 37°C in a 5% CO_2 atmosphere. Cells were routinely sub-cultured once a week with trypsin-EDTA (0.25%, 0.53 mM) and seeded at a density of $2x10^5$ cell/ml.

For gene silencing, Caco-2 intestinal epithelial cells ($2x10^5$ cell/ml) were seeded in 12 well tissue culture plates and grown until 70% of confluence. Then, medium was replaced with antibiotic-free medium, and cells were transfected with specific NOD1 (Santa Cruz Biotechnology sc-37279) and NOD2 (Santa Cruz Biotechnology sc-43973) small interfering RNA (siRNA) following the manufacturer´s instructions. A negative control siRNA (Santa Cruz Biotechnology sc-37007) was used as reference. The transfection and medium reagent used in this study were also obtained from Santa Cruz Biotechnology. After 48 h, the culture medium was replaced with fresh DMEM and NOD1 and NOD2 knockdown Caco-2 cells were stimulated with EcN or ECOR12 OMVs, at dose of 10 µg/ml, for 8 hours. To inhibit RIP2, Caco-2 cells were treated with 1µM of RIP2 tyrosine kinase inhibitor Gefitinib (Invivogen) for 1 hour prior the addition of OMVs (10 µg/ml), and then incubated in the presence of the inhibitor for an additional 8 hours. As a positive control Caco-2 cells (control, NOD1 silenced and Gefitinib treated cells), were stimulated for 8 hours with 1 µg/ml of M-TRiDAP (MurNAc-L-Ala-gamma-D-Glu-mDAP) (Invivogen), a PG degradation product found mostly in Gram-negative bacteria that is recognized by the intracellular receptor NOD1. In all cases, supernatant and cells were collected for ELISA and quantitative RT-PCR assays, respectively.

Analysis of NF-kB activation in Caco-2 cells

To measure activation of the nuclear transcription factor NF-kB, control Caco-2 cells as well as cells treated with the RIP-2 inhibitor or transfected with NOD1 siRNA were stimulated with 10 µg/ml of EcN and ECOR12 OMVs at different timings (20, 40, 90 120, 150 and 180 minutes). Then, cells were washed with 1X PBS and suspended in lysis buffer (50 mM Tris-HCl (pH 8.0), 170 mM NaCl, 0.5% NP_4O, 50 mM NaF, 25% glycerol, 10 mg/ml proteinase A, 10 mg/ml leupeptine, 1 mM DMSF, 0.1 mM Na_3, 20 mM β-glycerolphosphate and 1mM DTT) for one hour at 4°C. Cell lysate was clarified by centrifugation at 16.000 *x g* for 30 minutes at 4°C. Protein

concentration was measured by the Bradford Protein Assay (Bio-Rad). NF-kB activation was evaluated through IkBα degradation by Western blot. Samples (100 µg protein) were mixed with SDS-PAGE sample buffer, boiled for 5 minutes at 95 °C and electrophoresed on 10% SDS-PAGE gel. Proteins were transferred to a Hybond-P polyvinylidene difluoride membrane using a Bio-Rad Mini Transblot apparatus. The membrane was blocked in PBS-0.05% Tween-20 and 5% skimmed milk (blocking solution) for 1 hour at room temperature, and incubated with specific antibodies against IkBα (Santa Cruz Biotechnology), 1:500 dilution in blocking solution overnight at 4°C, followed by incubation with peroxidase-conjugated anti-mouse IgG antibody (1:7000) for 1 hour at room temperature. The protein-antibody complex was visualized using the ECL Plus Western blotting detection system (GeHealthcare). Control of protein loading and transfer was conducted by detection of the housekeeping NEK9 protein.

Confocal fluorescence microscopy

HT-29 cells were grown in 8-well chamber slider (Ibidi) until approximately 80% confluence and incubated with EcN and ECOR12 OMVs (10 µg/well) at 37°C. When indicated cells were incubated with BODIPYL-FL labelled OMVs. After stimulation, cells were washed three times in PBS. Nuclei were labbelled with DAPI (0.125 µg/ml, Sigma Aldrich). Then, samples were fixed with 3% paraformaldehyde for 30 minutes, permeabilized with 0.05% saponin (Sigma Aldrich) and blocked using PBS containing 1% bovine serum albumin for microscopy analysis. Unlabelled OMVs were stained using anti-*E.coli* LPS mouse monoclonal antibody (Abcam®) followed by AlexaFluor 488 conjugated goat anti-mouse IgG (Molecular Probes). NOD1 receptor was detected using anti-NOD1 policlonal antibody (Thermofisher) and AlexaFluor 633 conjugated goat anti-rabbit IgG (Molecular Probes). Endosomes were detected with mouse polyclonal antibody against EEA1 (Santa Cruz Biotechnology) and Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes).

Immunofluorescence labelling of cells was analysed by confocal microscopy using a Leica TCS SP2 laser scanning confocal spectral microscope with a 63x 1.32NA oil immersion objective and an image resolution of 0.232 x 0.232 x 0.488 µm/voxel (*x, y, z*, respectively). Images were captured with a Nikon color camera (8 bit). Fluorescence was recorded at 405 nm (blue; DAPI), 488 nm (green; BODIPYL-

FL, Alexa Fluor 488), 546 nm (red; WGA) and 633 nm (far-red; Alexa Fluor 633). Images were analyzed using Fiji image processing package (Schindelin et al., 2012). Colocalization was assessed by calculating the overlap coefficient (r) from quantitative data obtained from four confocal stacks using the JaCoP plugin. The value of this coefficient ranges from 0 to 1. Data were presented as mean ± standard error from three independent experiments. In all cases, the total number of cells analyzed was between 60 and 70.

Cytokine quantification

After stimulation, culture supernatants were collected and centrifuged at 16,000 *xg* for 20 minutes at 4 °C, and stored at -80 °C until assay. Secreted IL-8 and IL-6 were quantified by enzyme-linked immunosorbent assay (ELISA) sets (BD Biosciences) according to manufacturer´s instructions. The results were expressed as pg/ml.

RNA isolation and quantitative reverse transcription PCR (RT-qPCR) Total RNA was extracted from Caco-2 cells by using the Illustra RNAspin Mini kit (GE Healthcare) following the manufacturer's protocol. Purity and RNA concentration were measured by the absorbance ratio at 260 and 280 nm in a NanoDrop® spectrophotometer. RNA integrity was verified by visualization of 28S and 18S rRNAs after 1% agarose/formaldehyde gel electrophoresis.

RNA (1 µg) was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) in a final volume of 20 µl following manufacturer's recommendations. RT-qPCR reactions were performed in a StepOne Plus PCR cycler (Applied Biosystems) by using SYBR Green PCR Master Mix (Applied Biosystems) and specific primers for human IL-6, IL-8, NOD-1 and NOD-2 (Table 1). A control reaction was performed in the absence of RNA. The $2^{-\Delta\Delta Ct}$ method was used to normalize expression results. The values of the housekeeping β-actin gene were used to normalize the values obtained for each of the genes under study.

Statistical analyses

Statistical analysis was performed using SPSS (version 20.0, Chicago, IL, USA) software package. All assays were repeated at least three independent times in

triplicate. The values for all measurements are presented as the mean ± standard error (SEM). Differences between more than two groups were assessed using one-way ANOVA followed by Tukey's test. The *p* value less than 0.05 were considered statistically significant.

Genes	Primer sequences	References
NOD1	5´-ACGATGAAGTGGCAGAGAGTT-3` 5´-GGCAGTCCCCTTAGCTGTGA-3`	Boonyanugomol et al., 2013
NOD2	5´-GAAGTACATCCGCACCGAG-3` 5´-GACACCATCCATGAGAAGACAG-3`	Sabbah et al., 2009
IL-8	5´-CTGATTTCTGCAGCTCTGTG-3` 5´-GGGTGGAAAGGTTTGGAGTATG-3`	Martirosyan et al., 2013
IL-6	5´-AGCCACTCACCTCTTCAGAAC-3` 5´-GCCTCTTTGCTGCTTTCACAC-3`	Jiang et al., 2017
β-actin	5´-GCTCTGGCTCCTAGCACCAT-3` 5´-GCCACCGATCCACACAGAGT-3`	Takahashi et al., 2011

Table 1. Primer sequences used for RT-qPCR

RESULTS

EcN and ECOR12 OMVs activate the innate inflammatory response in an NOD1 dependent manner in intestinal epithelial cells

In non-invasive pathogens, internalized OMVs allow intracellular delivery of PG to activate NOD receptors upon their recruitment to early endosomes. Whether microbiota could activate these intracellular receptors through extracellular vesicles remains unknown. Thus, we sought to analyse whether OMVs released by the commensal ECOR12 and the probiotic EcN can trigger the NOD inflammatory response in intestinal epithelial cells.

First, we analysed by RT-qPCR the expression of NOD1 and NOD2 in two intestinal epithelial cells lines, HT-29 and Caco-2. Both intracellular receptors were constitutively expressed in the two cell lines, but their relative expression level was

higher in Caco-2 cells (**Figure 1A**). It must be stressed that NOD2 expression was very low. The threshold cycle (Ct) values for NOD2 detection were around 10 times higher than those for NOD1. These results are in accordance with the expression profile reported for these receptors in several epithelial cell lines evaluated by semi-quantitative RT-PCR (Kim et al., 2004).

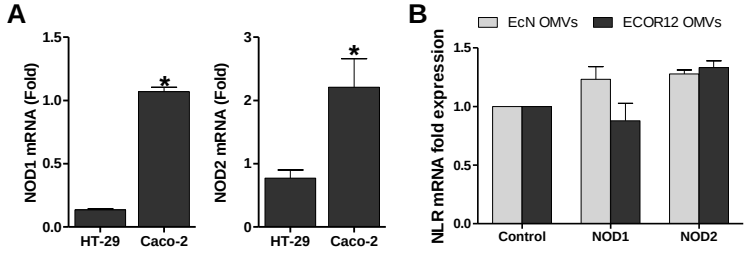

Figure 1. Expression analysis of NOD1 and NOD2 in intestinal epithelial cells. Relative mRNA levels of NOD1 and NOD2 were measured by RT-qPCR, using β-actin as the reference gene. **(A)** Relative mRNA levels of NOD1 and NOD2 in HT-29 and Caco-2 cells. Data are presented as relative expression in Caco-2 cells in comparison with mRNA levels in HT-29, which were set at 1 (n=3 independent biological replicates, **p*<0.010). **(B)** Relative mRNA levels of NOD1 and NOD2 in Caco-2 cells after 8 h-stimulation with OMVs isolated from EcN or ECOR12. Data are presented as fold-change compared to untreated control cells, whose normalized values were set as 1 and indicated by a dashed line (n=3 independent biological replicates, **p*≤0.05).

To approach functional studies, we selected Caco-2 cells as they express higher levels of intracellular NOD receptors than HT-29. In addition, we took advantage of the negligible expression of TLR4 and its co-receptor MD-2 in this cell line (Abreu et al., 2001; Kim et al., 2004). This avoids interference by the other main inflammatory pathways activated by vesicle components other than PG, such as LPS. NOD1 is constitutively expressed in intestinal epithelial cells, while NOD2 is upregulated by treatment with specific pro-inflammatory cytokines or by infection with invasive bacteria. To test whether NOD2 expression could be upregulated in intestinal epithelial cells by internalized OMVs from microbiota strains, the relative expression of both NOD receptors was assessed in Caco-2 cells challenged with EcN or ECOR12 OMVs (10 μg/ml). The basal NOD1 and NOD2 mRNA levels remained unchanged after 8 h stimulation (**Figure 1B**).

To analyze whether internalized OMVs elicit an inflammatory response through activation of NOD1 and/or NOD2 receptors, we examined OMV-induced responses in Caco-2 cells silenced for NOD1 or NOD2 expression. To this end, Caco-2 cells were transfected with siRNA specific sequences targeting these NOD receptors, or

with control scrambled siRNA (Scr). After two days, knockdown of NOD1 or NOD2 gene expression in these cells was assessed by qRT-PCR. The levels of NOD1 and NOD2 mRNA were significantly decreased by 50 and 55%, respectively, compared with scrambled transfected cells (**Supplementary Figure S1**). Then, transfected cells were stimulated for 8 h with OMVs from EcN or ECOR12, and the expression of IL-8 and IL-6 was quantified by RT-qPCR. Both bacterial vesicles induced significant levels of IL-6 and IL-8 mRNA expression in control cells (transfected with Scr siRNA). NOD2 silencing did not impair OMV-mediated upregulation of either IL-6 or IL-8, as mRNA levels remained similar to those of control Scr-transfected cells (**Figure 2**).

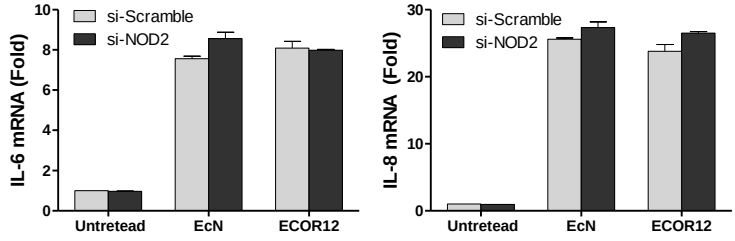

Figure 2. NOD2 is not essential for the OMV-mediated activation of IL-6 and IL-8 expression in Caco-2 cells. Caco-2 cells were transfected with siRNA against NOD2 or with control scrambled siRNA (Scr) as a control. After 48 h, cells were stimulated with OMVs (10 µg/ml) isolated from EcN or ECOR12 strains or left non-stimulated. Relative mRNA levels of the pro-inflammatory cytokines IL-6 and IL-8 were measured after 8 h by RT-qPCR, using β-actin as the reference gene. Data are presented as the mean ± SEM and expressed as fold-change compared to non-stimulated Scr-control cells (n=3 independent biological replicates).

In contrast, NOD1 siRNA-transfected cells displayed significantly reduced mRNA levels of IL-6 (7-fold decrease) and IL-8 (2-fold decrease) in response to OMV stimulation (**Figure 3A**). In NOD1-knockdown cells the observed changes in cytokine mRNA expression correlated with the secreted protein levels, as evidenced from the quantitative ELISA results (**Figure 3B**). In response to OMVs, secreted IL-6 and IL-8 levels were lower in NOD1-silenced cells than in Scr-control cells; although for IL-8 the results did not reach statistical significance (**Figure 3B**). Stimulations with Tri-DAP, a cognate activator of the NOD1 signaling pathway, were performed in parallel to assess functional NOD1 activity in these cells. Consistently, IL-6 and IL-8 expression was significantly reduced by NOD1-specific siRNA treatment at both mRNA and protein levels (**Figure 3A,B**). Overall, these

results indicate that NOD1, but not NOD2, is involved in the IL-6 and IL-8 responses triggered by internalized EcN or ECOR12 OMVs in intestinal epithelial cells, although NOD1-independent pathways also contribute to IL-8 production.

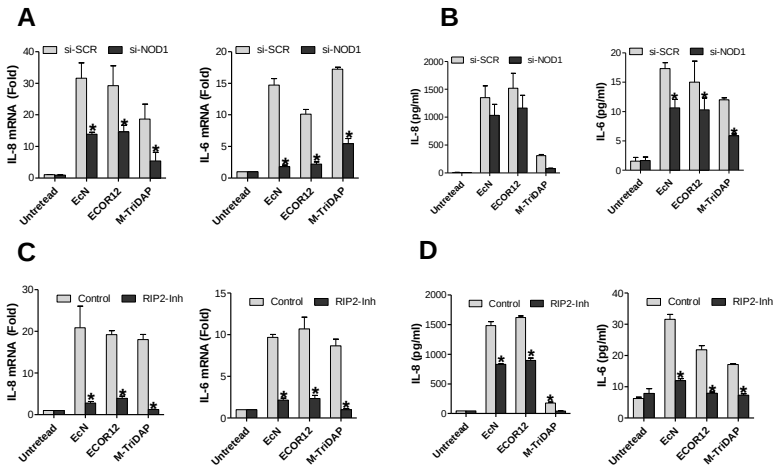

Figure 3. EcN and ECOR12 OMVs activate IL-6 and IL-8 expression in intestinal epithelial cells in a NOD1- and RIP2-dependent manner. Caco-2 cells were stimulated for 8 hours with OMVs (10 μg/ml) isolated from EcN or ECOR12 strains or with M-TriDAP (1 μg/ml) as a positive control of NOD1 activation. Control cells were left unstimulated for comparison. **(A-B)** Involvement of NOD1 in the OMV-mediated activation of IL-6 and IL-8 responses was assessed in NOD1-silenced cells. Two days before the stimulation with OMVs or with M-TriDAP, Caco-2 cells were transfected with siRNA against NOD1 or with control scrambled siRNA (Scr). Expression of these cytokines was quantified after 8 h incubation: **(A)** relative mRNA levels of IL-6 and IL-8 were measured by RT-qPCR, using β-actin as the reference gene, and **(B)** secreted IL-6 and IL-8 were quantified by ELISA. **(C-D)** Requirement of RIP2 in the OMV-mediated activation of IL-6 and IL-8 responses was assessed in Caco-2 cells treated with the RIP2 inhibitor Gefitinib. Prior to the addition of OMVs or M-TriDAP, cells were pre-treated with Gefitinib (1 μM) for 1 hour. Expression of IL-6 and IL-8 was quantified by **(C)** RT-qPCR and **(D)** ELISA as described for NOD1-knockdown cells. In all panels data are presented as mean ±SEM (n=3 independent biological replicates,* $p \leq 0.05$, *versus* non-stimulated controls). Relative mRNA levels (panels A, C) are expressed as fold-change compared to non-stimulated controls.

As RIP2 specifically mediates pro-inflammatory signalling from NOD1 bacterial sensors (**Parker et al., 2007**), we next sought to analyse IL-6 and IL-8 production in response to microbiota OMVs in Caco-2 cells treated with the RIP2 inhibitor Gefitinib. To this end, cells were pre-treated with Gefitinib (1μM) prior to the addition of EcN or ECOR12 OMVs, and the levels of IL-6 and IL-8 were measured after 8 h-incubation. Inhibition of RIP2 significantly reduced the expression and secretion of both pro-inflammatory cytokines in response to OMVs in Caco-2 cells compared to OMV-stimulated controls (**Figure 3 C,D**). These findings prove the

requirement of the RIP2 kinase in the inflammatory response to internalized microbiota OMVs, and therefore confirm the ability of microbiota OMVs to activate intracellular NOD1 and the downstream signalling pathway.

EcN and ECOR12 OMVs activate NK-κB through the NOD1-RIP2 pathway

Activation of RIP2 results in the phosphorylation and subsequent degradation of IκBα, which in turn allows activation and translocation of the NK-κB complex into the nucleus, where it enables the transcription of pro-inflammatory genes.

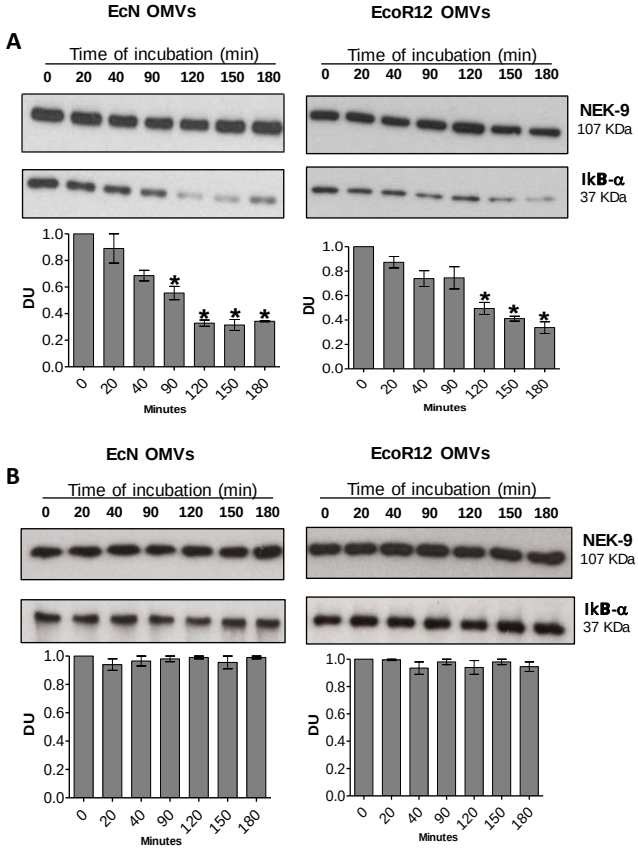

Figure 4. Inhibition of RIP2 impairs OMVs-induced NF-κB activation in Caco-2 cells. Activation of NF-κB was assayed by means of IκBα degradation. **(A)** Caco-2 cells were stimulated with EcN or ECOR12 OMVs (10 μg/ml) for 20, 40, 90, 120, 150 and 180 min, and IκBα levels were assessed by Western blot. For normalization, blots were probed with an anti-NEK-9 antibody. **(B)** Parallel stimulations were performed in Caco-2 cells treated with the RIP2 inhibitor Gentinib (1 μM) for comparison. Densitometric quantification of IκBα was performed with samples from three independent experiments. Representative immunoblots were shown. Normalized values from untreated control cells (time 0 min) were set as 1. Significance against untreated control cells (*$p \leq 0.05$)

To confirm activation of NK-κB in cells challenged with OMVs, levels of IκBα were assessed by Western blot at different post-treatment times. Stimulation of Caco-2 cells with EcN or ECOR12 OMVs caused significant degradation of IκBα. The time kinetics of IκBα degradation was somewhat slower for ECOR12 OMVs (**Figure 4**). Degradation of IκBα was not observed when cells were either transfected with NOD1 siRNA or treated with the RIP2 inhibitor Gefitinib (**Figure 4**). These data confirm that OMVs released by *E. coli* microbiota strains activate the NK-κB pathway in an NOD1-dependent manner.

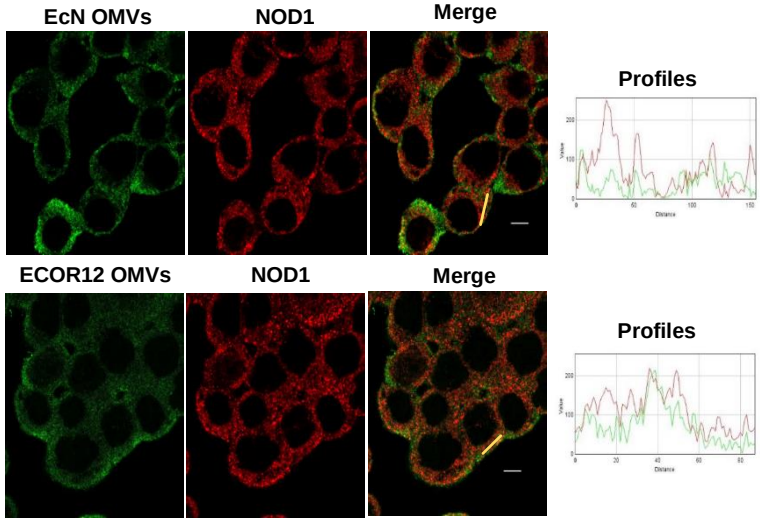

Figure 5. NOD1 colocalizes with EcN and ECOR12 OMVs. HT-29 cells were incubated with EcN or ECOR12 OMVs (10 μg) for 1 hour and analyzed using laser scanning confocal spectral microscope. NOD1 was stained using anti-NOD1 policlonal antibody and AlexaFluor 633-conjugated goat anti-rabbit IgG (red). OMVs were stained using anti-*E.coli* LPS mouse monoclonal antibody followed by AlexaFluor 488-conjugated goat anti-mouse IgG (green). Images are from a single representative experiment (n=3). Colocalization of the red and green signals was confirmed by histogram analysis of the fluorescence intensities along the yellow lines. Analysis was performed by laser scanning confocal spectral microscope with 63x oil immersion objective lens, and images were captured with a Nikon color camera (8 bit). Scale bar: 10 μm.

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EcN and ECOR12 OMVs associate with NOD1 and trigger its aggregation

NOD1 detection of PG contained in Gram-negative derived OMVs is required for activation of the downstream signalling pathway. Thus, association of NOD1 with OMVs from EcN and ECOR12 was evaluated by means of confocal fluorescence microscopy. OMVs were visualized by immunostaining with anti-*E. coli* LPS and

Alexa Fluor 546-conjugated anti-mouse IgG antibodies. After 1 h post-stimulation, OMVs from both strains colocalized with NOD1 in HT-29 cells (**Figure 5**). The degree of colocalization was estimated from the corresponding overlap coefficients (r), with values of 0.51 ± 0.03 for EcN and 0.48 ± 0.01 for ECOR12 vesicles. Colocalization of internalized vesicles with NOD1 was confirmed through experiments performed with HT-29 cells incubated with EcN OMVs fluorescently labelled with BODIPY®FL vancomycin (**Supplementary Figure S2**)

It is well-known that direct binding of PG ligand to NOD1 triggers self-association of the receptor via the NACHT domain. This is a key step for the recruitment of RIP2 to initiate the phosphorylation cascade that results in the assembly of large signalling complexes. To address whether microbiota OMVs induce changes in the NOD1 oligomerization state, confocal fluorescence microscopy analysis of NOD1 was performed in HT-29 at different post-incubation times (20, 60, 90 and 180 min). Non-stimulated cells were processed in parallel as a control. These analyses revealed that upon OMVs stimulation, NOD1 formed aggregates in the cell cytoplasm in a time-dependent manner, which may arise by oligomerization upon NOD1 activation (**Figure 6**).

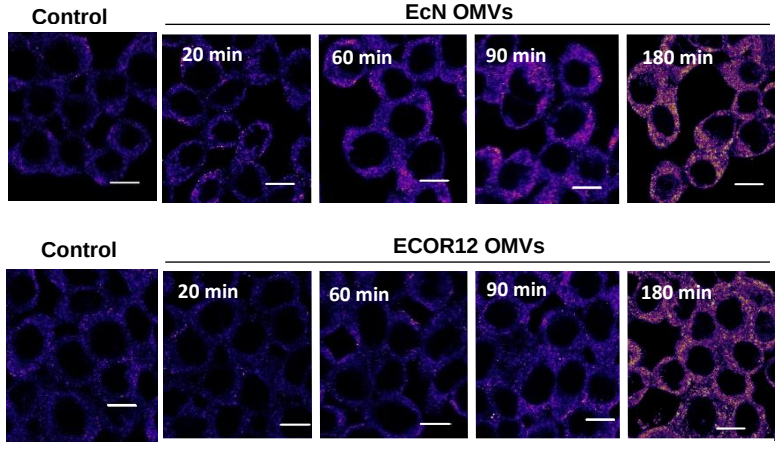

Figure 6. Time-course analysis of NOD1 aggregation pattern in HT-29 cells in response to EcN or ECOR12 OMVs. HT-29 cells were incubated with EcN or ECOR12 OMVs (10 µg) for the indicated times and analyzed using laser scanning confocal spectral microscope. NOD1 was stained using anti-NOD1 policlonal antibody and AlexaFluor 633-conjugated goat anti-rabbit IgG. Images are from a single representative experiment (n=3) and are presented in color-code Fire. Analysis was performed as described for Figure 5. Scale bar: 10 µm.

Remarkably, NOD1 activation timing profiles differed between OMVs. The pattern of NOD1 aggregation was already apparent at 20 min post-stimulation with

EcN OMVs, and gradually increased up to 180 min. However, changes in the NOD1 fluorescence signal pattern were delayed in cells treated with ECOR12 OMVs. At 180 min, both OMVs elicited a similar NOD1 aggregation level. These results are consistent with the different IκBα degradation profile observed in Caco-2 cells in response to these *E. coli* derived OMVs.

NOD1 is recruited to early endosomes by EcN and ECOR12 OMVs

It has been described that NOD1 detection of OMV-internalized PG takes place at early endosomes (Irving et al., 2014). In this context, we have previously shown that EcN and ECOR12 OMVs are sorted to early endosomes during their intracellular trafficking through clathrin-mediated endocytosis (Cañas et al., 2016).

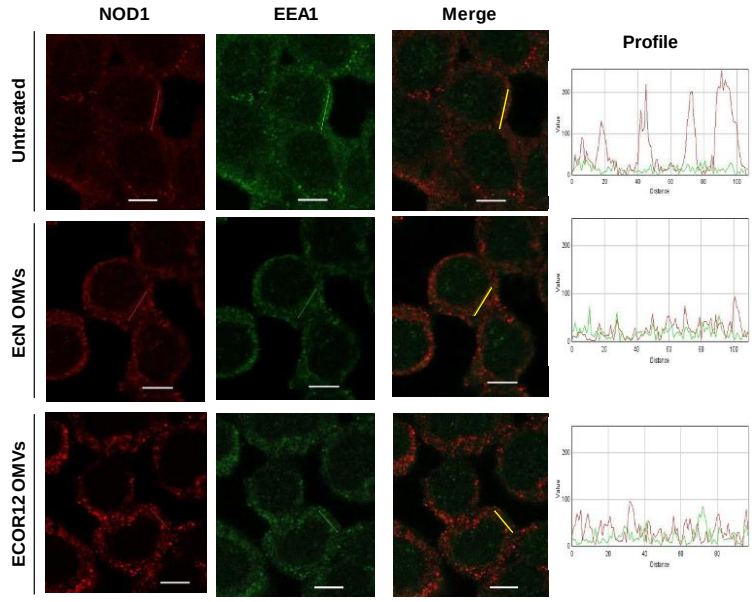

Figure 7. NOD1 colocalizes with EEA1-labelled endosomes in cells stimulated with EcN or ECOR 12 OMVs. HT-29 cells were incubated with EcN or ECOR12 OMVs (10 µg) for 1 hour and colocalization of EEA1/NOD1 **(A)** and EEA1/OMVs **(B)** was analyzed using laser scanning confocal spectral microscope. NOD1 was stained using anti-NOD1 policlonal antibody and AlexaFluor 633-conjugated goat anti-rabbit IgG (red). Endosomes were detected with mouse polyclonal antibody against EEA1 and Alexa Fluor 488-conjugated goat anti-rabbit IgG (green). Images are from a single representative experiment (n=3). Colocalization of the red and green signals was confirmed by histogram analysis of the fluorescence intensities along the yellow lines. Analysis was performed as described for Figure 5. Scale bar: 10 µm.

To assess whether NOD1 could be recruited to early endosomes by internalized microbiota OMVs, colocalization of NOD1 with the endosome-associated EEA1

protein was analysed by confocal fluorescence microscopy in HT-29 cells at 1 h post-stimulation. Non-treated control cells were analysed in parallel for comparison. Colocalization of NOD1 with the specific endosome marker was only observed in OMVs stimulated cells (**Figure 7**). No yellow spots were apparent in the merged images collected from control cells. In stimulated cells, the presence of OMVs in early endosomes was confirmed by their colocalization with EEA1 (not shown). Quantitative analysis revealed similar overlapping coefficient values for colocalizations of EEA1/NOD1 ($r = 0.38 \pm 0.03$ in EcN OMV-treated cells, and $r = 0.34 \pm 0.01$ in ECOR12 OMV-treated cells) and EEA1/LPS-OMVs ($r = 0.44 \pm 0.08$ for EcN OMVs, and $r = 0.39 \pm 0.04$ for ECOR12 OMVs). These results prove that NOD1 detection of PG internalized via microbiota OMVs also depends on its recruitment to early endosomes.

DISCUSSION

It is well-known that intestinal microbiota releases many regulatory signals that modulate the maturation and function of the intestinal immune system. To house this huge microbial population, the intestinal tract needs barrier and regulatory mechanisms to control reciprocal interactions between microbiota, the epithelium and the mucosal immune system, preventing aberrant responses and preserving homeostasis. In the gut, crosstalk between microbiota and the host mainly depends on secreted factors that can diffuse across the mucus layer and gain access to the epithelium. Among bacterial secreted factors, membrane vesicles play a key role in bacteria-host communication, allowing the delivery of effector molecules upon interaction and internalization into the host cells. OMVs released by Gram-negative bacteria enclose many ligands of pattern recognition receptors such as LPS (TLR4 ligand), PG (NOD1/NOD2 ligand) or DNA (TLR9 ligand). These receptors, which are expressed in epithelial and immune cells, are key components of innate immunity as they sense gut microbes and trigger appropriate immune responses.

NOD1 and NOD2 cytosolic receptors detect PG, a component of the cell wall of Gram-negative and Gram-positive bacteria. Many studies have shown the pivotal role of these immune receptors in defence against bacterial infections and in the modulation of host inflammatory responses (Caruso et al., 2014; Philpott et al.,

2014). In addition to this function as sensors of bacterial pathogens, recent contributions evidenced that NODs are also fundamental to maintain intestinal homeostasis and microbiota balance. In fact, mutations that affect NOD2 expression or activity have been associated with multiple chronic inflammatory and autoimmune diseases (Feerick and McKernan, 2016). In this context, boosting the stimulation of NOD receptors by microbiota has been proposed as a mechanism to ensure gut homeostasis by enhancing innate immunity (Clarke et al., 2010). Nevertheless, how gut microbiota, which is composed of non-invasive bacteria, can deliver PG into host cells is a rather unexplored issue. A plausible pathway is that PG fragments released into the intestinal lumen during bacterial cell division are internalized by epithelial cells through endocytosis or by oligopeptide transporters such as PEPT1 or PEPT2 (Swaan et al., 2008; Philpott et al., 2014). NOD-activating PG products have been found in *E. coli* culture supernatants (Pradipta et al., 2010), and PG fragments released by *L. salivarius* protect colitis progression in mice by upregulating IL-10 (Macho-Fernandez et al., 2011).

Another pathway for intracellular PG delivery is through bacterial vesicles. This a well-studied mechanism in Gram-negative pathogens such as *H. pylori*, *V. cholerae* and *A. actinomycetemcomitans* (Kaparakis-Lliaskos et al., 2010; Bielig et al., 2011; Thay et al., 2014). Studies performed with *H. pylori* revealed that internalized OMVs reach endosomal compartments, and that interaction with OMV-PG takes place at early endosomes (Irving et. al., 2014). With this information, we hypothesized that microbiota-released OMVs could mediate immunomodulatory effects through NOD activation by their PG. Previous contributions of our group showed that OMVs from commensal and probiotic *E. coli* strains are internalized in intestinal epithelial cells via clathrin-mediated endocytosis (Cañas et al., 2016) and elicit immunomodulatory and barrier reinforcement responses (Fábrega et al., 2016; Alvarez et al., 2016). Several vesicle cargo molecules and MAMPs can account for these effects, thus involving a number of immune receptors and signalling pathways. To evaluate involvement of the NOD signalling pathways in the immune response triggered by commensal *E. coli* strains we used the epithelial cell line Caco-2, which poorly responds to LPS due to low expression of TLR4 and their co-receptor MD-2 (Abreu et al., 2001; Kim et al., 2004).

Our results show that OMVs from the commensal ECOR12 and the probiotic EcN induce an innate immune response in Caco-2 cells by activating NOD1-

signalling cascades, which lead to activation of NF-κB and secretion of pro-inflammatory cytokines IL-6 and IL-8. Several factors led us to rule out the involvement of NOD2 in these responses: (i) intestinal epithelial cells express very low levels of NOD2, (ii) NOD2 expression is not upregulated by microbiota *E. coli* OMVs, and (iii) NOD2 silencing did not affect OMV-activated IL-6 and IL-8 responses. In contrast, NOD1 silencing and RIP2 inhibition significantly decreased expression of these pro-inflammatory cytokines at both mRNA and protein levels. Interestingly, EcN and ECOR12 OMVs elicited greater activation of IL-8 than the specific NOD1 ligand Tri-DAP (used at 1 μg/ml), thus indicating the relative contribution of MAMPs other than PG to OMV-mediated activation of IL-8 secretion. Activation by OMVs of other signalling pathways that lead to IL-8 expression may explain why IL-8 secreted levels were not significantly reduced by treatments that interfere with NOD1 levels (NOD1-kockdown cells) or RIP2 activity (Gefitinib-treated cells).

In cells stimulated with EcN or ECOR12 OMVs, NOD1-mediated activation of NF-κB was confirmed by measuring IκBα degradation. As expected, both OMVs triggered degradation of this IκB inhibitory protein, and the effect was not observed in RIP2 inhibited cells. Interestingly, both OMVs triggered IκBα degradation with different kinetics, which were faster for EcN OMVs. In cells treated with vesicles from the probiotic EcN, a reduction in IκBα levels was apparent at 40 min post-stimulation, with values that were statistically significant at 120 min and later times. However, vesicles from the commensal ECOR12 did not cause any significant changes in IκBα levels until 120 min. At 120 min, IκBα levels were reduced by more than 60% in cells treated with EcN OMVs, but by around 40% in cells treated with ECOR12 OMVs. At 180 min, no differences in IκBα levels were observed between EcN- or ECOR12-treated cells. The different IκBα degradation timing profile was compatible with the kinetics of NOD1 aggregation in OMVs-treated cells. NOD1 aggregation may be used as a sign of NOD1 activation that leads to NOD1 oligomerization and recruitment of other proteins to form large signalling complexes (Sorbara et al., 2013; Irving et al., 2014). Analysis by confocal fluorescence microscopy evidenced that the kinetics of NOD1 aggregation in response to internalized ECOR12 OMVs was slower than that triggered by EcN OMVs. Up to 90 min, the smaller number of NOD1 activated complexes observed

in the cytosol of ECOR12-treated cells correlated well with the lower IκBα degradation ratios estimated by Western blot at the same incubation times.

The intracellular compartment where the PG contained in OMVs internalized via clathrin-mediated endocytosis interacts with cytosolic NOD1 was elucidated through an elegant study performed with *H. pylori* OMVs. Results from this study revealed that such interaction takes place at the membrane of early endosomes, where NOD1 and RIP2 are recruited (Irving et al., 2014). Regarding microbiota-derived OMVs, confocal fluorescence microscopy analysis performed at 60 min post-stimulation confirmed that OMVs from both EcN and ECOR12 colocalize with the endosome marker EEA1 and with NOD1. At this incubation time, NOD1 was found associated with early endosomes only in OMVs-treated cells. The overlap coefficients calculated for the colocalization of OMVs with EEA1 match those reported previously at 30 min (Cañas et al., 2016), and are compatible with the transient location of vesicles in early endosomes during their trafficking to lysosomes. Similar overlapping coefficient values were obtained here for EEA1/NOD1 signals, which is consistent with the fact that NOD1 associates to early endosomes to interact with PG contained in OMVs.

It is known that NOD receptors recognize muramyl peptides derived from bacterial PG. These active degradation products can be generated by the action of amidases or lytic enzymes either of bacterial (muramidases) or from mammalian origin (lysozyme type enzymes) (Girardin et al., 2003). The study carried out with *H. pylori* OMVs did not reveal how NOD1-activating peptides are generated from PG-OMVs and how they are presented to NOD1 (Irving et al., 2014). In this sense, both endosomal enzymes and the acidic pH of endosomes (pH 5.5-6) seem to be essential for the processing of NOD1-activating peptides derived from bacterial PG (Lee et al., 2009). In addition, bacterial OMVs contain PG hydrolytic enzymes that can account for the release of muramyl peptides (reviewed in Jan, 2017). Enzymatic generation of these peptides into the endosomal compartment, together with the presence of a transport system for oligopeptides in the endosome membrane (SLC15A4), which acts as a gateway for the release of these active peptides to the cytosol (Lee et al., 2009), may help NOD1 interaction and its activation to initiate the phosphorylation cascade that leads to activation of NF-κB.

Clearly, the formation and processing of NOD1-activating muramyl peptides from internalized OMVs require bacterial and/or endosomal enzymes. Thus,

differences in the OMVs cargo or in the envelope vesicular structure may influence the availability of active muramyl peptides. As stated above, vesicles from the probiotic EcN and the commensal ECOR12 elicited similar IL-6 and IL-8 responses, although they differed in their initial kinetics of NOD1 and NF-kB activation. We may speculate that these differences could be attributed to different vesicular cargo, especially in the content of PG-degrading enzymes. OMVs from the probiotic EcN contain several lytic murein transglycosylases such as MtlA, MtlB and EmtA (Aguilera et al., 2014), but no information is available so far on the ECOR12 OMV-proteome. Other factors related with the vesicular envelope may also contribute to the different activating profile. For example, the probiotic EcN contains a K5 polysaccharide capsule that is absent in the commensal ECOR12. The presence of this capsule on the surface of EcN OMVs may influence the action of PG processing enzymes. The faster the active PG-derived peptides are produced the less time is needed for NOD1 activation.

CONCLUSION

In conclusion we provide evidence that OMVs released by beneficial gut bacteria provide a mechanism for PG delivery into the host cytosol, thus allowing the sensing of microbial products that steadily prime the innate immune system. By this mechanism microbiota OMVs contribute to the tolerogenic responses essential to maintaining intestinal homeostasis.

ABBREVIATIONS

DMEM: Dulbecco's modified Eagle medium, DSS: dextran sulphate sodium, DTT: dithiothreitol, EcN: *Escherichia coli* Nissle 1917, FCS: fetal calf serum, LB: Luria-Bertani broth, LPS: lipopolysaccharide, MAMPs: microbial-associated molecular patterns, NLR: NOD-like receptor, NOD: nucleotide oligomerization domain, OMVs: outer membrane vesicles, PAGE: polyacrylamide gel electrophoresis, PBS: phosphate buffer saline, PG: peptidoglycan; PRR: pattern recognition receptor, RIP2: receptor-interacting protein 2, RT-qPCR: quantitative reverse transcription PCR, SDS: sodium dodecyl sulphate, TLR- Toll-like receptor, ZO: zonula occludens.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

LB and JB conceived of the study with the participation of RG, MAC and MJF in experimental design. LB and JB wrote the manuscript and supervised the work. RG, MAC and MJF carried out data interpretation and statistical analysis. MAC and MJF performed the experimental work. All authors revised, read and approved the final manuscript.

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SUPPLEMENTAL MATERIAL

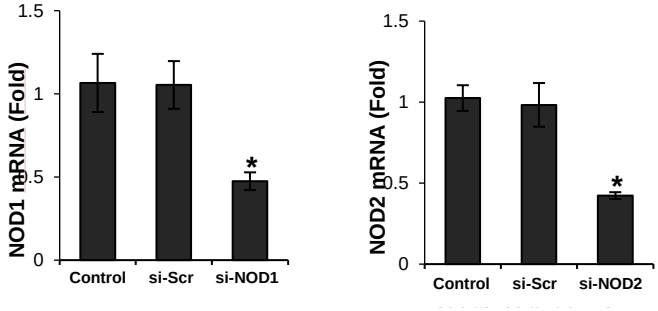

Figure S1. Quantitative PCR of NOD1 and NOD2 mRNAs in Caco-2 cells transfected with siRNA specific sequences targeting these NOD receptors, or with control scrambled siRNA (Scr).

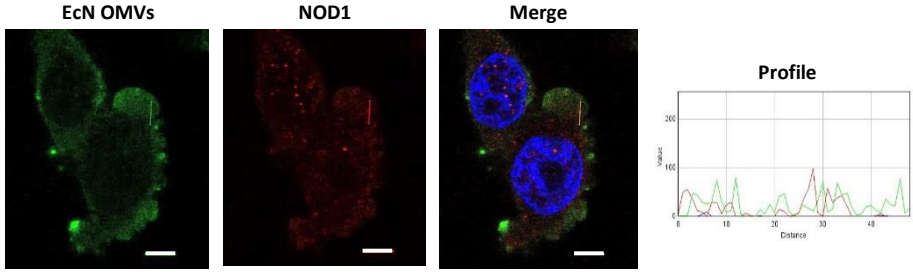

Figure S2. Colocalization of EcN OMVs with NOD1. HT-29 cells were incubated with BODIPY®FL-labelled OMVs for 1h and analyzed using laser scanning confocal spectral microscope. NOD1 was stained using anti-NOD1 anti-NOD1 policlonal antibody and AlexaFluor 633-conjugated goat anti-rabbit IgG (red) and nuclei with DAPI (blue). Images are from a single representative experiment (n=3). Colocalization of the green (PG-OMVs) and red (NOD1) signals was confirmed by histogram analysis of the fluorescence intensities along the yellow line. Analysis was performed by laser scanning confocal spectral microscope with 63x oil immersion objective lens, and images were captured with a Nikon color camera (8 bit). Scale bar: 10 µm.

6. CHAPTER 4

Outer membrane vesicles and soluble factors secreted by probiotic and commensal *E. coli* strains: effect on *Salmonella enterica* serovar Typhimurium and *Shigella flexneri* infection.

RESUMEN

En los últimos años, numerosos estudios científicos han demostrado que la microbiota intestinal, la cual incluye cepas probióticas y comensales, juegan un papel crucial en la resistencia del huésped frente a patógenos intestinales. La cepa probiótica *Escherichia coli* Nissle 1917 (EcN) ha sido ampliamente estudiada como antagonista de patógenos entéricos. Entre ellos se encuentran diferentes especies de *Salmonella,* si bien, aún existe un consenso común sobre cuál es el mecanismo de acción. En este trabajo, hemos analizado los efectos de factores secretados por EcN en la infección por *Salmonella* y *Shigella*, dos bacterias patógenas con alto impacto en la salud a nivel mundial. En paralelo, otras cepas no patógenas de *E. coli* (ECOR12, ECOR63, DH5α y MG1655) fueron usadas para su comparación. Los resultados demostraron que los sobrenadantes de *E. coli*, tanto de la cepa probiótica, como de las cepas comensales, reducen la invasión de *Salmonella*, sin afectar su adhesión epitelial o su crecimiento. También hemos demostrado que este efecto no es debido a vesículas de membrana externa (OMVs) liberadas por EcN, pero sí a otros componentes secretados. Usando tratamientos enzimáticos, confirmamos que estos factores activos secretados, no son proteínas, ni moléculas de ADN ni RNA. También observamos que los sobrenadantes de EcN reducen ligeramente la secreción de proteínas de invasión de *Salmonella*. Además, los efectos de OMVs de EcN también fueron analizados en un modelo de infección por *Shigella*, usando células epiteliales intestinales. Sin embargo, al igual que para *Salmonella*, en la infección por *Shigella*, las OMVs de EcN no fueron capaces de reducir el número de bacterias internalizadas. En conclusión, este trabajo sugiere que factores secretados por EcN podrían disminuir la invasión de *Salmonella* mediante la interferencia con el sistema de secreción T3SS, o bien modulando la expresión de genes reguladores implicados en la invasión de *Salmonella*. Este efecto parece no ser específico de EcN, pero sí común a otras cepas no patogénicas de *E. coli*. Más estudios son necesarios para confirmar la naturaleza de estos compuestos secretados y el mecanismo de acción frente a la invasión por patógenos bacterianos.

PRESENTATION

Part of the results presented in this thesis chapter comes from the work carried out during a four-month stay at the Institute of Biological and Molecular Infections (IMIB) at Würzburg (Germany), under the supervision of Dra Ana Eulalio. This group has wide experience in infection models of several enteric pathogens, such as *Salmonella*, *Shigella* and *Listeria*. Previous studies using the probiotic *Escherichia coli* Nissle 1917 (EcN), performed by the group of Dr. Oelschlägel, also located within the IMIB, showed the ability of this probiotic to inhibit *Salmonella* infection. The effect was attributed to secreted factors released by EcN as it was observed in the absence of direct contact between the probiotic and the intestinal epithelial cells (IECs) (Altenhoefer *et al*., 2004). This observation encouraged and prompted us to study whether this inhibitory effect could be due to EcN OMVs or alternatively to factors secreted in a vesicle-independent manner. This was in fact the aim of the research developed during this short stay abroad. In addition, the project was completed studying the effect of EcN OMVs on a *Shigella* infection model.

On the other hand, the group of Dra Ana Eulalio is one of the most international and powerful groups focus on the study of microRNAs (miRNAs) modulated by bacterial enteric pathogens during infection. In this work context, and during the period of the stay, I also had the opportunity to learn the methodology to study miRNAs. This knowledge was applied to evaluate the ability of EcN OMVs to regulate the expression of some inflammatory miRNAs, involved also in *Salmonella* infection.

The work performed during this short stay was continued and extended in our laboratory at the University of Barcelona, where the *Salmonella* infection model was replicated in other epithelial cell lines and extended to several non-pathogenic *E. coli* strains. In addition, experiments aimed at analysing the ability of EcN supernatants to interfere with the T3SS-mediated secretion of *Salmonella* invasion proteins were also approached in our laboratory.

Outer membrane vesicles and soluble factors secreted by probiotic and commensal *E. coli* strains: effect on *Salmonella enterica* serovar Typhimurium and *Shigella flexneri* infection

ABSTRACT

In the last years, increasing evidences have demonstrated that the gut microbiota, which includes probiotic and commensal strains, play a crucial role in host resistance against invading pathogens within the intestine. The Gram-negative probiotic *Escherichia coli* Nissle 1917 (EcN) has been widely studied as antagonist of enteric pathogens such as *Salmonella* spp., albeit no consensus has been reached on what the mechanism of action is. In this work we analysed the effect of secreted factors released by EcN on *Salmonella* and *Shigella* infection, two pathogenic bacteria with high impact on health society. In parallel, other non-pathogenic *E. coli* strains (ECOR12, ECOR63, DH5α and MG1655) were used for comparison. Results showed that *E. coli* supernatants from both probiotic and commensal strains reduce *Salmonella* invasion, without affecting *Salmonella* epithelial cell-adhesion or growth. We demonstrated that this effect is not due to outer membrane vesicles (OMVs) released by EcN, but to other secreted components. Enzymatic treatment confirmed that the active secreted factor is not a protein, DNA or RNA molecules. We also observed that EcN supernatant slightly reduces secretion of invasion *Salmonella* proteins. Moreover, the effect of EcN OMVs on *Shigella* infection was also analysed in intestinal epithelial cells. As for *Salmonella* infection, EcN OMVs did not reduce the number of internalized *Shigella* counts. In conclusion, this work suggests that EcN secreted factors might diminish *Salmonella* invasion by interfering with the T3SS secretion system or by modulating the expression of regulatory genes involved in *Salmonella* invasion. This effect seems not to be EcN specific but common to non-pathogenic *E. coli* strains. More studies are necessary to confirm the nature of these secreted compounds and the mechanism of action against pathogen bacterial invasion.

Keywords: *Probiotics, Salmonella, Invasion, EcN, OMVs.*

INTRODUCTION

Salmonella and *Shigella* genus are classified as members of *Enterobacteriaceae* family. These pathogens are responsible for significant morbidity and mortality worldwide, producing diarrhoea and a big range of associated symptoms. In human disease, the genus *Salmonella* is well-characterized; being food-borne acute gastroenteritis and enterocolitis the most common forms of *Salmonella* infection. On the other hand, shigellosis is a disease with a high infectivity rate. A minimum inoculum can cause shigellosis, and outbreaks are likely to occur because the natural reservoirs are mainly humans (Dekker and Frank, 2015).

Traditional treatments for salmonellosis and shigellosis include antibiotics. However, antibiotic consumption is a non-specific treatment, which also destroys commensal microbiota in the gut. This is also associated with a weakening of the immune system. Moreover, the use of antibiotics increases the risk of antimicrobial drug resistance by pathogenic bacteria. Thus, strains of *Salmonella* spp. with resistance to antibiotics are now widespread in both developed and developing countries (Threlfall, 2002).

In the last few years, the use of probiotic bacteria has been explored as an alternative to antibiotics for salmonellosis treatment. Remarkably, the Gram-negative probiotic *Escherichia coli* Nissle 1917 (EcN) is among these probiotics. This bacterium was isolated in 1917 by Dr. Alfred Nissle from stools of a soldier who survived an outbreak of shigellosis. Probiotic EcN is a good colonizer of the human gut with proven therapeutic efficacy in the remission of UC in humans. Moreover, it displays indirect antagonistic effects that control invasion of gut epithelial cells by enteroinvasive pathogens such as *Salmonella* (Sonnenborn, 2016).

Numerous studies performed with *Salmonella* and EcN strains have been performed trying to elucidate the mechanisms by which EcN exerts its action. In this context, it has been described that preincubation of intestinal epithelial cells (IECs) with EcN reduces both *Salmonella dublin*-induced cell death and IL-8 secretion (Otte and Podolsky, 2004). Additionally, *in vivo* experiments showed that EcN is a good colonizer of piglet's intestine. Experiments performed in porcine IECs revealed that EcN also reduces the invasion of *Salmonella enterica* (Kleta *et al*., 2006). A study carried out later in gnotobiotic piglets demonstrated that colonization with EcN interferes with translocation of *Salmonella enterica* serovar

Typhimurium into mesenteric lymph nodes or systemic circulation and ameliorates the clinical condition by decreasing ileum and plasma TNF-α levels (Splichalova *et al*., 2011). Two other studies suggested that EcN reduces intestinal *Salmonella* colonization by competing for iron, a limiting nutrient which is essential for *Salmonella* growth (Deriu *et al*., 2013; Weiss, 2013). Interestingly, and in agreement with previous reports, a study carried out in an *in vitro* 3-D culture model (which is closer to real host-microbial interactions) showed that EcN is effective inhibiting *Salmonella* adhesion (Costello *et al*., 2015). Recently, one study done in mice revealed that EcN limits the growth, expansion and colonization of *Salmonella* through release of microcins (Sassone-Corsi *et al*., 2016). Linked with this information, an *in vivo* study in turkeys reported the ability of a genetically modified EcN strain that expressed the antimicrobial microcin J25 to reduce *Salmonella* colonization and growth (Forkus *et al*., 2017). All these scientific contributions have been achieved analysing the effect of live probiotic suspensions.

Direct competition between EcN and *Salmonella* for adhesion to IECs is a key factor that reduces *Salmonella* invasion efficacy. EcN factors that mediate this effect are the F1C fimbriae and flagella (Schierack *et al*., 2011). In addition to this inhibitory effect on *Salmonella* infection, which is dependent on the physical presence of EcN bacteria, some studies show that probiotic secreted factors, released into the culture medium also contribute to diminish *Salmonella* infection ratio. In this context, a study using HeLa cells demonstrated that EcN supernatants (SN) were capable of diminishing *Salmonella enterica* invasion. The use of EcN mutants allowed to stablish that this inhibitory activity is not associated to EcN microcins nor to medium acidification, but to secreted components released by the probiotic that does not require direct contact with the epithelial cells (Altenhoefer *et al*., 2004). A new research brought to light a possible mechanism by which EcN affect *Salmonella* adhesion using an *in vitro* model with the porcine intestinal epithelial cell line IPEC-J2. This study revealed that EcN SN interfere with *Salmonella* invasion but not with intracellular *Salmonella* growth. Moreover, secreted factors present in the EcN SN promoted downregulation of proteins relevant for *Salmonella* adhesion and invasion, such as SiiE adhesion, although this effect might be also common for other *E. coli* strains. Authors conclude that in the case of the probiotic EcN these effects could be potentiated with the strong

adhesion of EcN to the epithelial cells, which is a pre-requisite for the probiotic role. (Schierack *et al*., 2011).

Based on this last information, the aim of this study was to define whether the inhibitory effect of EcN SNs on *Salmonella* invasion is mediated by released outer membrane vesicles (OMVs). We evaluated the activity of several EcN extracellular fractions (total bacteria-free SN, isolated OMVs and OMV-free SN) on *Salmonella enterica* serovar Typhimurium and *Shigella flexneri* infections. The analysis was extended to other non-pathogenic *E. coli* strains.

MATERIALS AND METHODS

Bacterial strains

The probiotic strain *Escherichia coli* Nissle 1917 (serotype O6:K5:H1) was provided by Ardeypharm (GmbH, Herdecke, Germany). The commensal *E. coli* strain ECOR12 was isolated from healthy human stool samples (Ochman and Selander, 1984). The *E. coli* K-12 strains DH5α and MG1655 were from the laboratory collection. The pathogenic *Salmonella enterica* serovar Typhimurium strain SL1344 and *Shigella flexneri* serotype 5 strain M90T were provided by Dra. Ana Eulalio (University of Würzburg). Both strains constitutively express the green fluorescent protein (GFP) from a chromosomal locus. All bacterial strains were routinely grown at 37 °C in Luria-Bertani broth (LB). The medium was supplemented with 20 µg ml-1 of chloramphenicol in the case of *Salmonella* and *Shigella* strains. When indicated bacteria were grown in DMEM. Bacterial cultures were grown aerobically with constant rotation (150 rpm). The growth was monitored by measuring the optical density at 600nm (OD600).

Cell culture and growth conditions

The human intestinal epithelial cell lines used in this study were HT-29 (ATCC) and T84 (CCL-248). HT-29 cells were cultured in DMEM GlutaMAX (Life Technologies) and T84 cells were cultured in DMEM/F12 Glutamax medium (Gibco-BRL). In both cases the medium was supplemented with 10% fetal bovine serum (Biochrom), 25 Mm HEPES, 1% non-essential amino acids, penicilin (100 U/ml) and streptomycin (100 µg/ml) (Gibco-BRL). Human monocyte THP-1 cells were grown in RPMI 1640 (Life Technologies) supplemented with 10 % fetal bovine serum. For

THP-1 differentiation into macrophages, cells were incubated with 50 µg/ml of phorbol-12-myristate-13-acetate (PMA) (Sigma Aldrich) for 48 hours, in RPMI medium with 10% of fetal bovine serum. All cell lines were maintained in a 5% CO2 humidified atmosphere at 37 °C.

Isolation of OMVs and culture supernatant fractions

EcN OMVs were isolated from culture SN as described previously (Aguilera *et al*., 2014). Briefly, LB or DMEM overnight cultures were centrifuged at 10,000xg for 20 min at 4 ^{o}C to remove bacteria; the resulting SN was filtered through a 0.22 µm-pore-size filter (Merck Millipore) and concentrated using a Centricon® Plus-70 filter device (Merck Millipore). This fraction (SN), which contains all bacterial secreted factors, was stored also at -20^{o}C until use. To separate OMVs, the concentrated SN was ultracentrifuged at 150,000xg for 1 hour at 4°C in an Optima ™ L-90K ultracentrifuge (Beckman Coulter). The SN from this step, which is devoid of OMVs, was used a source of soluble secreted factors (OMV-free SN). The pellet containing OMVs was washed once with 1X PBS and re-suspended in 300µl of PBS. All these fractions were used immediately or stored at -20^{o}C until use. Protein concentration of these samples was measured by the method of Lowry (Lowry *et al*., 1951).

Pathogen infections and CFU assay

T84 (2x10^{5} cells/ml) and HT-29 (2x10^{4} cells/ml) were seeded on 12-well plates and cultured until 80-90% confluence or until forming a monolayer differentiated (5-7 days). Infection with *Salmonella* (MOI 25) or *Shigella* (MOI 10) was carried out as described elsewhere (Maudet *et al*., 2014). Cultures of these pathogens grown overnight in LB-chloramphenicol were diluted 1:100 in fresh LB medium and grown until an OD600nm of 2. One millilitre of each culture was centrifuged at 12.000 rpm for 2 minutes, and the bacterial pellet was resuspended in one millilitre of DMEM. The number of bacteria to be added to the epithelial cells was calculated according to the number of eukaryotic cells in the well, which were counted previous infection, using a Countess ™ (Invitrogen) device.

For pathogen adhesion studies, after 15 minutes incubation with *Salmonella*, cells were carefully washed, and bacteria attached to cell surface were counted. For invasion assays, after 1 h incubation, the medium was replaced with fresh medium containing 100µg/ml of gentamicin (Sigma Aldrich) to kill extracellular

bacteria, and intracellular *Shigella* or *Salmonella* were counted after 30 minutes or 1h post-infection respectively. For replication assays, the number of intracellular bacteria was counted after 3h (*Shigella*) or 20h (*Salmonella*) post-infection.

To count the number of pathogenic bacteria attached or internalized, cells were washed 4 times with sterile phosphate-buffered saline (PBS 1X) and lysed with PBS containing 0.1% Triton-X-100 for 15 minutes at room temperature. Samples were then serially diluted in sterile PBS, and plated on LB-chloramphenicol agar plates. The number of colonies from the recovered bacteria was counted and compared with that obtained from the input bacteria used for infection. This technique is known as the CFU assay, and results are presented as the ratio of adhered or intracellular bacterial units compared with untreated infected control cells, which value was set at 1 (cells infected with *Salmonella* or *Shigella* in the absence of any stimulation with *E. coli* fractions).

Confocal image acquisition

T84 cells, HT-29 and THP-1-derived macrophages were grown on glass cover slips (12 mm Ø) placed in 24 well plates. After *Salmonella* or *Shigella* infection timing, cells were washed with 1X PBS 3 times, and fixed with paraformaldehyde 3% (PFA) for 30 minutes at room temperature. Nuclei were labelled with DAPI (ANAME SL) for 15 minutes. Cells were washed twice with PBS and cover slips were placed in a slide using medium mounting Fluoromount-G (ANAME SL). Samples were analysed microscopically to determine *Salmonella* or *Shigella* internalization. Confocal microscopy was carried out using a Leica TCS SP5 laser scanning confocal spectral microscope, using the 63x oil immersion objective lens. Images were captures with a Nikon colour camera (16bits). Fluorescence was recorded at 405 nm (blue; DAPI), and 488 nm (green; GFP *Salmonella* and *Shigella*). Briefly, cellular nuclei and bacteria were segmented based on the DAPI and green fluorescence, and Z-stack images were taken at 0.5 to 1.0 µm. Images were analysed using Fiji image processing package.

Stimulation with *E. coli* secreted fractions

Transwell co-culture model. T84 cells were seeded on the basolateral compartment of 12 mm polycarbonate Transwell cell culture devices (0.4 µm, Transwell Millipore) and grown as described above. Overnight cultures of *E. coli*

strains (EcN, EcoR12, EcoR63, DH5α and MG1655) were diluted 1:100 in fresh LB medium and grown aerobically until OD 0.5. One millilitre of each culture was centrifuged at 12.000 rpm for 2 minutes, and bacterial the pellet was resuspended in one millilitre of DMEM. A bacterial suspension (MOI of 100) was placed into the hanging insert of the Transwell system (also called apical compartment), and incubated at 37 ^{o}C in a 5% CO2 humidified atmosphere. In this model, the *E. coli* strains (apical compartment) are not in direct contact with T84 cells seeded on the basolateral compartment, but the secreted factors released by *E. coli* can diffuse through the 0.4 µm porous membrane and reach the basolateral compartment. After 1 h, T84 cells were challenged by *Salmonella* infection (MOI 25) in the basolateral side and incubated for 15 minutes (adhesion assay), 1 hour (invasion assay) or 20 hours (intracellular replication assay). At the indicated times T84 cells were collected and processed for CFU counting as described above. Experiments with confluent T84 cells (forming a polarized monolayer) and also with non-polarized T84 cells (70-80% confluency) were performed using this co-culture system.

When indicated, the basolateral medium which contains the factors released by EcN was collected after 1 h incubation and ultracentrifuged for 1 hour at 150.000xg to separate EcN OMVs from EcN SN containing soluble factors (OMV-free SN). Each isolated fraction was then incubated on a new T84 polarized monolayer for 1 h, followed by *Salmonella* infection (MOI 25). Cells were collected at 1 h post-infection and processed for CFU counting.

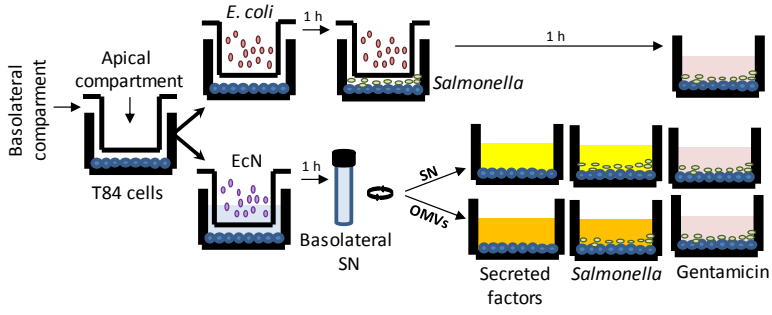

Figure 1. Schematic representation of *Salmonella* infection of T84 cells in the Transwell system following the co-culture model.

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In all cases positive controls of infection (infected cells in the absence of *E. coli* grown in the apical compartment) were used. The protocol is schematically represented in Figure 1.

Direct stimulation with OMVs and concentrated SN. IECs (T84 and HT-29) and immune cell lines (THP-1-macrophages) were challenged with isolated EcN OMVs or concentrated total culture SNs to evaluate the effect on *Salmonella* infection. T84 cells ($2x10^5$cells/ml) were grown in 24 well plates for 2-3 days (non-differentiated) or for 7 days (polarized cell monolayer). Then, cells were incubated for 2 hours with EcN concentrated SN (200 µg/ml) or with EcN OMVs (5, 10 and 50 µg/ml). Both extracellular fractions were obtained from LB or DMEM cultures. Then, T84 cells were infected with *Salmonella* (MOI 25). Positive controls without treatment were processed in parallel. HT-29 cells ($3x10^5$cells/ml) were seeded on 24 well plates and when they reached 70-85% confluency (after 24 hours approximately), they were pre-incubated with 0.1 and 1 µg/ml of EcN OMVs in the presence of gentamicin (5 µg/ml). After 24 hours at 37 ^oC, cells were infected with *Salmonella* (MOI 25), and collected at 1 and 20 hours post-infection to test the effect on *Salmonella* invasion and replication respectively. Confocal microscopy and CFU assays were performed to evaluate the infection ratio.

As an immune infection model, THP-1 human monocyte cells were seeded ($3,6x10^5$ cells/ml) on 24 well plates. Differentiation of monocytes to macrophages was achieved by incubation with PMA (50 µg/ml) for 48 hours. Then, cells were incubated with EcN OMVs (1 and 10 µg/ml) in the presence or absence of gentamicin (5 µg/ml) for 4 and 24 hours at 37°C, followed by *Salmonella* infection (MOI 10). Intracellular *Salmonella* was analysed by confocal microscopy and CFU assay at 1 and 20 hours post-infection.

Enzymatic supernatant treatment

The co-culture model using the Transwell system was used to evaluate the nature of the factor released by EcN that affects *Salmonella* invasion. Thus, after one hour of EcN incubation, the basolateral SN was challenged to different treatments including proteinase K, DNAse and RNAse to check the effect on *Salmonella* invasion at one-hour post-infection. After one hour of EcN growth and before *Salmonella* addition, the basolateral SN was treated during one hour with

100 mg/ml of DNase I (Life Technologies) or 100 mg/ml of RNase A (Sigma Aldrich). For protein analysis, the basolateral SN was incubated with 100 µg/ml of proteinase K (Thermo Scientific) for 1 hour followed by 30 min incubation with 0.5 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma Aldrich) at 37°C. PMSF is a non-specific inhibitor of multiple proteases. Untreated controls were used for comparison.

Bacterial growth inhibition

To test the ability of probiotic and commensal *E. coli* strains (EcN, EcoR63, EcoR12 and DH5α) to inhibit *Salmonella* growth (in the absence of eukaryotic cells), a Transwell system device was used to separate both non-pathogenic and pathogenic strains. Thus, $2x10^8$ CFU/ml of each *E. coli* strain, resuspended in DMEM, was added to the apical Transwell compartment, and incubated for 1 hour at 37 ºC in a 5% CO2 humidified atmosphere. Then, maintaining the basolateral medium, *Salmonella* $5x10^6$ CFU/ml was added into the basolateral compartment and incubated for an extra hour. Finally, *Salmonella* was quantified by CFU assay and results were compared with those from the positive control, which corresponds to the growth of *Salmonella* alone.

Isolation of secreted proteins in the culture medium

Overnight EcN and *Salmonella* cultures in LB were diluted 1:50 in the indicated culture media (100 ml final volume) and incubated at 37 ºC with shaking to an OD600 of approximately 1.0. Then, bacteria were removed by centrifugation (13.000 *xg*, 10 min at room temperature) followed by a 0.2 µm filtration step. When indicated, *Salmonella* overnight inoculum was diluted 1:100 in bacteria-free SN collected from EcN cultures, and grown to an OD600 of 1.0, as described above. As reference controls, single *Salmonella* and Nissle cultures grown to an OD600 of 1.0 were used. The proteins in the filtrated SN were precipitated by incubation with 10% of trichloroacetic acid (TCA) (Sigma Aldrich) at 4 ºC for 16 h. Then, samples were centrifuged (16.000xg at 4°C, 45 minutes) and the protein pellet was washed twice in 90% (v/v) ice-cold acetone, air-dried and re-suspended in 40 µl of 25 mM Tris-HCl buffer (pH 8.0), supplemented with 10 µl of 4X loading buffer (1M Tris-HCl, pH 6.8, 4% SDS, 8% β-mercaptoethanol, 20 % glycerol, 0.025 % bromophenol blue). Samples were kept at -20°C until use. For analysis, aliquots (10 µl) were heated at

98°C for 5 minutes and proteins were separated using sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970), followed by SYPRO® RUBY protein gel staining (Figure 8).

miRNAs analysis

To evaluate the ability of EcN OMVs to modulate microRNAs involved in *Salmonella* infection, two immune models (monocytes and macrophages) were used. In the monocyte model, THP-1 cells were seeded ($8x10^5$ cells/ml) in 24 well plates for 24 hours. In the macrophage model, THP-1 cells ($8x10^5$ cells/ml) were 2 days incubated with PMA (50µg/ml) until macrophage differentiation. Then, both cell types were incubated with EcN OMVs (10µg/ml) for 6 hours. Untreated negative controls cells were processed for comparison. Cells were collected for RNA extraction and miRNAs analysis.

Total RNA was isolated in TRIzol (Life Technologies) and extracted by phenol-chloroform followed by isopropanol precipitation. The protocol was adapted to preserve small RNAs (Mraz *et al.*, 2009). For miRNA quantification, reverse transcription was performed using the miRCURY LNA Universal cDNA synthesis kit (Exiqon) followed by RT-qPCR using miRCURY LNA SYBER Green master mix (Exiqon) and predesigned mercury LNA PCR primer set (Exiqon), according to the manufacturer´s instructions. The following primer sets were used: miR146a-5p, miR155-5p and miR21. Expression of each miRNA was normalized to U6, and the $2^{-\Delta\Delta Ct}$ method was used to calculate fold changes.

Statistical analysis.

The data are presented as the mean ± standard error (SEM) of at least three independent experiments. The exact number of independent biological experiments performed for each analysis is indicated in the corresponding Figure legend. Graphical and statistical analyses were performed using SPSS and Prism Software (GraphPad). For statistical comparison of datasets from two conditions, two-tailed Student´s t-test was used; for data from three or more conditions, ANOVA with Tukey´s or Dunnet´s post-hoc test was used. A p-value of 0.05 or lower was considered significant.

RESULTS

Factors secreted by *E. coli* strains inhibit *Salmonella* invasion without affecting bacterial growth

Polarized T84 cells grown on the basolateral compartment of a Transwell system were pre-incubated for 1 hour with non-pathogenic *E. coli* strains (MOI 100) seeded into the apical compartment (Figure 1). The *E. coli* strains analysed were the probiotic EcN, the intestinal isolates ECOR12 and ECOR63, and the laboratory K-12 strains DH5α and MG1655. Then, T84 cells were infected with *Salmonella* (MOI 25), and the number of intracellular bacteria was calculated by the CFU assay at 15 minutes post-infection (adhesion test), one-hour post-infection (invasion test), and 20 hours post-infection (replication test) as described in the methods section. This model avoids the direct contact or competition for adhesion to T84 cells between the two bacterial strains. Results are presented in Figure 2

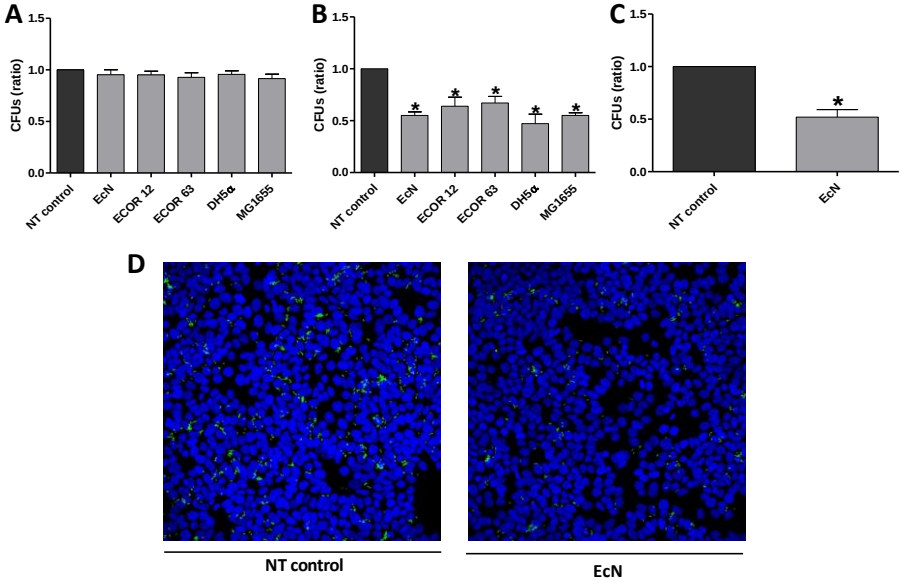

Figure 2. Released factors from *E. coli* strains diminish *Salmonella* invasion in T84 cells. Using a Transwell system, polarized T84 cells seeded on the basolateral compartment were incubated for 1 hour with secreted factors of different non-pathogenic *E. coli* strains grown in the apical compartment. A) Adhesion assay: The number of *Salmonella* attached to cell surfaces at 15 minutes post-infection was analysed by CFU assay. B) Invasion assay: intracellular *Salmonella* was analysed by CFU assay at 1-hour post-infection. C) Replication assay: Intracellular *Salmonella* counts were analysed by CFU assay at 20 hours post-infection. Values are means ± standard error from at least 3 independent experiments. Results significantly different from that of untreated control cells are indicated by an asterisk ($P < 0.05$). D) T84 cells incubated with EcN in the co-culture model were infected with *Salmonella*

(MOI 25) and intracellular bacteria were evaluated by confocal fluorescence microscopy at one-hour post-infection. Nuclei were stained with DAPI (blue), and *Salmonella*, which constitutively expressed GFP (green), was visualized in green.

At 15 minutes post-infection, none *E. coli* strain caused significant reduction in the number of internalized *Salmonella* (Figure 2A), thus indicating that *E. coli* secreted factors do not impair *Salmonella* adhesion to IECs. On the contrary, the CFU counts at 1 h post-infection showed great reduction in the number of internalized *Salmonella* for incubations carried out in the presence of *E. coli* strains. Internalized CFUs were significantly reduced by around 50% in the presence of EcN, DH5α and MG1655, and by around 40% in the presence of ECOR12 and ECOR63 secreted factors (Figure 2B). Invasion assays performed with non-differentiated T84 cells also showed a significant drop in the number of internalized *Salmonella* after EcN pre-incubation (data not shown). This information was confirmed by confocal fluorescence microscopy. Images showed less GFP-green fluorescence signal corresponding to *Salmonella* in cells incubated in the presence of EcN secreted factors than in the untreated controls (Figure 2D). The effect of *E. coli* secreted factors on the intracellular growth of *Salmonella* (replication assay) was evaluated only for the probiotic EcN strain. At 20 post-infection the number of intracellular *Salmonella* was significantly reduced by 49% (Figure 2C). This inhibition rate was similar to that calculated in the invasion test (Figure 2C), thus indicating that EcN secreted factors do not interfere with the replication of internalized *Salmonella*.

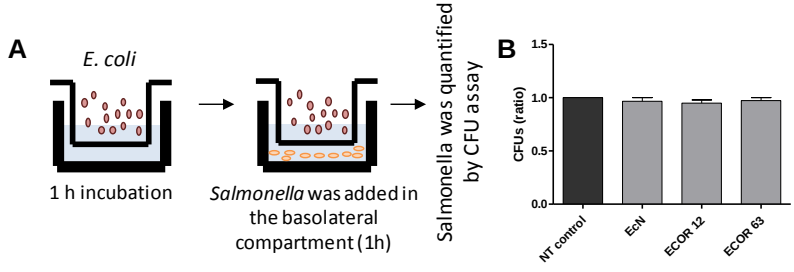

Figure 3. Inhibition of *Salmonella* growth by *E. coli* released factors. A) Schematic representation of the assay for *Salmonella* growth inhibition set up in the Transwell system. *E. coli* strains were seeded into the apical compartment of a Transwell device ($2.5x10^7$ bacteria/ml, which corresponds to the number of *E. coli* cells equivalent to a MOI of 100 in Figure 2). After 1 h growth, *Salmonella* ($1x10^8$ bacteria/ml, which corresponds to the number of *Salmonella* cells equivalent to a MOI of 25 in Figure 2) was added to the basolateral compartment and further incubated for 1 hour. B) *Salmonella* CFUs counts at the end of the experiment. Data are presented as means ± standard error from at least three independent

experiments. CFU values were not significantly different from that of untreated *Salmonella* controls ($P < 0.05$).

Additionally, we tested whether the inhibitory effect of *E. coli* released factors on *Salmonella* invasion was retained after PBS washing of T84 cells in the basolateral compartment. To this end, after one-hour incubation with EcN and DH5α strains grown in the apical side of the insert, the medium of the basolateral compartment was removed and T84 cells were washed with PBS prior to the addition of *Salmonella* in fresh medium. In this case, at 1-hour post-infection, the inhibitory activity on *Salmonella* invasion was neutralized as intracellular CFU values were similar to that of control infected cells (data not shown).

To test if the inhibitory activity of EcN secretory components on *Salmonella* infection was due to their ability to reduce *Salmonella* growth rather than *Salmonella* invasion, the same experimental Transwell model was used in the absence of T84 cells (Figure 3A). In this case, the CFU assay refers to *Salmonella* grown in suspension in the basolateral compartment. The results showed that *Salmonella* growth rate is not affected by EcN, ECOR12 or ECOR63 released factors (Figure 3B).

DNase, RNase and proteinase K treatments do not affect the inhibitory activity of EcN secreted factors on *Salmonella* invasion ratio.

To discover the nature of factors released by *E. coli* strains that interfere in *Salmonella* invasion, the same Transwell co-culture protocol was used. However, after 1-hour pre-incubation with EcN in the apical insert, the basolateral SN was treated with RNase A, DNase I or Proteinase K. As shown in Figure 4, none of these treatments abolished the inhibitory effect on *Salmonella* infection.

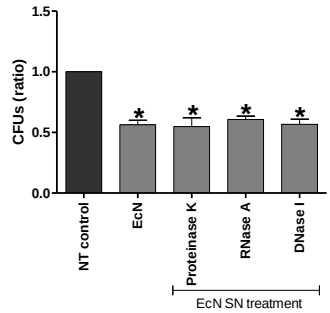

Figure 4. Effect of enzymatic treatment of the EcN conditioned medium on *Salmonella* infection. Using a Transwell system, polarized T84 cells seeded on the basolateral side were challenged with EcN (MOI 100, in apical compartment) for 1 hour. Medium from the basolateral compartment (conditioned media) was collected and treated with proteinase K (100 µg/ml), RNase A (100 mg/ml) and DNase I (100 mg/ml). These SNs were then added to new T84 cells and incubation was let to proceed for 1 hour, followed by *Salmonella* infection (MOI 25). At 1-hour post-infection, CFU assay was performed. Untreated conditioned EcN medium was used as a control for comparison. Values are means ± standard error from at least 3 independent experiments. Results significantly different from that of untreated control cells are indicated by an asterisk ($P < 0.05$).

Inhibition of *Salmonella* invasion is not mediated by EcN OMVs

Some factors secreted by bacteria to communicate with the host are delivered by outer membrane vesicles (OMVs), while other are secreted as single compounds in a vesicle-independent manner. To analyse independently the effect of EcN released factors on *Salmonella* infection concentrated culture SNs were fractionated into OMVs and soluble released factors (OMVs-free SN), and both fractions were tested on a *Salmonella* infection model.

To this end, EcN was applied in the insert device of a Transwell system. After 1-hour incubation the basolateral medium was separated into two fractions: EcN OMVs and OMVs-free SN. Both fractions were incubated separately on new T84 cell monolayers for 2 h, followed by *Salmonella* infection following the invasion assay. At 1 hour-post infection, numbers of intracellular *Salmonella* were counted. Results showed that EcN OMVs did not cause any reduction of the *Salmonella* invasion ratio, whereas the fraction corresponding to soluble released factors yielded a significant decrease in intracellular *Salmonella* counts (Figure 5A).

In addition to this *in situ* experiment (Transwell co-culture model), another experimental approach (direct stimulation model) was performed to prove that the inhibitory effect of EcN released factors is not mediated by OMVs. For this, EcN OMVs at three different doses (5, 10 and 50 µg/ml) and EcN concentrated SN (200 µg/ml), obtained from EcN cultures in two different media (LB or DMEM), were directly incubated with T84 cells for 1 hour, and then infected with *Salmonella* (MOI 25). At 1-hour post-infection, results from CFU counts confirmed the lack of inhibitory activity for EcN OMVs, as intracellular *Salmonella* CFUs did not differ from those of untreated control cells. As expected, incubation in the presence of EcN concentrated SN caused a decay in intracellular *Salmonella* numbers, although it was not statistically significant. (Figure 5B).

To validate these results in another intestinal epithelial cell line, non-polarized HT-29 cells were used as a model to analyse the effect of EcN OMVs on *Salmonella* invasion. After 24 hours pre-treatment with OMVs (1 and 10 µg/ml) cells were infected with *Salmonella* (MOI 25) and processed for CFU assay at 1 and 20 hours post-infection. Intracellular *Salmonella* counts in cells pre-treated with EcN OMVs did not significantly differ from the values of untreated control cells (Figure 5C). Moreover, as *Salmonella* is able to replicate into macrophages, the same experimental approach was applied to THP-1 derived macrophages, and *Salmonella* infection was analysed by CFU assay (Figure 5D) and confocal fluorescence microscopy (Figure 5E). Results showed that pre-treatment with EcN OMVs did not interfere with *Salmonella* invasion or replication in this immune cell model.

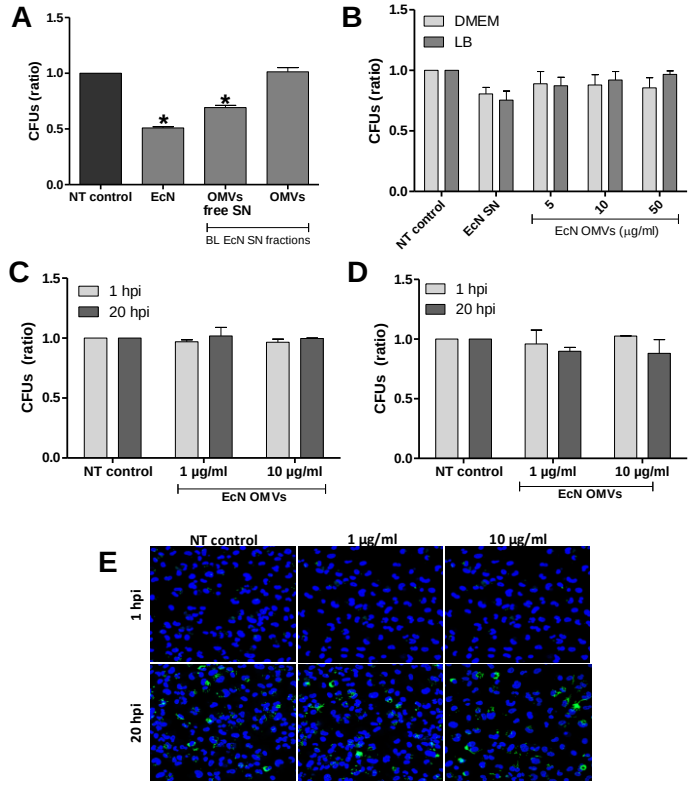

Figure 5. Inhibition of *Salmonella* invasion is not linked to EcN OMVs. A) The basolateral EcN SN obtained from a Transwell co-culture model was separated into OMVs and OMVs-free SN. Both fractions were incubated separately for 1 h on T84 polarized cells, followed by *Salmonella* infection (MOI 25). At 1-hour post-infection, CFU assay was performed. B) Differentiated T84 cells were treated for 1 hours with EcN OMVs (5, 10 and 50 µg/ml) or EcN

concentrated SNs (200 µg/ml) obtained from DMEM or LB cultures, and then infected with *Salmonella* (MOI 25). Intracellular *Salmonella* was calculated at 1 h post-infection. C) Non-confluent HT-29 cells were treated for 24 hours with EcN OMVs (1 and 10 µg/ml) isolated from overnight LB cultures, and then infected with *Salmonella* (MOI 25). Intracellular *Salmonella* was calculated at 1 h and 20 h post-infection. D) THP-1 derived macrophages were incubated for 24 hours with EcN OMVs (1 and 10 µg/ml) of and then infected with *Salmonella* (MOI 10). Intracellular *Salmonella* was calculated at 1 h and 20 h post-infection. E) Analysis by confocal fluorescence microscopy of *Salmonella* infections carried out in THP-1 derived macrophages and presented in panel D. A representative image of each condition is showed. Nuclei were stained with DAPI (blue), and *Salmonella*, which constitutively expressed GFP (green), was visualized in green. Values are means ± standard error from at least 3 independent experiments. Results significantly different *versus* untreated control cells are indicated by an asterisk ($P < 0.05$).

Shigella infection is not affected by EcN OMVs treatment

The pathogenesis of *S. flexneri* is based on the ability of this pathogen to invade IECs and to spread to the neighbour epithelial cells through TJ alterations. This mechanism allows *Shigella* replication and dissemination in the gut (Jennison and Verma, 2004).

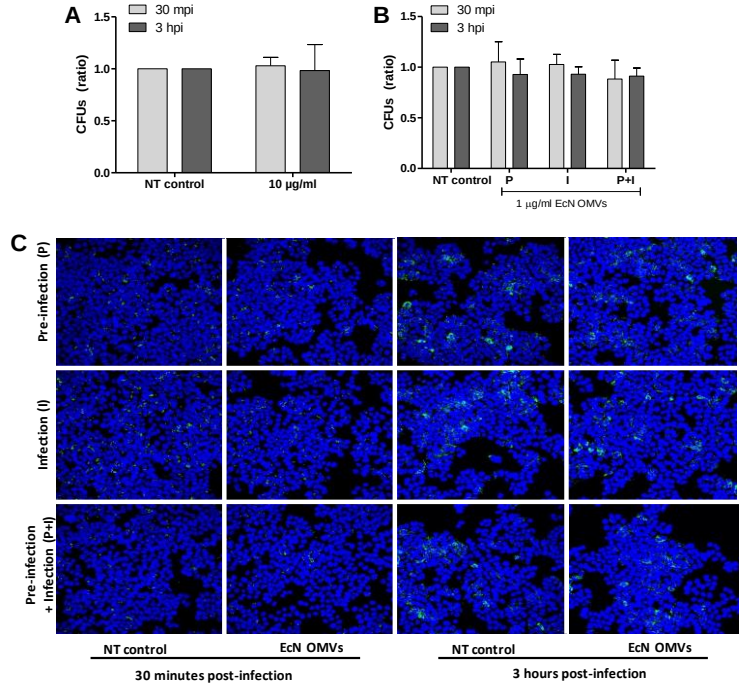

Figure 6. *Shigella* infection models in intestinal epithelial cells treated with OMVs. A) HT-29 cells were pre-incubated for 24 hours with EcN OMVs (10 µg/ml) and then infected with *Shigella* (MOI 10). B) Three models of *Shigella* infection were set up to evaluate the effects of EcN OMVs (1µg/ml) in HT-29 cells: (P) Pre-treatment model, OMVs were added 24 h prior to

the infection with *Shigella*; (I) Co-infection model, OMVs were added simultaneously with *Shigella*; and (P+I) OMVs were added 24 h prior to the infection with *Shigella* and were kept during *Salmonella* infection. In all cases CFU assay was performed at 30 minutes and 3 hours post-infection. Values are means ± standard error from at least 3 independent experiments ($P<0.05$, versus untreated control cells). C) Analysis by confocal fluorescence microscopy of each *Shigella* infection model. A representative image is shown for each condition. Nuclei were stained with DAPI (blue), and *Shigella*, which constitutively expressed GFP (green), was visualized in green.

Since EcN OMVs have the ability to strengthen the epithelial barrier through up-regulation of TJ proteins (Álvarez *et al*., 2016) we sought to analyse the effect of EcN OMVs on *Shigella* infection. In this context, HT-29 intestinal cells were pre-treated with EcN OMVs (10 µg/ml), followed by *Shigella* infection (MOI 10). CFU assays were performed at 30 minutes and 3 hours post-infection. Results did not show any significant variation compared with untreated control HT-29 cells (Figure 6A).

To evaluate the EcN OMVs effect at different times related to *Shigella* infection, EcN OMVs (1 µg/ml) were added to HT-29 cells following different schedules: (P) 24 hours prior *Shigella* addition and then removed; (I) added simultaneously with *Shigella*; and (P+I) 24 h before *Shigella* addition and in *Shigella* infection. Results were evaluated by means the CFU assay and confocal fluorescence microscopy at 30 minutes and 3 hours post-infection. No significant differences were observed between OMVs-treated or untreated control cells at any condition tested (Figure 6 B-C).

Effect of EcN released factors on the T3SS-mediated secretion of *Salmonella* invasion proteins

Results presented above showed that factors released by *E. coli* strains (other than OMVs) inhibit *Salmonella* invasion in IECs without affecting its adhesion or intracellular replication. As key proteins for *Salmonella* invasion are effectors of the T3SS secretion system we sought to evaluate whether EcN SNs might interfere with the secretion of *Salmonella* invasion proteins (Sip, Inv) by the T3SS. To approach this study, *Salmonella* was grown in pure DMEM culture or in DMEM SNs collected from exponential EcN cultures (EcN conditioned media). When the cultures reached an OD600 of 1, SNs were collected, and the secreted proteins analysed by SDS-PAGE (Figure 8A). EcN SN was processed in parallel as a control.

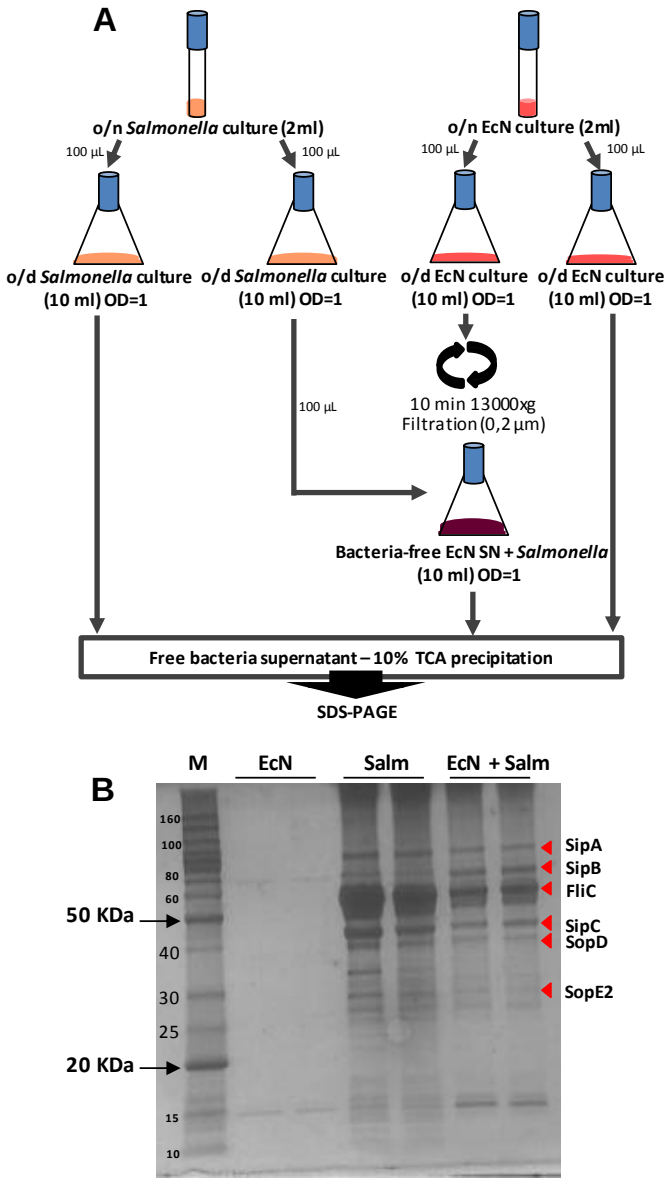

Figure 8. SDS-PAGE protein profile of *Salmonella* secreted proteins. A) Representative diagram of the protocol used to obtain the samples: EcN SN (EcN), *Salmonella* SN (Salm), and SN of *Salmonella* grown in EcN SN (EcN+Salm). All cultures were in DMEM. Proteins were isolated from SN by TCA precipitation. B) SDS-PAGE and SYPRO® Ruby protein gel staining of whole culture SNs. Two representative independent experiments (two lanes for each condition) were shown (n=4, number of independent biological replicates).

In this experiment, EcN SN was found to secrete barely few amounts of proteins. Only two faint protein bands of 15 kDa and 80 kDa were apparent (Figure 8B). In contrast, *Salmonella* secreted a large number of proteins. The observed protein profile matched the pattern reported for *Salmonella* secreted proteins (Sittka, 2007). By comparing protein band molecular mass, we could identify about three *Salmonella* invasion proteins (Sip A, B and C), and other T3SS effector proteins, such as SopD and SopE2 (Figure 8B). Interestingly, growth of *Salmonella* in EcN SN did not result in any remarkable difference in specific protein bands with respect to *Salmonella* control grown in pure DMEM, but a slightly reduction in all secreted proteins was observed (Figure 8B).

Expression of miRNAs changes upon EcN OMVs challenge.

Recent reports have shown the important role of miR-146a, miR-155, and miR-21 in innate immunity in relation to TLR and NF-κB signalling during *Salmonella* infection (Sharbati *et al*., 2012). Based on this information we decided to analyse whether EcN OMVs could modulate the expression of mature miR-146a, miR-155 or miR-21 transcripts in monocyte THP-1 cells and THP-1-derived macrophages. THP-1 monocytes were incubated with EcN OMVs (10 μg/ml) for 6 hours and expression of these miRNAs was measured by RT-qPCR. OMVs from this probiotic significantly induced miR-146a expression by approximately 35-fold compared to control cells. In contrast, OMVs treatment did not cause any change in miR-155 and miR-21 expression. In the THP-1 macrophage model upregulation of miR-146a by EcN OMVs was also observed, although to a lesser extent. In this case, data allowed estimating a 3.5-fold increase in miRNA-146a levels by EcN OMVs challenge, with respect to untreated control cells. Moreover, a slight increase was observed for miR-155 and miR-21, although for both miRNAS differences with untreated control cells did not reach statistical significance (Figure 9).

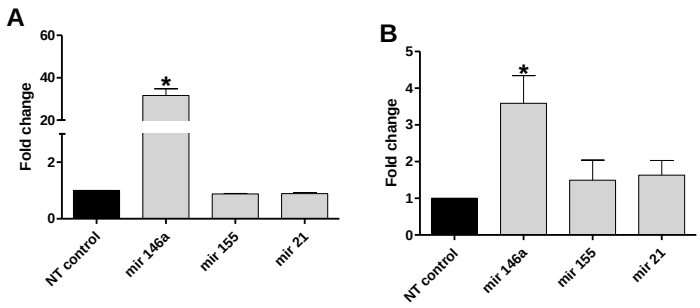

Figure 9. Effect of EcN OMVs on miRNA expression in THP-1 monocytes and derived macrophages. A) THP-1 cells were incubated with EcN OMVs (10 µg/ml) for 6 hours. Untreated cells were processed as a control. B) Macrophages derived from THP-1 by two incubation days with PMA were treated with EcN OMVs under the same conditions used for THP-1 monocytes. Quantification was performed by quantitative RT-qPCR and normalized by using U6 RNA levels. All results are presented as fold changes relative to the untreated control cells. Data are means ± standard error from at least three independent experiments performed in triplicate (* P<0.05, versus untreated control cells).

DISCUSION

Throughout life, the GI tract is colonized by a dense microbial community, known as commensal microbiota, whose major key role is maintaining the intestinal homeostasis, protecting from external factors, such as colonization and overgrowth of indigenous entero-pathogen bacteria (Kamada *et al*., 2013). Within this last group, there are two pathogenic strains, *Shigella* and *Salmonella* spp., that are responsible of a high number of clinical problems in society, especially in children, related to diarrhoeal infections. Antibiotics are commonly prescribed, but a high rate of resistance development has been associated (Beyene and Tasew, 2014). However, new scientific contributions have focused their attention on microbiota, demonstrating the positive impact of commensal and probiotic bacteria against invading pathogens within the intestine. Thus, some authors suggest that microbiota might affect or interfere with pathogens due to direct commensal-pathogen interaction. For instance, gut microbiota can produce specific metabolites such as SCFAs that downregulate the expression of virulence genes like Type 3 secretion system (T3SS) proteins in *Salmonella enterica*, or microbiota could alter oxygen tensions necessary for secretion of *Shigella flexneri* virulence factors (Gantois *et al*., 2006; Marteyn *et al*., 2010). Moreover, microbiota prevent pathogen colonization and infection enhancing host defence mechanisms,

which also reinforce intestinal mucosal barrier, the first line of defence against enteric infection (Santos, 2014).

Probiotic strain *Escherichia coli* Nissle 1917 (EcN), has been successfully used for prevention and treatment of different GI disorders, including infection by EPEC, *Shigella flexneri* and *Salmonella* spp. However, the accurate basis of the underlying mechanisms of action are still not clarified. Focusing on *Salmonella* infection, many are the proposed mechanisms by which EcN exerts its beneficial effect, such as the high potential of colonization by specific fimbriae or the competition for iron uptake (Deriu *et al*., 2013; Lasaro *et al*., 2009; Weiss, 2013). Nevertheless, recent *in vitro* researchers have suggested that this *Salmonella* inhibitory effect could be due to released factors by the probiotic EcN. As there is currently no information about these EcN compounds against *Salmonella* or *Shigella* infection, in the present study, we initially confirmed the probiotic effect of EcN cell free SN against *Salmonella*, and subsequently we tried to determine which is the secreted factor responsible of this effect. The first approximation was to study in a human intestinal epithelial cell model (T84), at which level along *Salmonella* infection, this or these EcN compounds are exerting their effects. The co-culture study (Figure 1), under different *Salmonella* incubation timings, suggested that EcN released factors interfere with invasion but not *Salmonella* adhesion, and they are in accordance with the previous adhesion results described with the *Salmonella* invasion mutant SL1344 *hilA::km* (Schierack *et al*., 2011). Moreover, this nearly 50% of invasion reduction is maintained along *Salmonella* infection, diminishing pathogenic bacterial replication number after 20 hours post-infection, data that do not correspond with intracellular replication results showed by Schierack *et al*, 2011. However, since *Salmonella* has been constantly in presence of EcN SN, which halved the entry of pathogenic bacteria, it is logical that after 20 hours, and independently of the *Salmonella* replication rate, the number of intracellular bacteria is less. Nevertheless, and according to other authors, this effect was not probiotic EcN-specific (Altenhoefer *et al*., 2004; Schierack *et al*., 2011), since other commensal (ECOR12 and ECOR63) and laboratory (DH5α and MG1655) *E. coli* displayed similar effects (Figure 2). Besides, it was also tested whether the inhibitory effect might be due *Salmonella* growth suppression by *E. coli* SNs. In this context, and in accordance with Shierack *et al*., extracellular *Salmonella* growth is not affected by *E. coli* released compounds (Figure 3).

On the other hand, it was proposed that *E. coli* SNs components might act binding to host cell surface. Thus, EcN SN pre-incubation experiments were assayed, and after extensive washing host cells, to dilute secreted factors components and detached those bind to host cells, *Salmonella* invasion was not inhibited. This result, in accordance with previous studies (Schierack *et al*., 2011), suggest that secreted *E. coli* factors could bind to epithelial cell surfaces or interact with virulence factors produced by *Salmonella*.

Focusing on EcN cell free SNs, our investigations went one step further, and different SN fractions were obtained and independently assayed on immune and IECs infected with *Salmonella*. Thus, the effect of EcN Outer Membrane Vesicles (OMVs) and OMVs free SNs was tested using a co-culture system, and also using quantified concentrated SNs and OMVs previously isolated from a big bacterial culture. EcN OMVs fraction did not show any effect on *Salmonella* infection, that was assayed in IECs (T84 and HT-29) and macrophages. This last immune model was evaluated because they are also central target cells during *Salmonella* infection. Nonetheless, others unknown secreted factors included in OMVs free SNs seems to be responsible of the *Salmonella* inhibitory effect. Moreover, it was also noticed that the effect of "fresh" OMVs free SN obtained in the co-culture system, compared with concentrated SNs (previously stored frozen), had a higher inhibitory potential, independently of the medium used for isolation (DMEM or LB). Probably, the activity of these secreted factors is affected by long-term storing or by temperature changes. Even so, EcN concentrated SN displayed a slight *Salmonella* decrease tendency (Figure 5).

Shigella flexneri like *Salmonella*, has a large virulence plasmid, encoding a Type III-secretion system (T3SS) and a set of secreted proteins, that inject virulence factors into host colonic epithelial cells (Dekker and Frank, 2015). Nevertheless, *Shigella* infection, a difference of *Salmonella*, requires a very small number (100-1000) of bacteria ingestion, which are able to be rapidly spreading through the intestinal epithelium, causing the infection (C. Sasakawa, 2009). One of the most important step in the intracellular life cycles of *Shigella* spp. is the formation of bacterial protrusions connecting cells, and the cell-cell spreading. This dissemination way is possible, recruiting host endocytic pathways and altering intestinal TJs (Ireton, 2013). On the other hand, recent research done by our group have demonstrated the internalization of EcN OMVs, and their ability to reinforce

intestinal epithelial barrier, modulating the expression of TJ proteins, upregulating ZO-1 and claudin-14, and maintaining the integrity of the intestinal epithelial barrier. Thus, in this pathogenic context, it was examined the ability of EcN OMVs to counteract *Shigella* infection. However, experimental results showed that EcN OMVs at dose of 10 µg/ml are not capable to reduce *Shigella* invasion, and neither to prevent or reduce the infections at lower doses of vesicles (Figure 6). Studies performed with OMVs are recently and published contributions are scarce. Thereby, doses of EcN OMVs used were extrapolated from others pathogen OMVs studies, in which the range to stimulate epithelial o monocytic cells were since 0.1 µg/ml to 10 µg/ml (Ismail *et al*., 2003; Jung *et al*., 2017). However, it is possible that in our model, these doses are not enough to exhibit an inhibitory response, and other concentrations and timings should be tested.

Additionally, and focusing on bacterial SN-dependent mechanisms, several probiotic and commensal bacterial species such as *Lactobacillus* spp. and Leuconostoc spp., have shown that secreted bactericidal compounds and pH decreasing of the medium are responsible for *Salmonella* inhibition. Nevertheless, in this study, like in others previously described, *E. coli* SNs do no exert its *Salmonella* inhibitory activity through microcins production and neither by acidification of the medium (Altenhoefer *et al*., 2004). Thereby, in this work, *E. coli* secreted factors were analysed more in-depth, trying to clarify the nature of these compounds. After different enzymatic treatments, our results displayed that the *Salmonella* inhibitory effect triggered by EcN SNs, are not mediated by proteins, DNA or RNA molecules. Consequently, this could suggest that others chemical structures such as lipids or carbohydrates could be involved. For instance, commensal strains such as *Bifidobacterium* are able to produce acetate to protect against enteropathogenic infection, reducing gut permeability and bacterial translocation (Fukuda *et al*., 2011). Moreover, it has also been reported that carbohydrate-like molecules, such as fucosylated oligosaccharides present in human breast milk are able to inhibit binding and disease by specific pathogens. Besides, they exhibit antimicrobial properties (Newburg *et al*., 2005).

Recent investigations have reported the key role of miRNAs in many biological processes, exerting post-transcriptional effects on gene regulation by controlling many signalling pathways, such as TLR pathways. Recently, miRNAs have been described as a possible strategy adopted by bacterial pathogens to survive inside

host cells (Schulte *et al*., 2011). Furthermore, changes in their expression are related to a multirange of diseases (Leung and Sharp, 2010). In particular, the inflammation-related miRNAs miR-146a (suppressor of NFκ-B), miR-155, and miR-21 have been included as key pieces in modulating TRL signalling, and controlling many inflammatory routes (Quinn and O´Neill, 2011). Besides, they are regulated during human monocyte differentiation as well as *Salmonella* infection (Sharbati *et al*., 2012). It is already known, by previous studies, that EcN bacterium upregulates miR-146a in a THP-1 model, as a negative-feedback loop regulating the same signalling system that is used for its own induction (Sabharwal *et al*., 2016). Here, and in accordance with this last study, monocytic and macrophage models were stimulated with EcN OMVs and results obtained were similar, with up-regulation only for miR-146a in both cell models (Figure 8). This might contribute to stablish an equilibrium between anti- and pro-inflammatory signals, as other authors suggest (Sabharwal *et al*., 2016). Remarkably, the suppression of miR-164a gene results in a higher production of inflammatory cytokines by macrophages (Boldin *et al*., 2011). Probably these vesicles are not properly skilled to show a physical change during *Salmonella* infection, but they are able to modulate intracellular responses within host cells through miRNAs expression, and this could be a possible approach to ameliorate a disease condition associated to inflammation context. However, these results need to be completed, studying the effect on miRNAs expression caused by EcN OMVs after *Salmonella* infection. Besides, another approach could be expanding the range of stimulations timings for miR-155 and miR-21 detection, to capture and ensure a possible change in miRNA fold increase.

Finally, studies performed with precipitated proteins from bacterial SNs, were assayed to check whether EcN released factors could affect to secretion of virulence proteins produced by *Salmonella enterica*. A similar approximation was also carried out by Kleta *et al*. 2006, with the entepathogenic *E. coli* strain EPEC, and they demonstrated that EcN SNs could block completely T3SS-secreted factors released by EPEC. On the contrary, our *Salmonella* SDS-PAGE results only showed a slight and general reduction for all *Salmonella* protein pattern when the pathogen was grown in presence of EcN cell-free SNs, compare with single *Salmonella* culture (Figure 9). It was not observed a complete inhibition of protein secretion. This contribution is in accordance with studies performed by Schierack *et al*., in 2011, who confirmed, through *lacZ* transcriptional fusion factors, that EcN SNs reduce

the expression of the invasion regulatory protein HilA, and also the expression of the adhesin SiiE, encoded in the SPI4. They proposed that this inhibitory effect could be due to SiiE regulation or by blocking SiiE-mediated adhesion by binding either to SiiE or to SiiE receptors on host cells. Thus, as HilA is an activator of SPI1 genes (encoding Sip and Inv proteins), in comparison with these results, in our study it was also observed downregulation of *Salmonella hilA* gene by *E. coli* SNs.

CONCLUSIONS

EcN is a probiotic strain extensively used in human and animal health, whose inhibitory activity against enteropathogenic bacteria has already been proven and reported. Besides the high colonizing and competitive potential, the probiotic effect is also partially due to secreted factors released by EcN. However, these key factors are not OMVs, proteins, DNA or RNA molecules. Although the nature is still unknown, these compounds are *E. coli* strain-specific, and affect to *Salmonella* invasion process. Probably they are acting on a central structure of host cell surface avoiding *Salmonella* internalization, or also they could interfere blocking *Salmonella* invasion proteins belonging to TSS3. Moreover, it is suggested that they also could modulate the expression of some virulence proteins. More studies focus on EcN specific factor responsible for the anti-invasive activity of EcN, and the mechanisms by which it exerts its action are needed.

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7. DISCUSION

Intestinal epithelium integrity is essential to preserve host immune tolerance against the adjacent intestinal microbiota. Thus, epithelial cells and commensal bacteria are physically separated by a mucus layer that avoids direct contact between them and a potential immune overreaction. However, despite this segregation border, communication between host and microbiota is necessary to maintain intestinal homeostasis and a healthy status (Pelaseyed *et al*., 2014)(Johansson *et al*., 2008). In this context, recent research on this filed has provided evidence that intestinal bacteria are able to stablish a long-distance communication with eukaryotic cells through releasing secreted bacterial compounds, which are able to diffuse through the mucus layer and deliver the bacterial effectors into the underlying intestinal and immune cells. Bacterial secreted factors can be divided into two main groups: soluble factors and extracellular membrane vesicles (Sánchez *et al*., 2008)(Kulp and Kuehn, 2010). Both seem to play specific roles. The main role of membrane vesicles is to act as a general secretory pathway of bacterial active compounds, which are protected from external degradation and can be directly delivered to the host cell (Kesty and Kuehn, 2004). Bacterial vesicles are important "information messengers" that allow communication intra and inter-kingdom avoiding direct cellular contact (Zargar *et al*., 2015).

The study of bacterial extracellular vesicles has been an emerging research field in the last 15 years, especially in pathogens, but relatively little is known about microbiota derived vesicles. Extracellular membrane vesicles (OMVs) were by the first time described in Gram-negative bacteria and their role on the interaction with the host has been mainly studied in pathogens (Greniert and Mayrand, 1987)(Kolling and Matthews, 1999) (Ellis and Kuehn, 2010). In the context of microbiota derived vesicles, studies started five years ago. Our group has been pioneer in demonstrating the key role of microbiota released vesicles on intestinal homeostasis, using as a model OMVs released by probiotic and commensal *E. coli* strains.

Most of the gut microbiota studies have been carried out by direct stimulation of immune or epithelial intestinal cells with live bacterial suspensions (Ivanov and Littman, 2011). In this context, the probiotic EcN is a bio-therapeutic agent generally recognized as a safe organism for human use, which is routinely used in clinical treatments for the remission of UC (Henker *et al*., 2008). EcN has been

widely studied and its beneficial effects have been proven in different models by direct stimulation with live probiotic (Boudeau *et al*., 2003) (Westendorf *et al*., 2005)(Schroeder *et al*., 2006)(Huebner *et al*., 2011)(Kandasamy *et al*., 2016)(Helmy *et al*., 2017). However, under physiological conditions associated with a non-damaged intestinal barrier, direct contact between commensal bacteria and the epithelium does not exist. Thereby, this model is far away from a normal physiological situation existing within the human gut.

Trying to set a closer model to the real gut situation, our group has focused on the study of OMVs isolated from gut microbiota strains, such as the probiotic *Escherichia coli* Nissle 1917 (EcN) and the commensal *E. coli* strains (EcoR12 and EcoR63). Our objective was to assess whether OMVs from these microbiota strains could mediate both barrier strengthening and immunomodulatory effects on IECs. Our first contribution was to demonstrate that OMVs are capable to diffuse through the intestinal mucus layer, and to be internalized into host IECs by clathrin-mediated endocytosis, getting access to lysosomes via endocytic compartments. Moreover, it has been proven that EcN and EcoR12 OMVs do not affect host cell viability nor cause oxidative damage on DNA (Cañas *et al*., 2016).

Using *in vitro* models of epithelial barrier, we have also described the positive potential of both soluble secreted factors and OMVs released by EcN and EcoR63 in the reinforcement of the intestinal epithelial barrier. Both extracellular fractions secreted by these strains are able to reinforce TJ proteins associated to a healthy intestinal barrier, promoting upregulation of ZO-1 and claudin-14 and downregulation of claudin-2. The TcpC protein is among the soluble secreted factors responsible for this effect. However, OMVs, which do not contain TcpC, should mediate reinforcement of the epithelial barrier through other effectors. These effects are strain-specific as OMVs released by the commensal ECOR12 did not increase the epithelial barrier function (Álvarez *et al*., 2016). Moreover, under conditions of intestinal barrier damage caused by pathogens like EPEC, preliminary studies have shown that EcN OMVs also protect from intestinal barrier disruption by counteracting downregulation of the TJ junction proteins occludin and claudin-14, and impairing membrane delocalization of ZO-1.

The main objective of this thesis has been to prove the immunomodulatory effects of EcN OMVs in different *in vitro* and *in vivo* models. For comparison, some studies have been also performed with OMVs released by the commensal ECOR12

strain. First studies, which correspond to chapter 1 of this work, were performed in *in vitro* cell cultures and *ex vivo* models to assay whether EcN and EcoR12 OMVs could mediate immune modulation. For this purpose, three different models that mimic damaged or intact intestinal epithelial barrier were used: a) direct stimulation of PBMCs as a model of damaged intestinal barrier, b) Co-culture of Caco- 2 (apical)/PBMCs (basolateral) as an *in vitro* model of intact intestinal barrier; and c) the *ex vivo* model of human colonic organ culture. In all these models, a representative group of inflammatory markers was analysed. Results from the co-culture model evidenced that underlying immune cells are stimulated by soluble factors released from epithelial cells challenged by apical exposure to OMVs. Remarkably, this study showed for the first time the ability of microbiota vesicles to mediate signalling events through the intestinal epithelial barrier. Likewise, *ex vivo* experiments with colonic fragments confirmed that EcN and ECOR12 OMVs elicit an immune response in the intestinal mucosa, triggering upregulation of MIP1α, IL-10, TNF-α, IL-6 and IL-8 expression. In colonic mucosa vesicles from these strains also promoted upregulation of host mediators that contribute to the reinforcement of the intestinal barrier such as the antimicrobial peptide hBD-2, and IL-22. This cytokine, mainly expressed by immune cells, targets epithelial cells and reinforces the intestinal barrier, thus limiting the access of microbial compounds and allergens to the systemic circulation. Overall, results from different approaches carried out in our laboratory indicate that EcN OMVs contribute to reinforcing the epithelial barrier integrity through several mechanisms, either directly through transcriptional regulation of TJ proteins (Cañas *et al*., 2016) or indirectly through regulation of IL-22 (this thesis, (Fábrega *et al*., 2016)).

Regarding the inflammatory cytokine profile elicited by both EcN and ECOR12 OMVs, the effects were highly comparable. However, the ratio of anti-inflammatory (IL-10) *versus* pro-inflammatory (IL-22 or TNF-α) mediators indicate that OMVs from the probiotic EcN display a better anti-inflammatory balance than OMVs from the commensal ECOR12. This fact, led us to test whether EcN OMVs could mediate the *in vivo* anti-inflammatory effects of this probiotic. In fact, the anti-inflammatory potential of EcN bacterium and its beneficial effects in the remission of UC have been extensively proven and described in different *in vivo* models from a long time ago (Grabig *et al*., 2006a)(Ukena *et al*., 2007)(Garrido-Mesa *et al*., 2011)(Olier *et al*., 2012)(Souza *et al*., 2016)(Arribas *et al*., 2009).

However, the specific mechanism by which this probiotic exerts its anti-inflammatory effect has not been yet completely elucidated. In this scenario, we went further and demonstrated by the first time, that the *in vivo* EcN anti-inflammatory effect is mediated through released OMVs (Chapter 2). Thus, we used the experimental model of DSS-induced colitis in mice as it is a well-proven model of UC. Mice were orally administered with 5 µg of EcN vesicles following an experimental protocol that included a 10-day pre-treatment period, followed by 5-day colitis induction and 5-day recovery periods. The OMVs dose was selected according to a previous study carried out with OMVs from the commensal *B. fragilis* (Shen *et al*., 2012). In this experimental model EcN OMVs exerted anti-inflammatory properties, reducing colonic damage and improving the health status of mice, thus confirming our hypothesis. Moreover, in addition to counteract altered expression of inflammatory cytokines, EcN OMVs were also able to reduce the expression of inflammation-linked enzymes like COX-2 and iNOS that are upregulated in colitis. Besides, EcN OMVs also ameliorated colitis damage by restoring some markers of barrier function. For instance, EcN OMVs increased the expression of the bioactive peptide TFF-3, involved in preserving the mucosal barrier. Nevertheless, opposite to this last result, at the dose of vesicles tested (5 µg/day) we did not observe significant changes in the expression levels of TJ proteins with respect to non-treated colitic mice. It should be stressed that in previous experiments in T-84 cell monolayers addressed to analyse the effect of EcN OMVs on TJ proteins, vesicles were used at higher doses (100 µg/ml). Under these conditions, EcN OMVs upregulate expression of claudin-14 and ZO-1. Accordingly, preliminary *in vivo* assays performed in the same DSS colitis model showed that higher doses of OMVs (20 µg/day), were able to prevent DSS-induced downregulation of occludin and ZO-1, although the imbalance in pro-inflammatory cytokines such as IL-12 could not be counteracted. In general, higher doses of EcN OMVs elicited a worst inflammatory balance in colitic mice. In this sense, and far from the real intestinal context, an excess of OMVs, which contain a big range of PAMPs such as LPS, could negatively affect the inflammatory response triggered in a colitis model. Thus, assessing and stablishing a correct OMVs dose is a key factor to achieve a beneficial response against inflammatory pathologies.

Regarding the effector molecule responsible for the anti-inflammatory effects of the probiotic EcN, some studies pointed to colibactin. Colibactin is a non-

ribosomal peptide-polyketide, synthesized by enzyme activities encoded in the *pks* island, which belongs to a largely uncharacterized family of small genotoxic molecules. Studies performed with an EcN mutant deficient in colibactin synthesis proved that colibactin is required for the *in vivo* anti-inflammatory effects of this probiotic, and that deficiency in colibactin biosynthesis leads to higher colitis severity in DSS-treated mice (Olier *et al*., 2012). For this reason, colibactin has been proposed to be an immunomodulin. How this toxin is exported remains unknown. Our results proving that EcN OMVs exert, like the probiotic itself, anti-inflammatory effects in the experimental colitis model suggest that colibactin could be secreted and delivered to host cells by OMVs. However, involvement of other probiotic specific OMV-associated factors cannot be discarded.

Most of the immunomodulatory responses that gut microbiota elicits on the underlying epithelium are triggered by external interactions of bacterial components with host cell receptors located in the plasma membrane, such as TLR4 or TLR5 that recognize LPS and flagellin respectively (Hayashi *et al*., 2001)(Takeuchi *et al*., 1999). However, also cytosolic receptors such as NLRs have an important role in modulating host cell responses to microbiota (Franchi *et al*., 2009). Activation of NOD receptors through interaction with bacterial PG has been widely studied in invasive pathogen models, in which pathogen internalization allows delivery of PG into the cytosol of the infected cells. Besides, apart from a direct entry of invasive bacteria, different pathways for PG delivery into host cells have been reported in non-invasive pathogens, such as OMVs uptake by endocytosis, injection of PG through bacterial secretion systems or uptake of PG fragments through pH sensitive transporters located on the host cell membrane (Kaparakis-Liaskos, 2015). The discovery of these non-invasive new routes for PG delivery has opened new perspectives to study the possible modulation of host functions through cytosolic receptors by gut microbiota. In fact, recent *in vivo* studies have evidenced the key role of NOD1 and NOD2 receptors in maintaining intestinal homeostasis (Philpott *et al*., 2013). In addition, we have shown that EcN and EcoR12 OMVs are internalized by host epithelial cells through clathrin-mediated endocytosis, thus getting access to the host cytoplasm. Once inside the target cell, OMVs can interact with cytosolic NLRs through their PG component and trigger specific signalling pathways that lead to the activation of the transcription factors NF-κB or AP-1, which are master regulators of the inflammatory response.

In fact, preliminary results obtained by our group showed that NOD-1 silencing through specific small interfering RNAs (siRNA), but not NOD-2 silencing, significantly reduces the OMVs induced expression of pro-inflammatory cytokines IL-6 and IL-8 in Caco-2 cells, thus indicating that NOD-1 is involved in the intracellular signalling activated by EcN and EcoR12 OMVs (Cañas Pachecho, 2017). It should be noted that Caco-2 cells express very little TLR4 and its co-receptor MD-2 (Abreu *et al*., 2001; Kim *et al*., 2004). Therefore, this cell line is suitable for studying specific NOD-activated pathways since interference of other main TLR dependent inflammatory pathways activated by vesicle components such as LPS is circumvented. Starting from these preliminary evidences, this thesis project was gone further to confirm activation of the NOD1 signalling pathway by EcN and ECOR12 OMVs. Chapter 3 of this thesis collects the new contributions to this study. First, we approached RIP2 inhibition analysis. RIP2 a key protein in the NOD signalling pathway that starts the phosphorylation cascade that leads to NF-κB translocation into the nucleus to activate transcription of pro-inflammatory cytokines and mediators. It is known that NOD receptors interact with OMV-associated PG on early endosomes. This interaction activates NOD, which self-associates forming oligomers that recruit RIP2, which in turn phosphorylates components of the IKK complex, resulting in the activation of the NF-κB inflammatory response (Irving *et al*., 2014). Results from this thesis confirm activation of this NOD-1 pathway by *E. coli* commensal and probiotic OMVs: (i) RIP2 inhibition abolishes, like NOD-1 silencing, the IL-6 and IL-8 responses induced by OMVs in Caco-2 cells, (ii) both NOD1 silencing and RIP2 inhibition impaired OMV-mediated activation of NF-κB (measured by degradation of IκBα), and (iii) visualization of activated NOD1 aggregates by confocal fluorescence microscopy in cells treated with EcN and ECOR12 OMVs. Although, at 6-hour post incubation both EcN and ECOR12 OMVs yielded same activation level of the IL-6 and IL-8 responses, the NOD1 activation timing profiles were different between both OMVs samples. Both NOD1 aggregation and IκBα degradation followed a different activation timing profile depending on whether OMVs were from the probiotic EcN or the commensal ECOR12, being slower for ECOR12. This fact suggests that other vesicular components, like for instance the K5 capsular antigen, may affect PG interaction with NOD1. In addition, co-localization analysis by confocal fluorescence microscopy corroborated that internalized OMVs are directed

towards early endosomes. In addition, these experiments showed that NOD1 is specifically recruited to early endosomes by internalized OMVs, and that interaction between the PG contained in OMVs and NOD1 takes place at the membrane of this endocytic compartment. Thus, this work demonstrates by the first time that like pathogenic OMVs, vesicles from commensal and probiotic strains that are internalized via clathrin-mediated endocytosis can modulate intestinal functions and exert their effects through intracellular signalling pathways that involve activation of NOD cytosolic receptors. These results are consistent with those from the co-culture model of intact epithelial barrier (chapter 1) that showed indirect activation of immunocompetent cells by apical stimulation of epithelial cells with EcN or ECOR12 OMVs. Internalized OMVs by epithelial cells activate NOD1 signalling pathways that lead to secretion of IL-8 and other chemokines, which in turn activate underlying immune cells.

In another context, it is well-known that the probiotic EcN exerts antagonistic effects on pathogens by different mechanisms like growth inhibition or interfering with the mechanism of pathogenesis. Regarding this last mechanism, it has been reported that EcN inhibits the secretion of virulence factors by certain pathogens such as enterohemorrhagic *E. coli* (EHEC) (Rund *et al*., 2013) or enteropathogenic *E. coli* (EPEC) (Kleta *et al*., 2014). Studies performed with EPEC showed that the effect was attributed to EcN supernatant (SN), and thus being probably mediated by a secreted factor (*Kleta et al*., 2014). In this context, preliminary results from our group (Álvarez Villagómez, 2017) have shown that both OMVs and soluble factors released by commensal and probiotic *E. coli* strains are able to protect against the intestinal epithelial damage caused by EPEC, reinforcing the intestinal barrier. Additionally, studies performed by Schierack *et al*. 2011, showed that secreted factors released by EcN bacterium and other *E. coli* strains were also able to interfere with invasion of some enteroinvasive pathogens (Schierack *et al*., 2011). Considering these approaches, we planned to analyse whether EcN OMVs could be able to prevent infection by the enteroinvasive pathogen *Salmonella*, and results are collected in Chapter 4. This study was carried out at the University of Würzburg (Germany), as part of a four month stay under the supervision of Dra. Ana Eulalio. However, experiments performed during this stay abroad did not provide positive results, as no effect of EcN OMVs against *Salmonella* invasion was evidenced. Thereby, and taking into account that EcN OMVs specifically reinforce TJ proteins,

we tested EcN vesicles in a *Shigella* model, since unlike *Salmonella*, its spreading during infection is through TJs disruption. Unfortunately, our results showed that other secreted factors, but not EcN OMVs, are responsible for inhibition of *Salmonella* and *Shigella* invasion. After this research period, this study was continued and extended in our laboratory at the University of Barcelona, using co-culture models to verify the beneficial effect of EcN secreted factors against *Salmonella* infection. As a new contribution, we demonstrated that these still unknown factors are not proteins, DNA or RNA molecules, and they inhibit *Salmonella* invasion without affecting adhesion to the host cell. Some authors have attributed this inhibitory effect to a transcriptional downregulation of some virulence proteins such as HilA the main activator of T3SS genes encoded in *Salmonella* pathogenicity island SPS-1 (Schierack *et al*., 2011). Proteins encoded in this gene island are essential for the invasion process. In this sense, we performed bacterial culture experiments in which *Salmonella* was grown either in fresh medium (DMEM) or in filtered EcN culture SNs (EcN conditioned medium), and the profile of secreted proteins were analysed by SDS-PAGE. Results showed a general, but very slight reduction, of secreted *Salmonella* virulence factors when this strain was grown in the presence of soluble factors released by EcN. This reduction might be compatible with downregulation of *Salmonella* HilA caused by EcN factors. However, in experiments carried out in the co-culture model, the incubation time of *Salmonella* in the presence of EcN soluble factors is very short (only 1 hour), and thus insufficient to explain the effect only on terms of transcriptional downregulation. In addition, inhibition of *Salmonella* infection by EcN SNs was lost by washing steps that remove the presence of EcN secreted compounds. Overall, these results suggest that the 50% reduction of *Salmonella* invasion observed in the co-culture model can be due to direct interaction of soluble EcN factors with components of the *Salmonella* T3SS, thus interfering with the secretion of virulence factors through T3SS, and consequently less *Salmonella* invasion effectors are injected into the infected host cell. Alternatively, direct blocking of host cell surface components by secreted EcN factors cannot be ruled out. Furthermore, these experiments performed with other *E. coli* strains yielded similar results, thereby this inhibitory effect is non-probiotic specific.

Although treatment with EcN OMVs did not promote any significant change in *Salmonella* invasion (assessed by intracellular bacterial counts) we hypothesized

that EcN OMVs could modulate immune responses in host cells that result in amelioration of the inflammatory response associated with *Salmonella* infection. This hypothesis was based on previous results that showed the immunomodulatory role of EcN OMVs in epithelial and immune cells, as well as in *in vivo* models of UC (Chapters 1 and 2). We have shown that EcN OMVs modulate expression of cytokines and chemokines, but miRNAs could also be target of the regulation mediated by EcN vesicles.

The group of Dra Ana Eulalio is a leading group in the study of host miRNAs modulated by pathogens. Thus, we took advantage of the research stay in Dra Eulalio laboratory to evaluate whether EcN OMVs could modulate miRNAs in immune cells (monocytes or macrophages), specifically key miRNAs linked to inflammatory responses. Results showed a significant upregulation of miR-146a, which is activated by LPS present in EcN OMVs through TLR4. According to previous results, EcN OMVs, through TLRs (cell membrane receptor) and NOD-1 (intracellular receptor) signalling pathways, activate NF-κB translocation into the nucleus and the transcription of pro-inflammatory cytokines, as well as miR-146a. This miRNA has been described to provide a feedback regulation of gut inflammation through negative regulation of TRAK-6/IRAK-1 and NALP3 inflammasome, thus controlling inflammation. Activation of miR-146a by EcN OMVs might be one of the multiple mechanisms used by EcN OMVs to exert their beneficial anti-inflammatory effects.

Research information presented in this thesis work has contributed to expand our knowledge regarding how intestinal microbiota is able to modulate host cell responses to keep gut homeostasis, preventing pathological disorders. In this scenario, we have demonstrated the key beneficial role of secreted factors and OMVs released by commensal EcoR12 and probiotic EcN strains, eliciting anti-inflammatory responses through the activation of the intracellular host immune receptor NOD1 (Figure 16). Unlike living bacteria, OMVs are non-replicative bacterial forms. This fact is a beneficial factor to consider regarding the use of probiotics in therapy. In this sense, administration of extracellular vesicles (instead of living probiotic) diminishes the risk of uncontrolled infection like sepsis, especially in immunocompromised patients.

Thereby, isolated EcN OMVs (either alone or in combination with secreted soluble factors) could represent a new therapeutic treatment with a high bio-safety

level to prevent and ameliorate inflammatory conditions associated with intestinal disorders. This potential formulation fits into the term postbiotic, a group of new potential treatments based on probiotic or microbiota products that is nowadays.

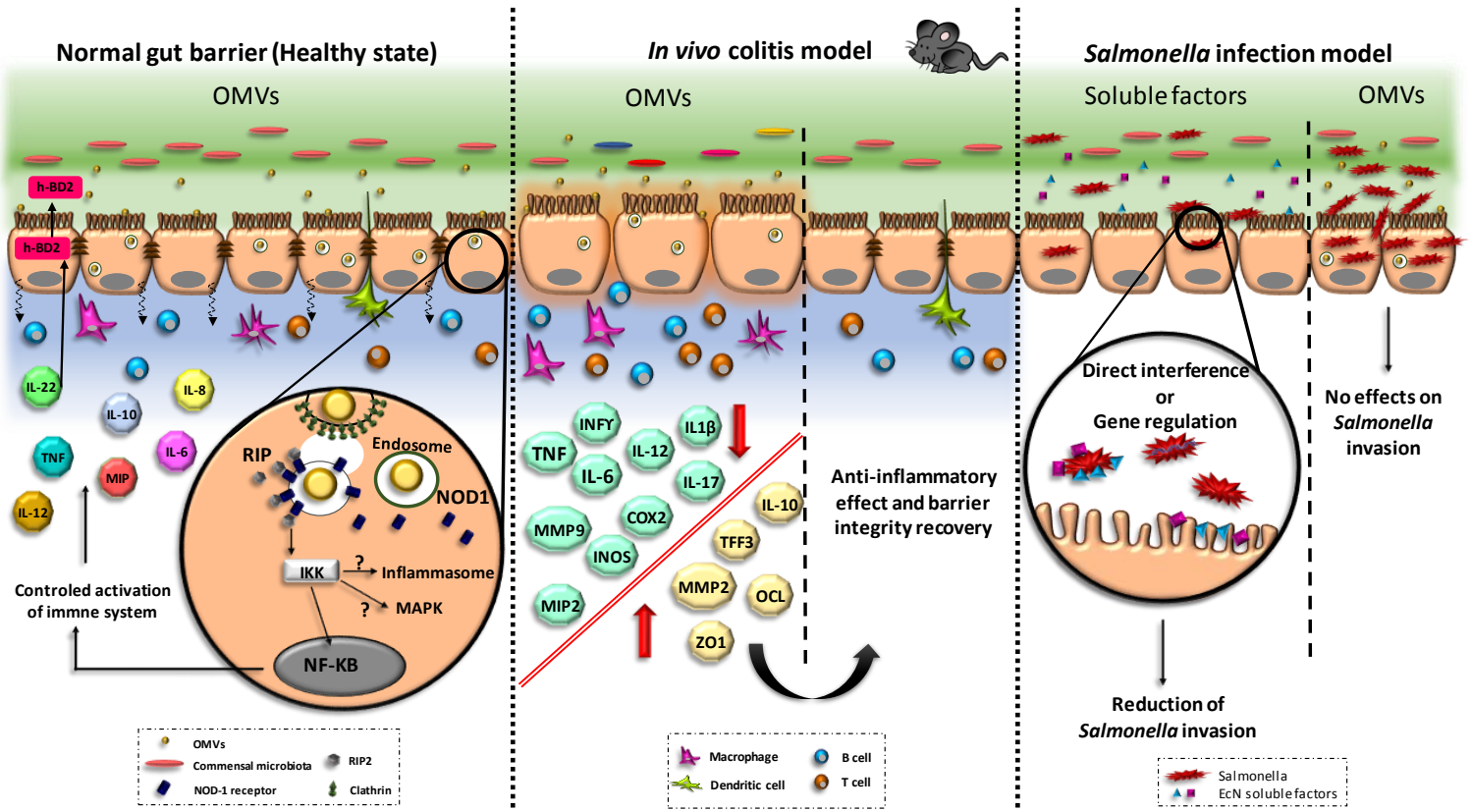

Figure 16. Graphical summary. This graphic summarizes and integrates the relevant contributions of this thesis work, including the immunomodulatory effects and signalling pathway triggered by EcN OMVs in cellular models (left panel), the *in vivo* anti-inflammatory effects in a DSS colitis model in mice (middle panel), and the implication of secreted soluble factors released by EcN on *Salmonella* infection model (right panel).

8. CONCLUSIONS

Immunomodulatory effects of ECN and ECOR 12 OMVs in cellular models of intestinal barrier

1. Direct stimulation of immune cells (a model of disrupted intestinal barrier) with OMVs released by the probiotic EcN or the commensal ECOR12 activates secretion of pro-inflammatory cytokines TNF-α, IL-6 and IL-8, the chemokine MIP1α, and the anti-inflammatory cytokine IL-10. In this model, bacterial lysates also trigger activation of these cytokines, even to a higher extent.

2. Apical stimulation of Caco-2/PBMCs co-cultures (an *in vitro* model that mimics intact intestinal barrier) with EcN or ECOR12 OMVs activates immunomodulatory responses in the underlying immunocompetent cells, whereas bacterial lysates fail to mediate any response.

3. Experiments with human colonic explants (an *ex vivo* model closer to the *in vivo* conditions) confirmed the great potential of EcN and ECOR12 OMVs to modulate the immune response in intact intestinal mucosa, being the expression profile for IL-10, MIP1α, TNF-α, IL-6 and IL-8 similar to that from the *in vitro* co-culture model. In addition, OMVs downregulated the pro-inflammatory cytokine IL-12.

4. Values of IL-10/IL-12 and IL-10/TNFα ratios indicate that OMVs from the probiotic EcN elicit better anti-inflammatory balance than ECOR12 OMVs.

5. Stimulation of colonic explants with EcN or ECOR12 OMVs also increases expression of mediators that reinforce the intestinal barrier such as IL-22 and the antimicrobial peptide β-defensin. In addition, OMVs from both microbiota strains downregulate expression of TGF-β and MUC-1, known to be increased in colon cancer.

Immunomodulatory effects of ECN OMVs in experimental models of colitis in mice

1. The anti-inflammatory effect of EcN OMVs was corroborated in the DSS-induced colitis model in mice. Oral administration of purified EcN OMVs (5 μg/day) significantly reduces weight loss and ameliorates clinical symptoms and histological scores.

2. In addition, OMVs treatment counteracts the altered expression of cytokines associated with colitis damage, specifically reduces the pro-inflammatory cytokines IL-1β, TNF-α, IL-6, MIP-2, INFϒ, IL-12 and IL-17, but increases expression of anti-inflammatory IL-10. EcN OMVs also neutralize the increased expression of the inflammatory enzymes COX-2 and iNOS.

3. EcN OMVs administration also improves the intestinal barrier function by increasing expression of TFF-3 and downregulating MMP-9.

Activation of immune cytosolic NOD receptors by EcN and ECOR12 OMVs

1. NOD1, but not NOD2, is essential for the immune responses mediated by EcN or ECOR12 OMVs in intestinal epithelial cells. Both NOD1 silencing and RIP2 inhibition significantly abolish OMV-mediated activation of NF-κB and the subsequent IL-6 and IL-8 responses.

2. Internalized OMVs, through their PG component, promote association of NOD1 to early endosomes. This intracellular trafficking allows NOD1 interaction with OMVs and thus activation of the NOD1 downstream signalling pathway.

Interference of EcN secreted factors with *Salmonella* infection

1. Soluble factors secreted by EcN reduce *Salmonella* invasion, without affecting its adhesion or intracellular growth in intestinal epithelial cells. Secreted OMVs do not contribute to this effect, which is shared by other *E. coli* strains.

2. The secreted interference factor(s) are neither proteins, nor DNA or RNA molecules. The active factor(s) interfere with the secretion of *Salmonella* invasion proteins through T3SS. Thus, reduced translocation of inv proteins across the membrane of the infected cell negatively impacts *Salmonella* invasion stages

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10. APPENDIX

Table 2. Classification of cytokines and chemokines. Information was collected from Turner, 2014; and Tato, 2008a, 2008b, 2008c and 2008d.

Inflammatory mediator	Source	Targets	Major Function
Pro-inflammatory cytokines			
IL-1α/β	Macrophages, B cells, T cells, DCs	Macrophages, NK cells, T cells, B cells, CNS, others	Inflammatory Promotes activation, co-stimulation and secretion of cytokines and other acute-phase proteins. Cell proliferation and differentiation Pyrogenic
IL-2	T cells	T, B, NK cells and macrophages	Proliferation and activation of target cells Enhancement of cytotoxicity INFγ secretion Antibody production
IL-3	T cells, NK cells, mast cells and eosinophils	Hematopoietic progenitors (stem cells), macrophages and mast cells	Hematopoietic precursor proliferation and differentiation Differentiation and survival of lymphoid and myeloid cells
IL-4	Th cells, mast cells	T cells, B cells, macrophages, monocytes	Proliferation of B and cytotoxic T cells Differentiation of Th2 Promotion of IgG and IgE production Inhibition of cell-mediated immunity Th17 development Enhance MHC class II expression
IL-5	Th2 cells	Eosinophils, B cells	Proliferation, maturation and activation Stimulation of IgA and IgM production Differentiation into plasma cells (IgG production) Hallmark of Th2 effector cells
IL-6	Macrophages, Th cells, fibroblasts and others	B cells, T cells, thymocytes, myeloid cells, osteoclast	Inflammatory and co-stimulatory action Induction of proliferation and differentiation of B cells (IgG production.

			Synergistic action with TGF-β to drive Th17
IL-7	Thymic stromal cells, epithelial cells, bone marrow and spleen	B cells, T cells, thymocytes (stem cells)	Homeostasis Differentiation Survival B and T cell growth factor
IL-9	T cells (Th2)	T cells, mast cells, neutrophils, epithelial cells	Growth and proliferation Promotion of Th2 cytokine secretion
L-11	Stromal cells	Hematopoietic stem cells, B cells, megakaryocytes	Differentiation and proliferation Induction of acute phase proteins
IL12 (p35+p40)	Macrophages, DCs, B cells, neutrophils, T cells	T cells, NK cells	Differentiation and proliferation Promotion of Th1 responses Cytotoxicity (activation of NK cells)
IL-13	T cells	B cells, macrophages, others	Globet cell activation in lungs and gut Proliferation and promotion of IgE production Regulation of cell-mediated immunity
IL-14	T cells	B cells	Promotion of B cell growth
IL-15	Broad expression in hematopoietic cells	T cells, NK cells, epithelial cells, others	Proliferation and survival Cytokine production
IL-16	T cells, eosinophils, mast cells	$CD4^+$ T cells	Recruitment of $CD4^+$ T cells
IL-17A	Th17 cells and others	Mucosal tissues, epithelial and endothelial cells	Pro-inflammatory Protective immunity in lung Tight junction integrity Mobilization of neutrophils and cytokine production by epithelial cells Promotion of angiogenesis
IL-18	Macrophages, others	Th1 cells, NK cells, B cells	Pro-inflammatory Induction of INF-γ
IL-19	Monocytes, others	Keratinocytes, other tissues	Pro-inflammatory
IL-20	Monocytes, others	Keratinocytes, other tissues	Pro-inflammatory
IL-21	Differentiated Th cells (Th2 and Th17 subsets)	T cells, B cells, NK cells, DCs	Proliferation of T cells Differentiation of B cells and NK cytotoxicity

IL-22	Th1 and Th17 cells, NK cells	Fibroblast, epithelial cells	Inflammatory Antimicrobial
IL-23 (p19+940)	Macrophages and DCs	T cells	Inflammatory Proliferation of Th17 cells
IL-24	Monocytes, $CD4^+$ T cells	Keratinocytes	
Il-27(p28+EBI3)	Activated DCs	T cells, others	Induction of early Th1 differentiation by stimulating expression of the Tbet transcription factor Inhibition of effector Th17 cell responses by inducing STAT-1-dependent blockade of IL-17 production
IL-31	Activated T cells	Myeloid progenitors, lung epithelial cells, keratinocytes	Pro-inflammatory
IL-33	Macrophages, DCs	Mast cells, Th2 cells	Co-stimulation Promotion of Th2 cytokines production
TNF-α	Macrophages, monocytes, T cells, others	Neutrophils, macrophages, monocytes, endothelial cells	Inflammatory Promotion of activation and production of acute-phase proteins (endotoxic shock) Phagocyte cell activation
G-CSF	Macrophages, fibroblast, endothelium	Committed progenitors (stem cells in bone marrow)	Differentiation, activation and production of granulocytes
GM-CSF	T cells, macrophages, fibroblast, others	Macrophages, granulocytes, DCs and progenitors (stem cells)	Inflammatory Induction of activation Differentiation, growth and survival Granulocyte, monocyte, eosinophil production
INF-γ	Th1 cells, NK cells, CD8 T cells	Macrophages, NK cells, T cells, others	Activation of APCs and cell-mediated immunity Increase MHC class II expression on cells Anti-viral activation Increase neutrophils and monocyte function
Anti-inflammatory cytokines			
IL-10	Differentiated Th cells, Treg cells, B cells, DCs and others	Macrophages, T cells, DCs, B cells	Immune suppression and anti-inflammatory (inhibition of cytokine production and mononuclear cell function) Decrease antigen presentation and MHC

			class II expression of DCs Downregulation of pathogenic Th1, Th2 and Th17 responses
TGF-β 1,2,3	T cells, DCs, macrophages, B cells, others	All leukocytes population (activated T and B cells)	Regulatory (inhibit T and B cell proliferation and haematopoiesis) Inhibition of growth and activation of Treg maintenance Synergism with IL-6 to promote Th17 Promotion of wound healing
Chemokines			
IL-8 (CXCL8)	Monocytes, macrophages, fibroblasts, keratinocytes, endothelial cells	Neutrophils, naïve T cells	Mobilization, activation and degranulation of neutrophils (chemotaxis) Pro-inflammatory Angiogenesis
MIP-1α	T cells, mast cells, fibroblasts	NK cells, T cells, basophils, DCs	Competition with HIV-1 Antiviral defence Promotion of Th1 immunity
MCP-1 (CCL2)	Monocytes, macrophages, fibroblasts, keratinocytes	Monocytes, NK and T cells, basophils, DCs	Activation of macrophages Basophil histamine release Promotion of Th2 immunity
RANTES (CCL5)	T cells, endothelium, platelets	Monocytes, NK and T cells, basophils, eosinophils, DCs	Degranulation of basophils Activation of T cells Chronic inflammation