Universitat Autònoma de Barcelona

Toll-like receptors as modulators of intestinal barrier function

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Toll-like receptors as modulators of intestinal barrier function

By

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A dissertation in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

Neuroscience Doctoral Program
Department of Cell Biology, Physiology and Immunology
Neuroscience Institute (Inc)
Universitat Autònoma de Barcelona

Advisor

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Bellaterra (Barcelona). September, 2016

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I hereby certify that the thesis entitled "*Toll-like receptors as modulators of intestinal barrier function*", submitted by **JAVIER ESTÉVEZ MEDINA** in partial fulfillment of the requirements for the degree of Doctor of Philosophy was carried out under my supervision and I authorize the submission to undertake its oral defense.

In witness whereof, I hereby sign this document.

Bellaterra (Barcelona). September, 2016.

Vicente Martínez Perea, DVM, PhD.
Ph.D. Advisor

This work has been funded by the Spanish “Ministerio de Ciencia e Innovación” (project BFU2009-08229), by Generalitat de Catalunya (grant 2014SGR789), and the FPU program of the Spanish “Ministerio de Educación, Cultura y Deporte” (reference FPU12/00620; personal support to Javier Estévez Medina).

"Si hay algo en nosotros verdaderamente divino, es la voluntad. Por ella afirmamos la personalidad, templamos el carácter, desafiamos la adversidad, reconstruimos el cerebro y nos superamos diariamente."

Santiago Ramón y Cajal (1852-1934)
Spanish neuroscientist.

Agradecimientos

Durante estos años en la Unidad de Fisiología de la Facultad de Veterinaria he aprendido muchísimas cosas, algunas inherentes a la experiencia de realizar una tesis doctoral, y otras ligadas a la propia vida. Los buenos momentos, y los no tan buenos, me han permitido crecer como investigador, como profesional y, sobre todo, como persona.

En primer lugar, quería dar las gracias a Vicente Martínez, el director de esta tesis, que no ha dudado ni un momento en ponerse la bata y echarme una mano cuando ha sido necesario. Me ha enseñado a tener criterio científico, y a él le debo la posibilidad de haber realizado este trabajo.

A Patri le agradezco el haberme descubierto en sus clases de Fisiología que otras salidas profesionales eran posibles tras licenciarse en Veterinaria, así como el haberme abierto las puertas del Departamento.

Agradecer la amabilidad del resto de profesores de Fisiología, Marcel y Maite, y a Ester sus consejos, su estima y sus charlas distendidas durante las comidas. Al personal administrativo, Pepe y David, agradecerles sus gestiones cuando han sido necesarias. A Antonio le agradezco su ayuda con los pedidos, su apoyo, las risas y las partidas de pádel, ¡que espero que continúen! A Emma su confianza y su ayuda en el laboratorio, su buen trato, el estar siempre dispuesta a escuchar las penas, y las risas a costa de tus “frases míticas”.

Los becarios y residentes, en general, han sido como una gran familia y me llevo muy buen recuerdo de todos. A Joan Antoni le debo la paciencia de enseñarme la técnica de las cámaras de Ussing, que no es poco… También sus infinitos consejos y los momentos de risas en los que daba alas a su humor irónico. A Burgueño le agradezco ser buena persona por naturaleza, estar en momentos importantes de mi vida, colarme en algún partido del Espanyol y, en definitiva, le doy las gracias por estar siempre ahí y ser un gran amigo. A Mònica, por ser mi “Sénior” y por enseñarme que, con ilusión y ganas, uno puede conseguir todo lo que se propone por difícil que pueda parecer. A Sergio Berdún le doy las

gracias por enseñarme que lo que realmente importa es tener claras las metas que uno se propone. A Ferran por las risas con Burgueño en el laboratorio. A Noe, por ser mi amiga y mi compañera durante el Máster y el doctorado, y sobre todo por corroborar que en la vida hay cosas más importantes que la ciencia y los éxitos profesionales, como por ejemplo ser feliz y vivir en paz con uno mismo. También quiero mencionar a Marina, a Eva, a Míriam, a Elena Tapia, a Sandra, a Elena Eyre, a Víctor, a Jakub, a Diana, a Sergio López y a Roberto, que han sido excelentes compañeros.

Quiero dar las gracias a la dirección de ITPSA por su confianza y por permitirme compaginar este último año de doctorado con mi labor en el Departamento Técnico. A Sergi, quiero agradecerle la confianza depositada en mí, su apoyo durante el último año de doctorado, su empatía y el haberme permitido seguir mi vida profesional en I+D+i.

Por supuesto quiero dar las gracias a la persona que más quiero, a mi mujer, Montse. Sin su apoyo jamás podría haber emprendido la aventura de realizar un doctorado. Gracias por quererme y por llevar dentro de ti a lo más bonito de nuestras vidas, nuestra hija Valeria, a quien dedico este trabajo.

Agradecer a mis padres haber luchado sin flaquezas por sus cinco hijos, su amor incondicional, haber sabido motivarnos para estudiar y labrarnos un futuro y, sobre todo, por inculcarnos vuestros valores y darnos todo lo que necesitábamos para ser felices. Estoy orgulloso de vosotros. Por último, dar las gracias a mis sobrinos y sobrinas por alegrarme los días difíciles, y a mis hermanos por su apoyo, su motivación, sus consejos, por confiar siempre en mis posibilidades y por estar orgullosos de mí.

Bellaterra. Septiembre de 2016.

Javier Estévez Medina

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Abbreviations

AJ: Adherens junctions

AJC: Apical junctional complex

APC: Antigen presenting cells

CCh: Carbachol

CD: Crohn's disease

CLR: C-type lectin-like receptors

DAMP: Damage-associated molecular patterns

DC: Dendritic cell

DMSO: Dimethyl sulfoxide

DSS: Dextran sodium sulphate

EBF: Epithelial barrier function

EGF: Epidermal growth factor

ENS: Enteric nervous system

FAE: Follicle-associated epithelium

FD4: FITC-dextran of 4 KDa

FITC: Fluorescein isothiocyanate

G: Conductance

GALT: Gut-associated lymphoid tissue

GCM: Gut commensal microbiota

GLP-2: Glucagon-like peptide-2

IBD: Inflammatory bowel disease

IBS: Irritable bowel syndrome

IEC: Intestinal epithelial cell

IEL: Intraepithelial lymphocyte

IFN: Interferon

IL: Interleukin

IgA: Immunoglobulin A

IgM: Immunoglobulin M

I_{sc}: Short circuit current

JAM: Junctional adhesion molecule

JC: Junctional complex

LPS: Lipopolysaccharide

MLC: Myosin light chain

MLCK: Myosin light chain kinase

NOD: Nucleotide-binding oligomerization domain

PAMP: Pathogen-associated molecular patterns

PCSK: Pam3CysSK4

PD: Potential difference

PRR: Pattern recognition receptors

RIG: Retinoic acid-inducible gene I

RLR: RIG-I-like receptors

SILT: Solitary isolated lymphoid tissue

TGF: Transforming growth factor

TJ: Tight junction

TLR: Toll-like receptor

TNF: Tumor necrosis factor

UC: Ulcerative colitis

VIP: Vasoactive intestinal polypeptide

ZO: Zonula occludens

Summary

Functional (irritable bowel syndrome, IBS) and inflammatory (inflammatory bowel disease, IBD) gastrointestinal disorders are characterized by an altered epithelial barrier function, with an increased permeability, and changes in the intestinal microbiota. Toll-Like Receptors (TLRs) participates in bacterial recognition within the intestine and in local neuro-immune control, thus participating in the regulation of intestinal epithelial barrier function.

The objective of this work has been to characterize the implication of TLR5 and TLR7 in the regulation of colonic epithelial barrier function. For this, colonic epithelial barrier function has been studied *in vitro* (electrophysiology and permeability to macromolecules in a Ussing chamber system), as well as in *in vivo* conditions (permeability to macromolecules), after the local over-stimulation of TLR5 and TLR7 with selective agonists, flagellin and imiquimod, respectively, in rats and mice. The effects on barrier function have been studied in normal conditions, under states epithelial permeabilization with DMSO, and in conditions of inflammation -dextran sulfate sodium (DSS)-induced colitis-. In order to characterize the mechanisms of action, dynamics of tight junction (gene expression -RT-qPCR- and cellular distribution -immunohistochemistry- of tight junction proteins) and the presence of a local immune activation (gene expression of pro-inflammatory cytokines) were assessed.

The results obtained indicate that the *in vivo* over-stimulation of colonic TLR7 improves epithelial barrier function in rats in physiological conditions, with a dose-dependent reduction in epithelial permeability to macromolecules, as assessed in Ussing chambers. However, under conditions of epithelial permeabilization with DMSO, the over-stimulation of TLR7 deteriorates barrier function, as assessed *in vivo.* In mice, the *in vitro* over-stimulation of colonic TLR7 was without effects. However, in a model of DSS-induced colitis, imiquimod reduces inflammation-induced increased epithelial permeability. Therefore, specie-specific differences seemed to exist for the barrier effects associated to the over-stimulation of colonic TLR7, leading to either protective or damaging actions on epithelial barrier function, depending upon the experimental conditions.

The over-stimulation of colonic TLR5 aggravates the barrier dysfunction associated to inflammation (DSS-induced colitis) in mice, increasing the permeability to macromolecules. However, the direct addition of flagellin to the Ussing chambers did not affect epithelial barrier function, neither in physiologic conditions nor during inflammation.

Regardless the conditions considered, TLR5/7-mediated modulatory actions on barrier function were not associated to changes in gene expression of the main tight junction-related proteins (claudin-2, claudin-3, occludin, tricellulin, junctional adhesion molecule 1 and Zonula Occludens 1). Moreover, no changes in the cellular distribution of tight junction proteins (claudin-2, claudin-3 y ZO-1) was observed. Likewise, TLR5/7 over-stimulation was not associated to changes in the expression of the barrier-modulating factors myosin light chain kinase and proglucagon (precursor of glucagon-like peptide 2). Finally, TLR-specific immunomodulatory effects were also observed. Over-stimulation of TLR7 revealed potential protective effects, reducing the expression of the pro-inflammatory cytokine IL12-p40. In contrast, over-stimulation of TLR5 tended to increase the expression of pro-inflammatory markers, thus suggesting pro-damaging effects.

In conclusion, these results provide evidence of the importance of TLRs-dependent host-microbial interactions in the control of intestinal epithelial barrier function. Colonic TLR5 and TLR7 should be considered potential therapeutic targets for the control of barrier function and local immune responses in functional and gastrointestinal disorders, such as IBD and IBS.

Resumen

Las patologías funcionales (síndrome del intestino irritable, IBS) e inflamatorias gastrointestinales (enfermedad inflamatoria intestinal, IBD) se caracterizan por alteraciones de la función barrera epitelial, con un aumento de la permeabilidad, y cambios en la microbiota intestinal. Los receptores de tipo *Toll* (TLRs) participan en el reconocimiento bacteriano en el intestino y en el control neuroinmune local, estando, por tanto, implicados en la regulación de la función barrera del epitelio intestinal.

El objetivo de este trabajo ha sido caracterizar la implicación de los receptores TLR5 y TLR7 en la regulación de la función barrera epitelial del colon. Para ello se ha caracterizado la función barrera epitelial del colon, tanto en condiciones *in vitro* (electrofisiología y permeabilidad a macromoléculas en un sistema de cámaras de Ussing), como *in vivo* (permeabilidad a macromoléculas), tras la sobre-estimulación local de los receptores TLR5 y TLR7 con agonistas selectivos, flagelina e imiquimod, respectivamente, en rata y ratón. Los efectos en la función barrera se han caracterizado en condiciones normales, en estados de permeabilización del epitelio con DMSO, y en condiciones de inflamación (colitis inducida por dextrano sulfato de sodio -DSS-). Con la finalidad de definir el mecanismo de acción, se ha valorado la dinámica de las uniones estrechas epiteliales (expresión génica de proteínas -RT-qPCR- y distribución celular -inmunohistoquímica-) y la activación inmune local (expresión de citoquinas pro-inflamatorias).

Los resultados obtenidos muestran que la sobre-estimulación del TLR7 del colon *in vivo* mejora la función barrera epitelial en la rata en condiciones fisiológicas, observando una reducción dosis-dependiente de la permeabilidad epitelial a macromoléculas evaluada en las cámaras de Ussing. No obstante, en condiciones de permeabilización del epitelio con DMSO, la sobre-estimulación del TLR7 causa un empeoramiento de la función barrera valorada *in vivo*. En ratones, la sobre-estimulación del TLR7 cólico *in vitro* no tiene efecto. Sin embargo, en un modelo de colitis inducida por DSS, reduce el aumento de la permeabilidad epitelial causado por la inflamación. Por tanto, parecen existir diferencias especie-específicas en los efectos de la sobre-estimulación del TLR7 cólico, pudiéndose observar tanto acciones promotoras como lesivas de la función barrera epitelial.

La sobre-estimulación del TLR5 cólico agrava la disfunción de la barrera asociada a la inflamación (colitis inducida por DSS) en el ratón, incrementando la permeabilidad a macromoléculas. Sin embargo, la adición del agonista del TLR5, flagelina, en las cámaras de Ussing no afecta a la función barrera epitelial, ni en condiciones fisiológicas, ni durante la inflamación.

En ningún caso, estos efectos moduladores de la función barrera se asociaron a cambios en la expresión génica de las principales proteínas de las uniones estrechas (claudina-2, claudina-3, ocludina, tricelulina, molécula de adhesión de la unión de tipo 1 y *Zonula Occludens* 1) ni a su distribución celular (claudina-2, claudina-3 y ZO-1). De la misma forma, los factores moduladores de la barrera, quinasa de la cadena ligera de la miosina y pro-glucagón (precursor del péptido similar al glucagón de tipo 2), tampoco presentaron cambios en su expresión asociados a la sobre-estimulación del TLR5 o del TLR7. Finalmente, se observó un efecto inmunomodulador receptor-específico. La sobre-estimulación del TLR7 reveló efectos potencialmente protectores al reducir la expresión de la citoquina pro-inflamatoria IL12-p40. Por el contrario, la sobre-estimulación del TLR5 tendió a aumentar la expresión de marcadores pro-inflamatorios, sugiriendo, por tanto, efectos pro-lesivos.

En conclusión, estos resultados muestran la importancia de las interacciones microbiota-hospedador mediadas por TLRs en el control de la función barrera epitelial intestinal. Tanto el TLR7 como el TLR5 cólicos pueden considerarse potenciales dianas terapéuticas para el control de la función barrera y las respuestas inmunes locales en desórdenes funcionales e inflamatorios gastrointestinales como el IBD y el IBS.

Resum

Les patologies funcionals (síndrome de l'intestí irritable, IBS) i inflamatòries gastrointestinals (malaltia inflamatòria intestinal, IBD) es caracteritzen per alteracions de la funció barrera epitelial, amb un augment de la permeabilitat, i canvis en la microbiota intestinal. Els receptors de tipus *Toll* (TLRs) participen en el reconeixement bacterià a l'intestí i en la regulació de la funció barrera de l'epiteli intestinal.

L'objectiu d'aquest treball ha estat caracteritzar la implicació dels receptors TLR5 i TLR7 en la regulació de la funció barrera epitelial del colon. Amb aquesta finalitat, s'ha caracteritzat la funció barrera del colon, tant en condicions *in vitro* (electrofisiologia i permeabilitat a macromolècules en un sistema de cambres d'Ussing), com *in vivo* (permeabilitat a macromolècules), després de la sobre-estimulació local dels receptors TLR5 i TLR7 amb agonistes selectius, flagelina i imiquimod, respectivament, en rata i ratolí. Els efectes en la funció barrera epitelial del colon s'han caracteritzat en condicions normals, en estats de permeabilització de l'epiteli amb DMSO, i en estats d'inflamació (colitis induïda per dextrà sulfat de sodi -DSS-). Amb la finalitat de caracteritzar el mecanisme d'acció, s'ha valorat la dinàmica de les unions estretes epitelials (expressió gènica de proteïnes -RT-qPCR- i distribució cel·lular -immunohistoquímica-) i l'activació immune local (expressió de citoquines pro-inflamatòries).

Els resultats obtinguts mostren que la sobre-estimulació del TLR7 del colon *in vivo* millora la funció barrera epitelial en la rata en condicions fisiològiques, observant una reducció dosi-depenent de la permeabilitat epitelial a macromolècules avaluada a les cambres d'Ussing. No obstant això, en condicions de permeabilització de l'epiteli amb DMSO, la sobre-estimulació del TLR7 causa un empitjorament de la funció barrera valorada *in vivo*. En ratolins, la sobre-estimulació del TLR7 còlic *in vitro* no té cap efecte. En canvi, en un model de colitis induïda per DSS, redueix l'augment de la permeabilitat epitelial causat per la inflamació. Per tant, semblen existir diferències espècie-específiques en els efectes de la sobre-estimulació del TLR7 còlic, observant-se tant accions promotores com lesives de la funció barrera epitelial.

La sobre-estimulació del TLR5 còlic agreuja la disfunció de la barrera associada a la inflamació (colitis induïda per DSS) en el ratolí, incrementant la permeabilitat a macromolècules. No obstant això, la addició directa de flagelina a les cambres d'Ussing no va afectar la funció barrera epitelial, ni en condicions fisiològiques ni durant la inflamació.

En cap cas aquests efectes moduladors de la funció barrera es van associar a canvis en l'expressió gènica de les principals proteïnes que composen les unions estretes (claudina-2, claudina-3, ocludina, tricelulina, molècula d'adhesió de la unió de tipus 1 i *Zonula Occludens* 1) ni a la seva distribució cel·lular (claudina-2, claudina-3 i ZO-1). De la mateixa manera, els factors promotors de la barrera, quinasa de la cadena lleugera de la miosina i pro-glucagó (precursor del pèptid similar al glucagó de tipus 2), tampoc van presentar canvis en la seva expressió associats a la sobre-estimulació del TLR5 o del TLR7. Finalment, es va observar un efecte inmunomodulador receptor-específic. La sobre-estimulació del TLR7 va revelar efectes potencialment protectors, reduint l'expressió de la citoquina pro-inflamatòria IL-12p40. Pel contrari, la sobre-estimulació del TLR5 va tendir a augmentar l'expressió de marcadors pro-inflamatoris, suggerint, per tant, efectes pro-lesius.

En conclusió, aquests resultats mostren la importància de las interaccions microbiota-hoste dependents de TLRs en el control de la funció barrera epitelial intestinal. Tant el TLR7 como el TLR5 còlics poden considerar-se potencials dianes terapèutiques per al control de la funció barrera i les respostes immunes locals en desordres funcionals i inflamatoris gastrointestinals, com són l'IBD i l'IBS.

Introduction

Within the intestine, a large number of pathogens, toxins, antigens and other harmful elements may be present. The organism has to combine the protection against them and the maintenance of intestinal functions in a homeostatic equilibrium. Overall, these harmful elements are located within the intestinal lumen, being the epithelium the barrier that determines their contact with the internal milieu. Thus, a proper intestinal epithelial barrier function (EBF) is essential to maintain homeostasis, allowing an adequate immune response to luminal antigens and, at the same time, the selective passage of molecules through the epithelial layer. Barrier dysfunctions have been related to the pathogenesis of different intestinal pathologies, including functional and inflammatory diseases, mainly Inflammatory Bowel Disease (IBD) and Irritable Bowel Syndrome (IBS), where the permeability of the epithelium is generally increased.

1. The intestine: structure and function

1.1. Anatomy and histology of the intestine

The intestine, classically divided in small and large intestine, presents different physiological functions along its length. The small intestine is divided in different parts that present functional or/and histological differences: duodenum, jejunum, and ileum. The main function of the small intestine is the chemical digestion and the absorption of the nutrients present in the ingested food.[1,2]

The large intestine connects the ileum with the anus and is composed by the cecum, the colon and the rectum. The general and main

function of the large intestine is the resorption of water and the excretion of the non-digestible components of aliments.[1,2] However, other functions such as bacterial digestion and absorption of some fatty acids or nutrients are also performed by this intestinal segment.[1,2]

From a histological point of view, the entire intestinal wall shares the same basic structural organization, composed by the mucosa, the submucosa, the tunica muscularis and the serosa (Fig. 1).[3–5]

Focusing in the large intestine, the mucosa consists in an epithelium, a supporting layer of conjunctive tissue called lamina propria and a thin layer of smooth muscle named muscularis mucosae (Fig. 1).[3,4] The colonic epithelium is simple and columnar, mainly formed by enterocytes (here named also colonocytes) but also by goblet cells and enteroendocrine cells, forming short villi.[3,4] Epithelial cells renews every 3–5 days from pluripotential stem cells, which are located in the crypts and migrate to the villus where final differentiation occurs to assure epithelial functions.[6]

The lamina propria is a reticular fiber framework composed by loose connective tissue which lies beneath the epithelium, surrounding the mucosal crypts, being the support and the nutritive source of the epithelium (Fig. 1). It contains fibroblasts, blood vessels, lymphatic tissue, and immune cells, thus constituting the first immunological barrier of the intestine. This immunological barrier includes resident immune cells (mainly lymphocytes, eosinophils, mast cells, macrophages and neutrophils) and organized lymphoid structures named gut-associated lymphoid tissue (GALT). These immune structures present a higher development in the large intestine vs. the small intestine, reflecting the

higher amount of antigenic contact related with the colonic microbiota.[3,4,7,8]

The *muscularis mucosae* is formed by a layer of smooth muscle cells (Fig. 1).[3,4,8] Its function is to facilitate the secretory and absorptive function of the epithelium.[4]

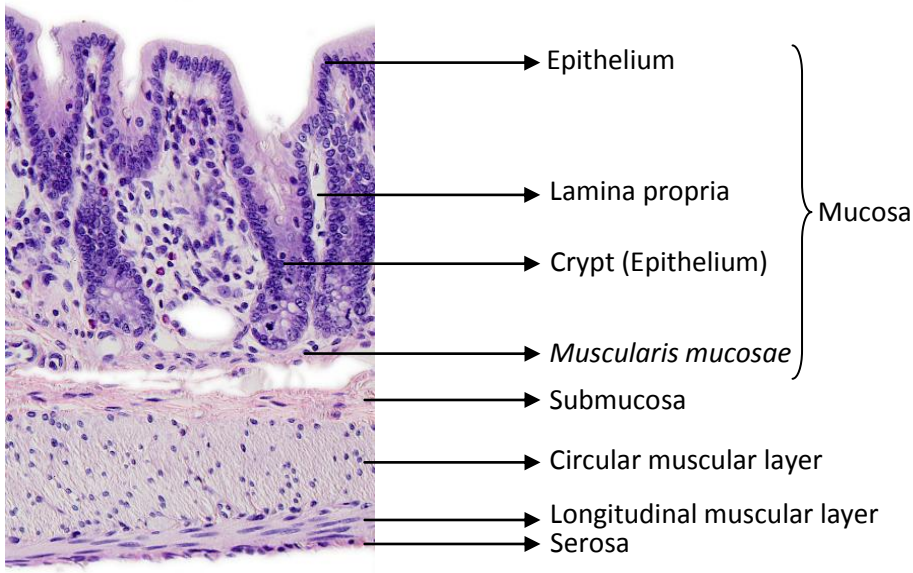

Figure 1. Histological structure of the colon. Microphotography of a rodent colonic histological preparation (Hematoxylin/eosin staining). Adapted from 9.

The submucosa is a dens connective tissue containing lymphatic and blood vessels and a wide nervous net, called the submucous plexus (Fig. 1).

Below the submucosa, the smooth muscle of the intestine is found: the *tunica muscularis*. The *tunica muscularis* has a similar structure along the gastrointestinal tract, being formed by two layers of smooth muscle:

the internal circular layer and, the external longitudinal layer, according to the spatial disposition of their muscle fibers (Fig. 1).[4] Between both muscular layers lays a neural network forming the myenteric plexus.

The serosa, the most external histological layer of the intestine, is formed by a thin layer of loose connective tissue coated by a simple squamous epithelium (mesothelium) (Fig. 1).

1.2. The Enteric Nervous System

The enteric nervous system (ENS) consists on the ensemble of neurons and glial cells that are found in the gastrointestinal wall, forming the intrinsic nervous system of the gastrointestinal tract.

Within the gastrointestinal wall, the ENS is structured in two ganglionated plexuses: the submucous plexus and the myenteric plexus. They are located between the submucosa and the inner circular muscle layer and between the circular and longitudinal muscle layers, respectively, as described above. In general, it is considered that the submucous plexus controls epithelial functions and blood flow, whereas the myenteric plexus is related to the regulation of smooth muscle contraction. However, the enteric nervous system is implicated in the regulations of other gastrointestinal functions, such as local immune responses, sensorial mechanisms and epithelial proliferation and differentiation.[10–13]

1.3. The Intestinal Immune system

The mucosal surface of the intestine is in direct contact with the external environment and exposed to a large variety of food- and

microbial-derived antigens. Therefore, the mucosa represents a first-line immune defense within the gastrointestinal tract. Immune cells, as an important element of the epithelial barrier, are organized forming the GALT. The GALT is composed by the organized immune inductive sites and, on the other hand, by the diffuse effector sites.[14,15]

1. *Diffuse effector sites:*

   The diffuse effector sites are composed by two quite distinct compartments: the intraepithelial lymphocytes (IELs), basically T cells between epithelial cells acting as first-line guards,[14,15] and lamina propria lymphocytes, which co-exist with other immune cells, including eosinophils, dendritic cells (DCs), mast cells, macrophages and plasma cells.[14,15]

2. *Organized immune inductive sites:*

   The organized immune inductive sites are constituted by lymphoid structures formed by the aggregation of immune cells. They include:

   a. *Mesenteric lymph nodes (MLNs).*
   b. *Organized lymphoid follicles:*

      Submucosal structures composed by clusters of B cells and T cells and whose covering epithelium, named follicle associated epithelium (FAE), contains microfold cells (M cells).[12,14,15,18] They can be: i) Macroscopic lymphoid aggregates: Peyer's patches, in the small intestine; caecal patches, around the ileo-caecal valve; and colonic patches in colon and rectum;[45] or ii) Microscopic lymphoid aggregates: commonly known as solitary isolated lymphoid tissue (SILT).[18,19]

Within the gut, the detection of luminal antigens can occur by different mechanisms. A predominant detection system is mediated by M cells localized in organized lymphoid follicles, as mentioned above. The main function of M cells is to monitor the gut lumen with the detection, capture, and transport of luminal antigens to put them in contact with antigen presenting cells (APC), such as DCs.[15,20] Once the antigen is processed by an APC, it is presented to B cells of the lymphoid follicle triggering their differentiation and the expression of immunoglobulins (IgM and IgA).[14,21]

However, the detection of antigens by APCs can be direct, without the intervention of M cells,[22] and the passage of antigens can occur via transcellular or paracellular pathways across the epithelium.[17] Moreover, intestinal epithelial cells (IECs) have been seen to participate in the modulation and initiation of anti-pathogen and tolerance immune responses after sensing luminal antigens by mechanisms dependent on different Pattern Recognition Receptors (PRRs) and mediated by the local production of immunomodulatory factors, such as cytokines.[15,23]

The equilibrium between the activation of immune responses, with induction of pro-inflammatory mediators and immunoglobulins, and T-cell-related mechanisms of tolerance against certain luminal antigens[15] is essential for assuring a proper intestinal barrier function. Altered tolerance responses with exacerbation of local immune response are common component of the pathogenesis of gastrointestinal inflammatory disorders, like celiac disease or IBD, where an altered response to luminal (dietary and microbial) antigens is commonly observed.[20]

2. Intestinal barrier function

The intestinal barrier function is the combination of elements that control the passage of antigens from the lumen to the internal milieu, while allowing the intestine to develop its physiological functions, thus ensuring a homeostatic state.

2.1. Function and components of the intestinal barrier

Although this work is focused in the epithelial paracellular permeability, the intestinal barrier function, as a whole, is formed by different levels of protection or components that influence the passage of macromolecules, or pathogens, to the internal milieu. From the gut lumen to deeper levels, the components of the intestinal barrier include:[24–30]

- Gastrointestinal, pancreatic and biliary secretions
- Gut Commensal Microbiota (GCM)
- IECs-derived antibacterial peptides
- Immunoglobulin A (IgA)
- Mucus layer (secreted by goblet cells)
- Epithelial lining

Just below the epithelial mucus layer we find the intestinal epithelium, a polarized layer of columnar cells (enterocytes, or colonocytes in the colon) joined by intercellular junctions separating the internal milieu from the external environment. This epithelial lining constitutes an essential component of the intestinal barrier function,

acting as a physical barrier to prevent the free passage of pathogens, antigens and other *noxae*. However, other functions of the intestinal epithelium include water secretion and absorption of dietary nutrients, water and electrolytes, thus implying that the epithelial barrier must be selectively permeable.

Several pathways have been described for the passage of solutes and water through the epithelium (Fig. 2). In general, these can be classified in two basic routes: transcellular and paracellular.

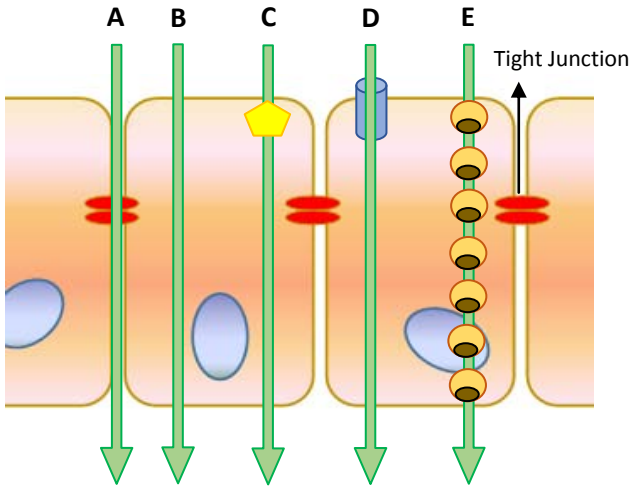

Figure 2. Passage routes across the intestinal epithelium. **A**: Paracellular route, **B**: Transcellular passive diffusion, **C**: Transcellular active carrier-mediated transport, **D**: Transcellular route via aqueous pores, **E**: Transcytosis. Adapted from reference 31.

An example of transcellular passage is what occurs with lipid soluble and small hydrophilic molecules, which may pass through the cellular lipid bilayer via passive diffusion or using aqueous pores. Other molecules use different transcellular ways, like active transport through carriers

(amino acids, sugars and other nutrients) or transcytosis mediated by endosomal vesicles (big molecules, some peptides, proteins and large particles).[31]

In contrast, many medium-sized hydrophilic molecules pass through the intercellular space, using the paracellular route. For instance, several microbial- and dietary-related products have been shown to use this pathway to enter the internal milieu. These mechanisms are of particular interest in the context of the present work because paracellular permeability has been shown to be altered in inflammatory and functional gastrointestinal disorders, thus allowing an increased passage of luminal antigens and the development of abnormal immune responses.[31,32]

Paracellular permeability occurs throughout the paracellular space. The paracellular space is formed by the tissue between epithelial cells, including the specialized structures that joint adjacent epithelial cells, forming the so called junctional complex (JC) (Figs. 3 and 4).[33] JCs are structures formed by:

1. Tight junctions (TJ): the most apical structure of the JC (Figs. 3 and 4) and the main component involved in the control of the paracellular passage of molecules to the internal milieu.[34,35]

2. Adherens junctions (AJ): formed by adhesion molecules of the cadherin family, mainly e-cadherin, and catenins (a, b and c), which acts as binding proteins with actin filaments of the cytoskeleton. AJ are located just below the TJ and their function is to provide an extracellular stabilized adhesive contact to held epithelial cells together.[36,37] Altogether, TJs and AJs form the apical JC (AJC), which,

among other functions, keeps the polarization of the epithelial cells, delineating their apical and their basolateral zone.[14]

3. Desmosomes: dense adhesions between cells, connected with the intermediate filaments of the cytoskeleton.[31] Although desmosomes can be found all over the lateral cell surface, these JC structures are usually concentrated below AJs.

4. Gap junctions: formed by tubular proteins (connexins), these structures allow the communication and interchange of ions and small molecules among adjacent cells.[31]

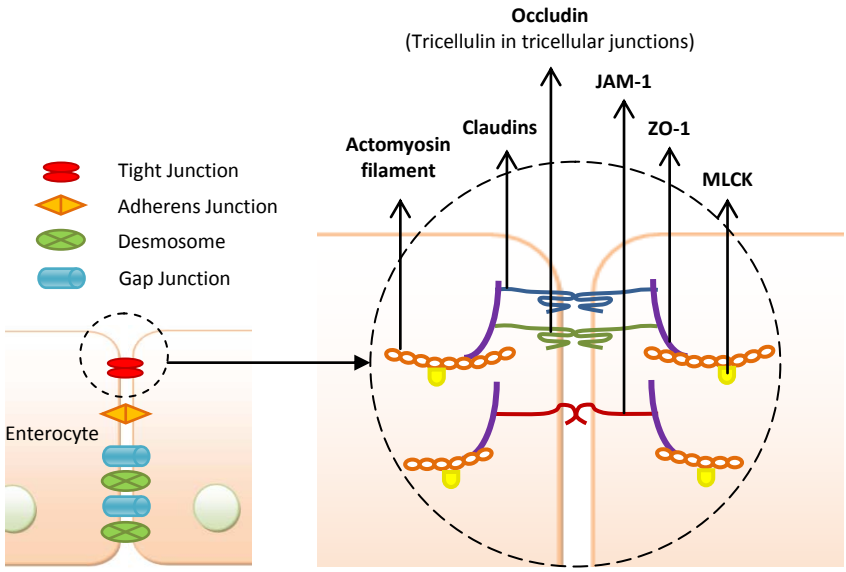

Figure 3. Scheme of the structures composing the junctional complex of the intercellular junctions in the intestinal epithelium.[38–41]

Besides the epithelial lining and the TJ, the last and the most inner component of the intestinal barrier function is the intestinal immune system. As commented above, the lamina propria contains a large number of immune cells that trigger innate and adaptive immune responses, in coordination with the enteric nervous system and the epithelium, to face any *noxae* arriving from the luminal content.

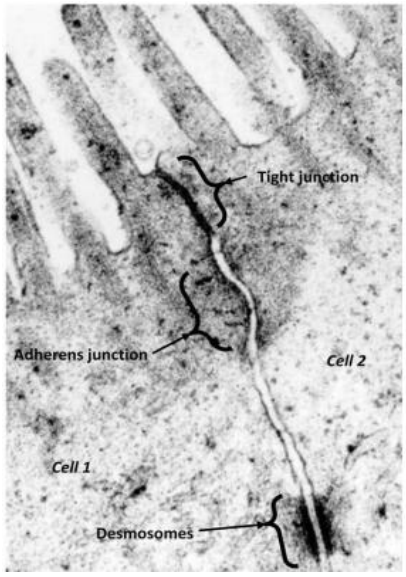

Figure 4. Electron micrograph of the apical junctional complex between two intestinal epithelial cells of the human ileal mucosa.[42]

2.2. Tight Junctions

Tight junctions (TJ), also named zonula occludens, are the most relevant intercellular junctions of the AJC involved in the control of paracellular permeability. As the paracellular permeability is the most important pathway for transepithelial transport, TJs are key structures in the control of intestinal barrier function.[31,43]

As mentioned above, TJs are also essential to maintain the polarity of IECs, preventing the diffusion of membrane components, like membrane receptors and transporters, from the apical to the basolateral cellular side, and *vice versa*. Moreover, TJs coordinate signaling and trafficking molecules related to cell differentiation, proliferation and polarity, thereby serving as a multifunctional complex.[44,45]

TJs are composed by more than 50 proteins, which, according to their distribution can be intracellular or transmembrane proteins:[45]

1. *Transmembrane TJ proteins:*

   Transmembrane TJ proteins mediate the adhesion between epithelial cells, sealing the paracellular space. These proteins can be classified in:

   a. Tetra-span proteins: claudins, occludin and tricellulin belong to this group.[44,46]

      I. Claudins: Claudins are considered the structural basis of the TJ (Fig. 3). There are 24 members of this family, although not all of them have been detected in the gut. Indeed, claudins -2 and -3, which have been the focus of this work, are the most highly expressed in the gastrointestinal tract.[47]

         Claudins are key proteins in the control of intestinal barrier function. In the intestinal epithelium, most claudins have sealing functions and promote a tighten TJ (table 1). However, some of them, particularly claudin-2, are pore-forming proteins, thus promoting paracellular permeability. Claudin-2 is highly expressed in the intestine[48] and it has

been shown to be up-regulated in patients with inflammatory and functional gastrointestinal disorders as well as in different animal models of these pathologies (table 1).[14,48–51]

Table 1. Claudin-type proteins and their functions.

Claudin	Functions	Disorder/disease	Specie
1	Barrier-forming	↑in UC. ↓ in IBS, AC, FA	H/R
2	Cation-selective and water pore-forming	↑in UC, CD, CeD, IBS[3]	H/R
3	Barrier-forming	↓ in UC, CD, CeD, AC	H/R
4	Barrier-forming	↓ in UC, MC, IBS, AC	H/R
5	Barrier-forming	↓ in CD, CeD, AC	H/R
7	Anion-selective pore-forming	↓ in UC, CeD	H/R
8	Barrier-forming	↓ in CD	H/R
10a	Anion-selective pore-forming	-	R[1]
10b	Cation-selective pore-forming	-	R
12	ND	-	H/R
13	ND	-	R
15	Cation-selective pore-forming	↑in CeD	H/R
18-2	Barrier-forming	-	R[2]
20	ND	-	R
21	ND	-	R
23	ND	-	R

Obtained from references 46,51,52. [1] In humans claudin 10 has been described. [2] In humans claudin 18 has been described. [3] Only in jejunum. **H**: human. **R**: rodent. **↑**: increased expression. **↓**: decreased expression. **UC**: ulcerative colitis. **CD**: Crohn's disease. **CeD**: celiac disease. **IBS**: inflammatory bowel syndrome. **MC**: microscopic colitis. **AC**: acute colitis.

II. Occludin: Occludin, forming hetero-polymers with different claudin proteins, seem to be a key protein maintaining the

integrity of TJs and regulating paracellular diffusion of small molecules.[42,49,50] Occludin expression has been shown to be down-regulated in animal models intestinal inflammation.[45]

III. Tricellulin: Tricellulin, as indicated by its name, is a TJ protein that stabilizes the union among three epithelial cells. Its down-regulation results in a detrimental barrier function.[54]

b. Single-span proteins: Within TJs, single-span proteins are represented by junctional adhesion molecules (JAM), being JAM-1, also called JAM-A,[55] the most abundant (Fig. 3).[46] JAM-1 has been implicated in the regulation of cell polarity, growth, and differentiation, and also in the construction and assembly of TJs, the regulation of intestinal permeability and inflammation.[14,56,57] For instance, in ulcerative colitis and Crohn's Disease patients JAM-1 is down-regulated in the intestinal mucosa, thus indicating that this TJ protein might be essential in the maintenance of a proper intestinal barrier function.[57]

2. *Intracellular TJ proteins:*

Intracellular TJ proteins are scaffolding proteins that anchor the transmembrane TJ proteins to the cellular cytoskeleton (Fig. 3).[58] Intracelullar TJ proteins include zonula occludens (ZO)-1, -2 and -3. However, only ZO-1 and ZO-2 seem to be important for EBF, since they participate in the reorganization of the cytoskeleton (Fig. 3).[59–61]

The cytoskeleton, and in particular actin and myosin II, can be regarded also as an active component related to TJs. In fact, actin and myosin II form ring-like structures (the so called perijunctional actomyosin ring) that encircle the apical pole of the IECs. This structures give flexibility to the TJs allowing its contraction, controlling the opening of the paracellular space and, therefore, affecting paracellular permeability.[62,63]

Considering the composition of TJs, they should be regarded as dynamic structures able to adapt to different regulatory mechanisms and stimuli.

2.3. Control of intestinal epithelial paracellular permeability: Dynamics of tight junctions and barrier-modulating factors

As described above, TJ-related proteins are essential for the control of intestinal epithelial barrier function.[31,34,43,62,63] The control of paracellular permeability is complex and depends upon several mechanisms that mediate fast/transitory or slow/long-lasting changes. These responses are crucial intermediate pathways of external stimuli to modify the assembly, disassembly, and maintenance of TJ structure and, thus, EBF.[64]

A fast mechanism mediating changes in TJ permeability is the redistribution of TJ proteins, promoting their junctional or cytoplasmic redistribution or the assembly/disassembly of the TJ.[41,65–68] Protein phosphorylation-dephosphorylation seems to be a basic mechanism affecting the structure and assembly of TJs, having dichotomous effects on EBF. For instance, the phosphorylation of claudin-3 decreases its assembly in the TJ. On the other hand, the phosphorylation of claudin-1

and claudin-4 is needed for their assembly into TJ.[69] In the case of occludin, phosphorylation promotes its assembly within the TJ, whereas dephosphorylation results in its release from the TJ to the cytoplasm.[14]

Slow/long-lasting mechanisms include transcriptional changes of TJ proteins, increasing or decreasing the expression of occludin, ZO proteins or claudins. Ultrastructural epithelial changes and apoptosis are also examples of slow/long-lasting changes affecting EBF.[14]

The underlying mechanisms behind TJs dynamics, particularly as it relates to paracellular permeability, include several endogenous and exogenous modulatory factors. Main barrier modulatory factors include the gut regulatory peptide Glucagon-Like Peptide-2 (GLP-2), myosin light chain kinase (MLCK), neurotransmitters from the ENS, and cytokines.

a) MLCK as a barrier modulating-factor

MLCK is a kinase that leads to fast/transitory changes in paracellular permeability by phosphorylation of myosin II regulatory light chain. This process induces the contraction of the actomyosin ring and, as a consequence, the increase in paracellular permeability.[34] Moreover, MLCK is also involved in the assembly and the regulation of TJs since it has been shown that its promotion induces changes in the cellular distribution of ZO-1 and occludin.[64,70]

b) GLP-2 as a barrier-modulating factor

GLP-2 recovers and maintains EBF integrity by up-regulating the expression of ZO-1, claudin-1 and occludin, as shown both *in vitro* (Caco-2 cells) and *in vivo*.[71–73] Moreover, in states of impairment of the EBF gene

expression levels of proglucagon, the precursor of GLP-2, are down-regulated[48] while an up-regulation can be observed in states of enhanced barrier function.[74] Overall, these observations clearly show the role of intestinal GLP-2 as a pro-barrier factor.

c) Enteric nervous system-derived factors as barrier modulators

Neurotransmitters from the ENS can also modulate TJs functionality. As previously mentioned, the ENS and the intestinal epithelium interact for the maintenance of several physiological functions, including EBF. A clear example is the modulatory role of submucosal neurons on intestinal permeability. For instance, vasoactive intestinal polypeptide (VIP) released by submucosal neurons improves EBF, like through the up-regulation of ZO-1, as shown *in vitro* in a colonic cell culture.[75] Similar protective effects have been associated to the stimulation of α7 nicotinic acetylcholine receptors, thus suggesting that acetylcholine might also mediate neuronal pro-barrier effects at this level.[76]

d) Immune system-derived factors as barrier modulators

Several cytokines and immune-related factors are able to affect TJs and their permeability (table 2), and have been proposed to be important in the pathophysiology of functional and inflammatory gastrointestinal disorders, food allergies, and celiac disease. Cytokines are considered to be responsible for the perpetuation of intestinal barrier dysfunction. [77]

Table 2. Cytokines and growth factor with modulatory effects on epithelial barrier function.

Cytokine	Effect on EBF	Mechanisms detected
IFNγ	↓	Myosin II-dependent vacuolization, internalization of JAM-A, occludin, claudin-1 and claudin-4
TNFα	↓	ZO-1 ↓
IL-1β	↓	MLCK ↑; pMLC ↑; Claudin-2 ↑; ↓ occludin
IL-4	↓	Claudin-2 ↑
IL-6	↓	Claudin-2 ↑
IL-13	↓	Claudin-2 ↑ / Pro-oxidative processes
IL-10	↑	Neutralization of IFNγ
IL-17	↑	Claudin-1 ↑
TGFβ	↑	Claudin-1 ↑; Claudin-4 ↑; Neutralization of IFNγ
EGF	↑	Restoration of occludin and ZO-1 distribution, and actin cytoskeleton assembly.

Obtained from references 39,77–81.↑: increased expression (mRNA or protein). ↓: decreased expression (mRNA or protein). **TGF**: Transforming growth factor. **EGF**: Epidermal Growth Factor.

Main cytokines implicated in EBF alterations are interferon-γ (IFNγ), tumor necrosis factor-α (TNFα) and interleukin- (IL-) 1β (particularly relevant in the pathophysiology of CD). [66,77,79,81,82]

IFNγ- and TNFα-dependent effects on EBF are mediated through the redistribution of ZO-1, JAM-1, occludin, claudin-1 and -4 and the phosphorylation of MLC. In fact, both cytokines cause the internalization of occludin and JAM-1 in sub-apical vesicle-like structures in active ulcerative colitis, as well as in acute dextran sodium sulfate-induced colitis.[65,66,83] Moreover, IFNγ and TNFα can also alter the expression of

claudins and occludin evidencing the multiple mechanisms involved in the immune modulation of EBF.[84]

2.4. Techniques for the study of epithelial barrier function

Techniques commonly used to study EBF and the electrolytic transport across the intestinal epithelium can be classified in *in vitro/ex vivo* and *in vivo*.

a) *In vitro/ex vivo* techniques

These techniques include the use of cell cultured monolayers, in which the cell line monolayer (frequently Caco-2, T-84 or HT-29) grows in a semi-permeable support, allowing the formation of the compartmentalization in basolateral and apical sides.[42,85] The main limitation of these systems to assess EBF is the absence of other components of the barrier besides the epithelial lining (ENS, immune system, etc.). Nevertheless, these techniques represent a good approach, since they can be used to detect changes in transepithelial electrical resistance (a measure of epithelial tightness)[86], and to study the paracellular permeability using different markers such as mannitol, inulin, dextran and lucifer yellow, among others, in particular when mounted in a Ussing chamber system.[85]

The Ussing chambers system is the most useful, versatile and common technique used for functional assessment of the intestinal EBF and electrolytic transport in live excised intestinal segments from human or animals (Fig. 5).

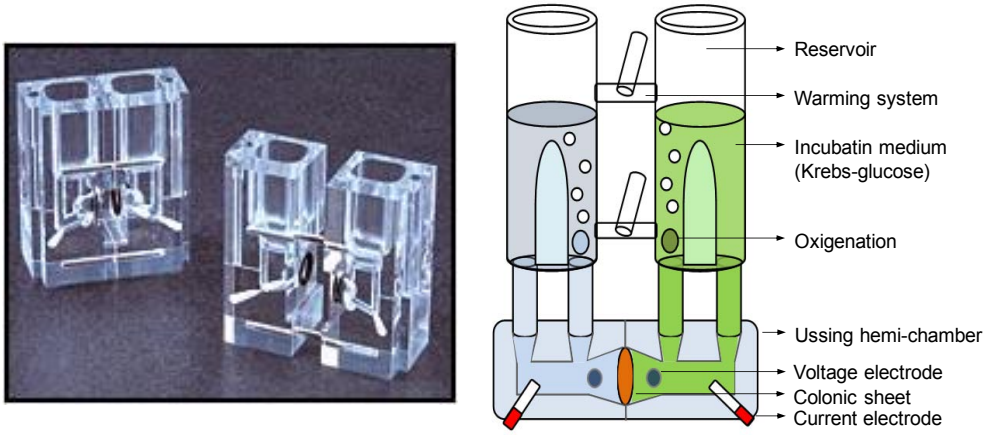

Figure 5. Ussing chamber system. To the right, scheme of a Ussing chamber.

Ussing chambers were developed over 50 years ago by the Danish biologist Hans H. Ussing for the study of epithelial active NaCl transport. As used in the present work, the technique consists in mounting a sheet of intestine between the two half-chambers. The tissue is previously stripped to separate and reject the serosa and muscular layers, to mount the epithelium and the underlying submucosal elements. The intestinal sample is nourished with a physiological buffer, with a specific pH, temperature and well oxygenated, which circulates through the chambers.[87–89] The system is coupled to two pairs of electrodes (one pair per chamber). In each pair, one electrode detects changes in voltage and the other serves as a stimulating electrode to apply electric currents. The system allows the recording of several electrophysiological parameters that serve as basis to define epithelial barrier function:

- *Potential difference (PD):*

  PD, in mV, is defined as the capability of an epithelium to separate charges.[89]

- *Short-circuit current (I_{sc}):*

  I_{sc} corresponds to the electric current (expressed usually as $\mu A/cm^2$) applied through the stimulating electrode to fix the voltage between the basolateral and the apical sides of the epithelial sheet (PD) to zero, thus allowing the equilibrium between the electric forces at both sides. Therefore, once the hydrostatic, the osmotic, the chemical and the electric forces are equalised, the I_{sc} can be considered as the measure of the electrogenic active transport of ions across the epithelium.[87,89]

  I_{sc} can be assessed after the administration of drugs to inhibit or stimulate specific ion transport pathways/channels, or to study the implication of different physiological systems.[89]

- *Conductance (G):*

  G (expressed usually as mS/cm^2) is the inverse of the transepithelial resistance and is calculated using the Ohm's law ($G = \Delta I_{sc} / \Delta V$), after applying a small voltage pulse.[87] Because of the leaky characteristic of the intestinal epithelium, >90 % of the total transepithelial ionic conductance correspond to paracellular transport, making this parameter a good indicator of paracellular permeability.[87]

As mentioned, these electrophysiological parameters reflect mostly hydroelectrolitic transport. However, ionic permeability and permeability to macromolecules do not always have the same behavior. Therefore, specific studies of permeability to macromolecules are necessary to correctly assess paracellular permeability. In these studies, specific markers (radioactive macromolecules, markers detected with enzymatic

reactions or dextrans conjugated with a fluorescent molecule) are used.[48,90–92]

Overall, the Ussing chamber system represents a useful technique to evaluate intestinal barrier function. After stripping, intestinal samples mounted in the Ussing chambers contain the mucosa and the submucosa, including important components related to the control of the EBF, such as the submucosal plexus or components of the resident immune system.[42] However, the Ussing chamber technique has also some limitations, for instance, the absence of systemic regulatory components, such as endocrine factors or the intestinal extrinsic innervation.

Alternative *in vitro* techniques to evaluate the ionic and macromolecular transepithelial transport are, for example, the assessment of the absorption of sodium or the passage of fluorescein isothiocyanate- (FITC-) dextran from confocal images from isolated intestinal glands, or the everted gut sacs technique.[93–95]

b) *In vivo* techniques

In vivo techniques to evaluate intestinal barrier function allow performing functional studies with the complexity of the whole organism and the presence of regulatory components absent in *in vitro/ex vivo* conditions. With different adaptations, these techniques assess the passage of a marker, administered into the intestinal lumen, to the internal milieu (commonly to blood and/or urine). An increased recovery of markers in blood and/or urine is interpreted as an increase in intestinal permeability.

Markers used range from different fluorescent-labelled dextrans,[48] to radioactive substances (such as ^{51}Cr-labelled molecules)[96] or non-absorbable sugars (such as lactulose and/or mannitol).[97] For instance, we have used orally-administered fluorescent-labelled dextrans to assess intestinal permeability in a model of *T. spiralis* infection-induced IBS-like intestinal alterations in rats[48] and sugars have been extensively used to test intestinal permeability in humans.[98,99]

3. Functional and inflammatory gastrointestinal disorders

An altered intestinal barrier function is a common finding of some functional and inflammatory gastrointestinal disorders, such as IBD and IBS. This state, known as "leaky gut", is characterized by an increased passage of luminal antigens to the internal milieu.[32,78] However, whether or not the alteration of the barrier function is the initial cause or just a step in the pathogenesis of these diseases needs to be clarified.

3.1. Inflammatory Bowel Disease and Irritable Bowel Syndrome

IBD is represented by two not completely discrete pathological entities, ulcerous colitis (UC) and Crohn's Disease (CD). The main feature of IBD is the chronic uncontrolled immune-mediated inflammation of the gastrointestinal tract in genetically susceptible individuals exposed to environmental risk factors.[100–102]

UC and CD share some clinical manifestation, such as diarrhea, abdominal cramps and pain, severe bowel movement, tenesmus, fever, loss of appetite, weight loss and fatigue, among others.[103] However, they present some clinical, histopathological and epidemiological differences. For instance, whereas UC is commonly characterized by a diffuse superficial inflammation restricted to colon, CD tends to manifest with discontinuous transmural asymmetric lesions that can affect any part of the gastrointestinal tract.[102–106]

Irritable Bowel Syndrome (IBS) is a highly prevalent functional gastrointestinal disorder characterized by abdominal pain and/or discomfort, diarrhea and/or constipation and bloating.[107,108] About 10-20% of adult and adolescent population have IBS-compatible symptoms, finding a female predominance twice higher than in males.[107,108]

The pathogenesis of IBD and IBS is an unclear aspect still to elucidate. A complex confluence of genetic predisposition, environmental factors and an altered immune response seems to be behind their appearance. What seems clear is that these patients present a defect in the intestinal epithelial barrier function. The barrier dysfunction allows the passage of luminal antigens that exacerbate the immune response and induce the release of cytokines and other inflammatory mediators, and the recruitment of immune cells. Altogether these changes lead to further epithelial alterations, generating a self-maintaining cycle and the perpetuation of the inflammatory state, that manifests with the characteristics symptoms of these diseases.[78,109]

3.2. *Animal models of IBD and IBS*

Animal models of diseases are instrumental for the study of the underlying pathophysiological mechanisms and the discovery of new therapeutic targets.

1) Animal models of IBD

Animal models of IBD can be classified in five types according to the origin of the inflammatory reaction (Table 3):

1. Spontaneous colitis models: Models in which the pathology develops without any exogenous factor. Some examples are specific mouse strains with natural genetic abnormalities, which develop spontaneously intestinal inflammation under certain conditions.[110]

2. Genetic models: Models in which spontaneous colitis appears as a result of defects in particular genes or as a result of gene targeting or introduction of a transgene. Most of these models are knockout mice of some gene which codifies an essential component of the epithelial barrier function or of the immune system, causing a defect in the regulation of mucosal immune responses and triggering, as a consequence, intestinal inflammation.[110–112] The main limitation of these models is the fact that, in humans, multiple genes seem to be implicated in the development of the disease.[110]

3. Immunological models: in these models, the induction of colitis is obtained via deregulation of the immune response by transferring T-cell populations lacking regulatory lymphocytes into immune-incompetent animals (table 3). This shifts the immune response towards a pro-inflammatory profile, causing the induction of an intestinal inflammation[110] (table 3).

4. Infectious models: Models in which the inflammation of the intestine is induced by a biological agent (usually a bacteria or a parasite). These models are particularly useful to study host-bacterial interactions, focusing on anti-pathogen host-defense. Among them, the most common models are the murine models of *Citrobacter rodentium*-induced *colitis* and *Salmonella (Salmonella enterica* Typhimurium)-induced colitis in streptomycin-treated mice.[113,114] The

models of parasitic infection with *Trichinella spiralis* or with *Nippostrongylus brasiliensis* are classically classified as a model of post-infectious IBS, although they shares some pathophysiological features with IBD.[48]

5. Chemically-induced colitis models: Models in which the inflammatory reaction is induced by a chemical agent. Depending upon the inductor used, the mechanisms can range from damage-induced to a more immunologically mediated inflammation.[110–112]

A well characterized and accepted model of chemically-induced colitis is the dextran sodium sulfate (DSS)-induced colitis in rodents.[115–119] The DSS model resembles human IBD, reflecting clinical and histopathological features of mainly UC, but also some of CD.[115] Moreover, several different studies have confirmed the predictive validity of the model for the identification and validation of pharmacological targets for IBD.[115]

The DSS model and IBD have a defect in the epithelial barrier function as a common component. DSS exerts its toxicity on IECs, increasing the apoptosis, reducing the epithelial cell replacement, reducing the expression of the TJ-related protein ZO-1, causing a redistribution of occludin, ZO-1, claudin-1, -3, -4 and -5, and inducing the expression of pro-inflammatory cytokines (TNF-α, IL-1β, IFN-γ, IL-10, and IL-12), most of them with damaging effects on the colonic EBF.[110,115]

Table 3. Animal models of IBD.

Typology	Examples
Spontaneous models	C3H/HeJBir; SAMP1/YitFc
Genetic models	Mdr1a KO; Dominant negative N-cadherin transgenic; Gai2 KO; JAM-1 KO; TLR5 KO; NEMO^{IEC-KO}; TAK1^{IEC-KO}; XBP1^{IEC-KO}; AP1M2 KO; RBP-J^{IEC-KO}; MUC2 KO; MUC2 mutant (Winnie and Eeyore strains); POFUT1^{IEC-KO}.
Immunological models	Transference to SCID mice of: CD4+αβ T cells; CD4+CD45RB^{high}; CD4+CD45RB^{low} from donor mice sensitized with ovalbumin; CD4+CD25-; CD62L+CD4+; hsp 60-specific CD8+ T cells. CD3ε transgenic (Tgε26) mice.
Infectious models	Mice infected with *Citrobacter rodentium*; *Salmonella*-induced colitis in streptomycin-treated mice; rats infected with *Trichinella spiralis*
Chemical-induced models	DSS-induced colitis; TNBS-induced colitis; Indomethacin-induced enteritis; carrageenan-induced colitis

Obtained from references 48,110,113,114,120. KO: Knock-out; IEC-KO: Intestinal Epithelial Cell-specific ablation; heat shock protein (hsp); severe combined immunodeficiency (SCID); TNBS: 2,4,6-Trinitrobenzenesulfonic acid.

2) Animal models of IBS

Animal models of IBS are more limited than those of IBD because the unspecific mechanisms of IBS difficult the full reconstruction of the disease in an animal model.[110] IBS models can be classified according to the pathogenic factor that triggers the intestinal alteration in those that

are initiated by psychosocial factors (central nervous system-directed) and those that are initiated by a physical stressor (gut-directed).[111]

- *Central nervous system-directed models:*

  Models based on psychosocial factors and the contribution of the brain-gut axis dysfunction to the pathophysiology of IBS. Are frequently based in the application of psychosocial stressors during the neonatal period (maternal separation stress model) or during adulthood (water avoidance stress or restraint stress models).[121] Moreover, some genetic models affecting different neuro-endocrine systems (CRH, GABA, noradrenergic and endocannabinoid systems) can be included in this group.[111]

- *Gut-directed models:*

  Models based in the application of inflammatory stimuli. They include neonatal inflammation/pain models (such as those induced by colorectal distention), and, in the adulthood, post-inflammatory models of visceral hypersensitivity (induced by chemical irritation, biological agents, such as *T. spiralis* or *N. brasiliensis* infection, or food-related allergens, such as the exposure to ovoalbumin).[48,111,122,123]

4. Host-bacterial interactions

4.1. Gut Commensal Microbiota

Most of the epithelia of the organism act as a support and interface surface to a high diversity of microorganisms, including viruses, bacteria, *archaea*, fungi and protozoa. The human digestive tract contains approximately 100 trillions of commensal microorganisms, being the intestine the anatomical structure with a higher population, with about 10^{14} microorganisms. These are mostly bacteria, with up to 1000 different species, with a dominance of anaerobic species, and Bacteroidetes and Firmicutes as the dominant phyla.[124–127] Gut commensal microbiota (GCM) composition varies depending on environmental conditions[128,129] and bacterial density changes along the gastrointestinal tract. Overall, the lower the area of the gastrointestinal tract, the higher the bacterial concentration, varying from 100 cells/milliliter in the stomach to 10^5, approximately, in the upper small intestine, and up to 10^{12} in the colon.[15,130]

The functions of GCM include the production of micronutrients, like short chain fatty acids or some vitamins,[131,132] the control of epithelial renewal, the modulation of visceral sensitivity[129,133,134] and local immune-related responses (including immunotolerance mechanisms[19,135,136] and protection against pathogen colonization[137]), and the control of the intestinal barrier function,[138] among others.

Alterations in the normal composition of GCM, named dysbiosis, have been associated with several pathologies, including gastrointestinal disorders like IBD or IBS[32,138,139] and also psychiatric disorders[140], obesity

and other metabolic diseases,[141,142] allergies and autoimmune diseases.[143] IBD and IBS patients accompany their pathological features with a microbial dysbiosis characterized by reduced bacterial diversity and depletion of some commensal species,[144–146] thus suggesting that the microbiota and its interaction with the host (host-bacterial interactions) are components of the pathophysiology of these alterations.

4.2. Host-bacterial interactions: Toll-Like Receptors

Several evidences suggest that the microbiota and its interactions with the host are important in the pathogenesis of multiple diseases. Within the gastrointestinal tract, host-bacterial interactions depend upon different components of the innate and the adaptive immune system. These mechanisms, working coordinately, recognize the microbiota, tolerating commensal microorganisms and reacting against pathogens. Recognition of the microbiota is mediated by several systems, being pattern recognition receptors (PRRs) a key component. PRRs are a series of receptors, expressed mainly by intestinal epithelial cells and immune cells, devoted to sense microorganisms through pathogen-associated molecular patterns (PAMPs) and to detect potentially damaging signals through damage-associated molecular patterns (DAMPs).[147] PRRs include four major families of receptors: Toll-like receptors (TLRs), Nucleotide-binding oligomerization domain receptors (NODs), C-type lectin-like receptors (CLR) and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs).[148–150]

TLRs are the best characterized PRRs, being essential in host-bacterial interactions, and participating in inflammatory and homeostatic processes. So far, the number of TLRs identified in mammals is 13, 10 in

humans (TLR1-10), 12 in mice (TLR1–9 and TLR11–13) and 10 in rats (TLR1-7, 9, 10 and 13)[151,152]. Overall, TLRs can recognize Gram-positive and Gram-negative bacteria, mycobacteria, RNA and DNA viruses, fungi and protozoans (Table 4).

Table 4. Characteristics of rodent and human TLRs.

	Agonist/Ligands	Localization	Functions/Effects	Change in colonic gene expression
TLR1 (TLR2/1 heterodimer)	Bacteria: Triacyl lipopeptide	IEC. Cell membrane	Co-receptor of TLR2. Promotion of protective intestinal TH17 immunity. Pro-inflammatory IL-17 T cell responses	
TLR2	Bacteria: Lipomannan. Lipoteichoic acid. Peptidoglycan. Porins. Atypical LPS. Lipoarabino-mannan. Virus: Hemagglutinin proteins Fungi: Zymosan Protozoa: Glycosyl – phosphatidyl-inositols. Glycolipids.	Low expression in adult ileum and colon. Mainly in crypts. Apical in villi and crypts; apical and basolateral in FAE	Preserved tight junction structure; increased TFF3 expression. Chemokine and cytokine production; RELA phosphorylation; ZO1 redistribution and TFF3 expression	↑ in UC

	Agonist/Ligands	Localization	Functions/Effects	Change in colonic gene expression
TLR3	Virus: dsRNA	Intracellular compartments. IEC: Basolateral in ileum and colon; top of colonic crypts. Neurons and glia of myenteric and submucous plexuses. DRG (primary sensory neurons).	Blocking of TLR3 reduces IL-15 production. Enhancement of colonic barrier function	↑ in IBD
TLR4	Bacteria: Lipopolysaccharide (LPS) Virus: Envelope and fusion proteins. Fungi: Mannan. Protozoa: Glycosyl – phosphatidylinositols Plants: Taxol	Basolateral in ileal crypts (fetus); basolateral in colon (low); apical in active Crohn's disease (ileum and colon) IEC: Apical pole. Apical in terminal ileum; basolateral in colon (low); intracellular in fetal small intestine. Neurons and glia of myenteric and submucous plexuses. DRG (primary sensory neurons)	Cell growth, chemokine and cytokine production, phagocytosis and translocation of bacteria, and uptake of microparticles by M cells; expression leads to increased TNF production, apoptosis and NF-κB activation; lack of expression leads to decreased TNF production and protects against NEC. Deterioration of intestinal barrier function	↑ in IBS, ↑ in UC

	Agonist/Ligands	Localization	Functions/Effects	Change in colonic gene expression
TLR5	Bacteria: Flagellin	IEC: Basolateral and intracellular in colon. Basolateral in ileum and colon; apical in FAE (small intestine)	Chemokine expression	↑in IBS, ↓ in UC
TLR6 (TLR2/6 heterodimer)	Bacteria: diacyl lipopeptides.	IEC. Cell membrane	Co-receptor of TLR2. Promotion of tolerogenic responses and IL-10 release	
TLR7	Viral ssRNA and bacterial RNA Sinthetic compounds: Imidazoquinolines. Loxoribine. Bropirimine	IEC: Intracellular compartments. Neurons and glia of nervous plexuses. DRG (primary sensory neurons). DCs, B cells, T cells	Cytokine production	↓ in IBS
TLR8	ssRNA (viral and no viral)	Intracellular compartments. Top of colonic crypts in ulcerative colitis and Crohn's disease; not in normal intestine	Chemokine secretion	↓ in IBS, ↑ in IBD

	Agonist/Ligands	Localization	Functions/Effects	Change in colonic gene expression
TLR9	Bacteria: CpG DNA	Intracellular compartments. IEC: Apical and basolateral in colon. Apical and basolateral (ileum); in granules and cytoplasm of Paneth cells	IL-8 secretion (whole biopsy). Protects against NEC; signaling causes degranulation of Paneth cells. Enhancement of colonic barrier function	↑ in UC
TLR10	ND	Cell membrane	Co-receptor of TLR2	ND
TLR11	Bacteria: Uropathogenic *E. coli* and *Salmonella* components Protozoa: Profilin	Cell membrane	Cytokine production	
TLR12	Protozoa: Profilin	ND	ND	
TLR13	Bacterial RNA	Probably in intracellular compartments	Cytokine production	

Obtained from references 13,147,153–178. ND: Not determined. DRG: Dorsal Root Ganglia. NEC: Necrotizing Enterocolitis. ssRNA: Single Stranded RNA.

Within the gut, TLRs are found mainly in IECs, although they can be also found in immune cells and enteric neurons. TLRs are located both in the cell surface and intracellularly (table 4).[13,160,179,180] TLRs activation lead to receptor-subtype-specific signaling cascades resulting in the production of immune mediators (cytokines and chemokines) and the transcription of other genes important for controlling infections and inflammatory responses.

In patients with inflammatory and functional gastrointestinal disorders, as well as in animal models, the intestinal expression of TLRs is altered (Table 4).[153–158,179,181] Moreover, different TLRs polymorphisms have been associated with a predisposition to intestinal inflammation.[147,182] Overall, these observations, together with the existing evidences of dysbiosis, further supports the hypothesis that altered TLR-mediated host-bacterial interactions significantly contribute to the pathophysiology of IBS and IBD.

4.3. Toll-Like Receptors and epithelial barrier function

As mentioned, a common finding in IBD and IBS is the presence of epithelial barrier alterations leading to a state known as “leaky gut” with an increased passage of luminal antigens to the internal milieu.[32,78] Some evidences implicate TLRs in this barrier dysfunction (table 4).[183,184]

Current evidences implicate TLR2, TLR3, TLR4, and TLR9 in the control of EBF, with both detrimental and enhancing effect in a receptor-specific manner.[178,179,184,185] For instance, the activation of TLR4 with bacterial lipopolysaccharides (LPS) increases TJs permeability *in vitro* and *in vivo* by a mechanism unrelated to changes in the distribution of TJ-related proteins (occludin, ZO-1, claudin-1, claudin-3 or claudin-5).[186] On the other hand, activation of TLR2 with the selective agonist Pam3CysSK4 (PCSK) is able to induce a rapid enhancement of EBF *in vitro* (Caco-2) and in different models in *in vivo* conditions.[179,183,184,187] Pro-barrier effects of TLR2 were associated to a modulation of TJs in *Citrobacter rodentium*- and DSS-induced colitis in mice.[160,179,187] Although with limited evidences,

some studies suggest that intestinal TLR3 and TLR9 mediate also pro-barrier effects within the GI tract.[177,178]

The potential role of other TLRs on EBF has not been assessed in deep. In this work we have focused on the potential role of TLR5 and TLR7 on EBF. The expression of both TLR5 and TLR7 is altered in states of dysbiosis[133] and in IBS/IBD patients,[157,181] thus supporting their implication in the functional alterations associated to these conditions. In particular, TLR7 has been shown to modulate colonic neuroimmune responses[188,189] and to improve DSS-induced colitis in mice,[190] thus suggesting a potential protective role that can affect also EBF. As it relates to TLR5, contradictory reports indicate that it might exhibit either protective or damaging effects on the epithelial barrier depending upon the experimental conditions and the models considered.[191–197]

Hypothesis & Objectives

From the previous background it can be concluded that TLRs-mediated host-bacterial interactions are important in the regulation of gastrointestinal functions, both in normal and pathological conditions. In particular, several evidences indicate that gut commensal microbiota modulates epithelial barrier function in a TLR-dependent manner and that TLRs, in a receptor-type specific manner, can elicit either protective o detrimental effects on barrier function.

Therefore, we **HYPOTHESIZED** that TLRs, in particular TLR5 and TLR7, are implicated in the local regulation of intestinal epithelial barrier function, affecting the passage of luminal molecules to the internal milieu and, therefore, the development of local immune responses within the gut.

To proof this hypothesis, we characterized colonic epithelial barrier function (electrophysiological parameters and permeability to macromolecules) in *in vitro* (Ussing chamber system) and *in vivo* conditions under the selective over-stimulation of TLR5 or TLR7 in different experimental conditions in rats and mice. Furthermore, we also assessed the potential implication of epithelial tight junctions in the responses observed.

Taking this into account, the specific **OBJECTIVES** of this work were:

- To determine if over-stimulation of colonic TLR7 affects colonic epithelial barrier function in normal conditions, in states of epithelial permeabilization, or during colitis.
- To determine if over-stimulation of TLR5 affects colonic epithelial barrier function in basal conditions or during colitis.

- To characterize if over-stimulation of colonic TLR5 and TLR7 affects the expression of tight junction-related proteins and barrier-modulating factors.
- To determine if over-stimulation of colonic TLR5 and TLR7 leads to a local immune activation.

Chapter 1

Local activation of Toll-Like Receptor 7 (TLR7) modulates colonic epithelial barrier function in rats

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Abstract

Background/Aims: Toll-like receptors (TLRs)-mediated host-bacterial interactions participate in the microbial regulation of gastrointestinal functions, including epithelial barrier function (EBF). We evaluated the effects of TLR7 stimulation on colonic EBF in rats.

Methods: Stimulation of colonic TLR7 with the selective agonist imiquimod (100 or 300 μg/rat, intracolonic), with or without the intracolonic administration of dimethyl sulfoxide (DMSO), was performed. Thereafter, colonic EBF was assessed *in vitro* (electrophysiological parameters and permeability to macromolecules in a Ussing chamber system) and *in vivo* (passage of macromolecules to blood and urine). Changes in the expression (RT-qPCR) and distribution (immunohistochemistry) of tight junction-related proteins were determined. Expression of proglucagon, precursor of the barrier-enhancer factor glucagon-like peptide 2 (GLP-2) was also assessed (RT-qPCR).

Results: Intracolonic imiquimod enhanced EBF *in vitro*, thus indicating a pro-barrier effect of TLR7. However, the combination of TLR7 stimulation and DMSO had a detrimental effect on EBF, manifested as an increased passage of macromolecules to blood and urine. DMSO alone did not affect epithelial permeability. Modulation of EBF, elicited by either imiquimod alone or combined with DMSO, was not associated to changes in gene expression or epithelial distribution of the main tight junction-related proteins. Moreover, no changes in the expression of proglucagon were observed.

Conclusions: These results show that TLR7-dependent host-bacterial interactions leads to the modulation of colonic EBF, having beneficial or detrimental effects depending upon de state of the epithelium. The underlying mechanisms remain elusive, but seem to be independent of the modulation of the main tight junction-related proteins or the barrier-enhancer factor GLP-2.

Key words: Epithelial barrier function; Host-bacterial interactions; Intestinal permeability; Tight junctions; TLR

Introduction

The disruption of the epithelial barrier function (EBF) with increased intestinal permeability is a common finding in several gastrointestinal disorders, including inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS).[1–3] The intestinal epithelium represents a functional barrier between the lumen and the internal milieu, allowing selective exchange processes (secretory and absorptive) and acting at the same time as a protective barrier against luminal antigens.[4] Gut commensal microbiota, considered for long time a passive component of the gastrointestinal tract, has recently emerged as a dynamic player in intestinal homeostasis.[5–7] Gut microbiota and microbial-derived products act as luminal factors that interact continuously with the host. In physiological conditions, these interactions contribute to the maintenance of intestinal homeostasis. However, in states of dysbiosis, host-microbial interactions change and might lead to the activation of the immune system and the generation of a persistent inflammatory state with functional changes, including alterations in EBF. In this sense, dysbiosis and altered epithelial permeability are co-existent features of IBS and IBD.[1,3,8–12]

Host-microbial interactions are largely mediated through pattern recognition receptors (PRRs). PRRs are expressed within the gut and recognize conserved microbial components named pathogen-associated molecular patterns (PAMPs).[13] In particular, recognition of gut microbiota depends largely on the interaction with a subgroup of PRRs named Toll-like receptors (TLRs), with 13 members described (TLR1-13).[14,15] The relative expression of the different TLRs differs depending upon the cell

type and the cellular localization.[15,16] Within the gut, some of the mainly expressed TLRs are TLR2, TLR3, TLR4, TLR5 and TLR7, being present in several cell types, including epithelial cells.[14] TLRs directly interact with luminal microbial components and, upon activation, elicit neuroimmune responses that affect intestinal functions.[17–21] For instance, we have shown that during antibiotic-induced dysbiosis, host-bacterial interactions are altered, including changes in the expression of TLRs, and there are local changes in immune- and sensory-related systems within the gut.[22,23] Similar alterations might be elicited when TLRs are over-stimulated, mimicking a state of dysbiosis with altered host-bacterial interactions. In this sense, we have shown that direct stimulation of colonic TLR7 with the selective agonist imiquimod leads to immune- and sensory-related changes similar to those observed during intestinal dysbiosis.[24,25]

Taking into account these observations, the aim of the present work was to directly assess the effects of colonic TLR7 stimulation on EBF in rats. For this, we assessed the effects of the over-stimulation of colonic TLR7 with the selective agonist imiquimod on epithelial electrical parameters and on epithelial permeability to macromolecules in *in vitro* (Ussing chamber system) and *in vivo* conditions. In order to characterize the underlying mechanisms mediating potential TLR7-dependent modulation of EBF, we also assessed changes in the expression and epithelial distribution of the main tight-junction (TJ)-related proteins (occludin, tricellulin, claudin-2, claudin-3, JAM-1 and ZO-1). Moreover, changes in the expression of the barrier-enhancer factor glucagon-like peptide 2 (GLP-2, assessed through the expression of its precursor, proglucagon)[26] was also assessed. Finally, the induction of a colonic

inflammatory-like state associated to the over-stimulation of TLR7 was assessed at the macroscopical and molecular levels.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (6-9 week-old at arrival; Charles-River Laboratories, Lyon, France) were used. On arrival, animals were housed in pairs in standard plastic cages under conventional controlled environmental conditions (20–22ºC, 40–70% humidity and 12 h light/dark cycle) and fed with a standard pellet diet (Panlab SL, Barcelona, Spain) and tap water *ad libitum*. All experimental procedures were approved by the ethical committees of the Universitat Autònoma de Barcelona and the Generalitat de Catalunya (protocols 1420 and 6333, respectively).

Tissue sampling

At the time of the experiments, animals were euthanized by decapitation, except when otherwise stated. A laparotomy was performed and the colon gently dissected and placed in ice-cold oxygenated Krebs buffer ([in mM] 115.48 NaCl, 21.90 $NaHCO_3$; 4.61 KCl; 1.14 NaH_2PO_4; 2.50 $CaCl_2$; 1.16 $MgSO_4$ [pH: 7.3–7.4]) containing 10mM glucose. Colonic segments were used to perform *in vitro* epithelial barrier function studies (mid colon) or preserved for morphological or molecular biology studies (mid-distal colon). Samples for histological and immunostaining studies were fixed in 4 % paraformaldehyde in phosphate buffer for 24 h. Thereafter, fixed samples were processed routinely for paraffin embedding and 5µm sections were obtained for

hematoxylin and eosin (H&E) staining or immunohistochemistry. Samples for molecular biology studies were immediately frozen in liquid nitrogen and stored at -80ºC until processed.

Measurement of Electrophysiological Parameters (Ussing Chambers)

Colonic segments were stripped of the outer muscle layers and myenteric plexus, opened along the mesenteric border, and divided into 1 cm^2 flat segments, approximately. Epithelial sheets were mounted in Ussing chambers (World Precision Instruments, Aston, UK) with an exposed window surface area of 0.67 cm^2. Tissues were bathed bilaterally with 5 ml of oxygenated and warmed (37 ºC) Krebs buffer (in mmol/L: 115.48 NaCl, 21.90 $NaHCO_3$, 4.61 KCl, 1.14 NaH_2PO_4, 2.50 $CaCl_2$ and 1.16 $MgSO_4$; pH: 7.3–7.4). The buffer also contained 10 mmol/L glucose as energy source. The electric equipment of the Ussing chambers consists of two voltage-sensitive electrodes (EKV; World Precision Instruments) to monitor the potential difference (PD) across the tissue, and two Ag-AgCl current passing electrodes (EKC; World Precision Instruments) to inject the required short-circuit current (I_{sc}) to maintain a zero potential difference, as registered via an automated voltage/current clamp (DVC-1000; World Precision Instruments). Ohm's law was used to calculate tissue conductance (G), using the change in I_{sc} when a voltage step of 1 mV was applied at 5 min intervals. Tissues were allowed to stabilize for 15–25 min before baseline values for PD, I_{sc} and G were recorded. Data were digitized with an analog-to-digital converter (MP150; Biopac Systems, Goleta, USA) and measurements were recorded and analyzed with Acqknowledge computer software (version 3.8.1;

Biopac Systems). Short circuit current and G were normalized for the mucosal surface area.

In vitro epithelial permeability to macromolecules

Paracellular permeability was evaluated following protocols previously described by us.[9] The mucosal-to-basolateral flux of fluorescein isothiocyanate (FITC)-labeled dextran (FD) with a mean molecular weight of 4 kDa was assessed in colonic epithelial sheets mounted in Ussing chambers. After stabilization (20-25 min), baseline electrophysiological parameters were assessed and FD4 was added to the mucosal reservoir to a final concentration of 2.5×10^{-4} M. Basolateral samples (250 µL, replaced by 250 µL of buffer solution with glucose) were taken at 15-min intervals during the following 60 min for measurement of FD4. Concentration of fluorescein in the samples was determined by fluorometry (Infinite F200; Tecan, Crailsheim, Germany) with an excitation wavelength of 485 nm (20 nm band width) and an emission wavelength of 535 nm (25 nm band width), against a standard curve. Readings are expressed as percentage (%) of the total amount of FD4 added to the mucosal reservoir.

In vivo colonic permeability to macromolecules

To assess colonic permeability in *in vivo* conditions, the passage of intracolonically administered FD4 to blood and urine, as well as the accumulation of FD4 in the colonic wall, was determined. For this, animals were anesthetized with isoflurane (Isoflo®) and FD4 was administered intracolonically (10 mg/animal, 0.2 mL) with a plastic cannula (8 cm from the anus, corresponding to the mid colon). FD4 administration was performed slowly (30 s – 1 min) to avoid any reflux.

Thereafter, animals were returned to their home cages and 30 min later were deeply anesthetized with isoflurane and blood collected via intracardiac puncture. Then animals were euthanized by a thoracotomy, and urine (intravesically) and colon samples collected. Fluorescence was determined in urine (1:10 dilution in distillate water), serum (obtained from blood centrifuged at 3000 G for 10 min, 4 ºC) and colon homogenates as described above. When assessing fluorescence in colonic samples, tissues were washed thoroughly with saline solution to eliminate any luminal remaining of FD4.

Experimental protocols

1. Effects of imiquimod on epithelial electrical parameters

Animals were deeply anesthetized with isoflurane (Isoflo®, Esteve Veterinaria, Barcelona, Spain) and treated with intracolonic imiquimod (100 or 300 µg/rat) or vehicle (hydroxypropylmethyl cellulose; 5mg/mL; 0.2 mL/rat). Five h later animals were euthanized and colonic sheets were obtained and mounted in Ussing chambers as described above. After a 20-25 min stabilization period, electrical parameters (Isc, PD and G) were assessed for an additional 60 min period.

In some cases, the effects of the acute exposure to imiquimod (direct addition to the Ussing chambers) on electrical parameters were assessed in colonic sheets obtained from naïve animal. In this case, after a 20-25 min stabilization period basal electrical parameters were recorded for a 10 min period and thereafter imiquimod (300 µg) was added to either the apical or the basolateral side and changes in electrical parameters recorded for an additional period of 60 min. Addition of vehicle in some tissues served as control.

At the end of the experiments, a single concentration of CCh (100 μM) was added to the basolateral side of the chamber in order to assess the viability of the tissues.

2. Effects of imiquimod on epithelial permeability in vitro

Isoflurane-anesthetized animals were treated with intracolonic imiquimod (100 or 300 μg/rat) or vehicle (hydroxypropylmethyl cellulose; 5 mg/mL; 0.2 mL/rat). Five h later animals were euthanized and colonic sheets were obtained and mounted in Ussing chambers as described above. After a 20-30 min stabilization period, baseline electrophysiological parameters were assessed and FD4 was added to the mucosal reservoir (2.5×10^{-4} M) and the passage of marker evaluated, as described above, for the following 60 min.

3. Effects of imiquimod on epithelial permeability in vivo

Rats were anesthetized with isoflurane and treated intracolonically with imiquimod (300 μg/rat) or its vehicle (0.2 mL) and returned to their home cages. 6 h later, animals were anesthetized again and (FD4; 10 mg/rat, 0.2 mL) was administered intracolonically, and permeability tested 30 min later, as described above.

In some cases, imiquimod treatment was combined with a permeabilization of the colonic epithelium. In these cases, 4 h after imiquimod administration, animals were anesthetized with isoflurane and the colonic epithelium was challenged with dimethyl sulfoxide[27] (DMSO, 100%, 0.2 mL) and 2 h later FD4 was administered intracolonically to assess permeability.

In all cases, animals received a single intracolonic treatment. For this, animals were deeply anesthetized with isoflurane (Isoflo®, Esteve Veterinaria, Barcelona, Spain) and treatments were applied intracolonically with a plastic cannula (8 cm from the anus, corresponding to the mid-distal colon). Thereafter, animals were returned to their home cages and 3 h (gene expression), 5 h (Ussing chamber studies) or 6.5 h later (*in vivo* permeability) were euthanized and tissue samples obtained as described above.

Quantitative Real Time reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA was extracted from colonic tissue samples using Ribopure RNA Isolation Kit (Applied Biosystems, CA, USA) and quantified with Nanodrop (ND-100 spectrophotometer, Nanodrop Technologies, Rockland, DE, USA). For cDNA synthesis, 1 µg of RNA was reverse-transcribed in a 20 µl reaction volume using a high capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA). TaqMan gene expression assays for occludin (Rn00580064_m1), tricellulin (Rn01494284_m1), claudin-2 (Rn02063575_s1), claudin-3 (Rn00581751_s1), JAM-1 (Rn00587389_m1), ZO-1 (Rn02116071_s1), IL-6 (Rn01410330_m1), IFNα (Rn02395770_g1), TLR7 (Rn01771083_s1) and proglucagon (Rn00562293_m1) were used (all from Applied Biosystems). Actin-β (Rn00667869_m1) was used as endogenous reference gene. The PCR reaction mixture was incubated on a 7500 Fast Real Time PCR system (Applied Biosystems). All samples, as well as the negative controls, were assayed in triplicates. The cycle threshold for each sample was obtained

and, thereafter, all data were analyzed with the comparative $2^{-\Delta\Delta CT}$ method, with the control group serving as the calibrator.[28]

Immunohistochemistry for tight-junction-related proteins

Paraffin embedded tissue sections (5 µm thick) were deparaffinized and rehydrated with a battery gradient of alcohols. Antigen retrieval for claudin-2 and claudin-3 was achieved by processing the slides in a microwave (2 cycles of 5 min, 800 W) in 10 mM Tris Base, 1 mM EDTA solution (pH 9). Epitope retrieval for ZO-1 was performed using a pressure cooker (at full pressure for 6 min) in 10 mM citrate buffer (pH 6). Thereafter, samples were incubated for 40 min in H_2O_2 (5% in distilled water) for inhibiting endogenous peroxidases and with the reagents of the *Avidin/Biotin Blocking Kit* (SP-2001; Vector Laboratories, Burlingame, CA) for inhibiting the endogenous avidin and biotin. Finally, a 1 h incubation at room temperature with horse, rabbit or goat serum, as appropriate, was performed for blocking unspecific unions before incubating the slides (overnight at 4ºC) with their respective primary antibodies: mouse monoclonal anti-Claudin-2 antibody (1:2000; ref.: 32-5600. Invitrogen, Camarillo, CA), goat polyclonal anti-Claudin-3 antibody (1:500; ref.: SC-17662. Santa Cruz, Dallas, TX), and rabbit polyclonal anti-ZO-1 (1:500; ref.: SC-10804. Santa Cruz). The following day, sections were incubated with the respective secondary antibody for 1 h at room temperature: biotinylated horse anti-mouse IgG (1:200, ref.: BA-2000, Vector Laboratories), biotinylated rabbit anti-goat IgG (1:200, ref.: SC2774. Santa Cruz) or Biotin-XX Goat Anti-Rabbit IgG (H+L) (1:200, ref.: B2770. Invitrogen). In all cases, an avidin/peroxidase kit (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) was used for detection, and the antigen-antibody complexes were revealed using 3,3'-diaminobenzi-dine

(SK-4100 DAB; Vector Laboratories). The slides were counterstained with toluidine blue or with hematoxylin. In all cases, the specificity of the staining was confirmed by omitting the primary antibody.

For claudin-2 and ZO-1 immunohistochemistries, 10-20 representative microphotographs (x 200) were taken per animal with a Nikon Eclipse 90i microscope. The intensity of the staining was measured using the software Image J (National Institute of Health, Bethesda, MD) and a mean value obtained. Claudin-3 immunoreactivity at the epithelial surface was quantified applying a semi-quantitative score (0: no immunoreactivity; 1: scarce, low intensity, granules in some epithelial cells; 2: clear, but not-organized granules in abundant epithelial cells; 3: clear and well-organized granules, drawing a chain in the zone corresponding to epithelial TJs, in most cells). In this case, for quantification, 15 randomly selected fields covering the whole thickness of the jejunal mucosa, from at least two tissue sections for animal, were scored by two independent observers. A final score (0-3) for each animal was calculated as the mean of the scores assigned by each observer. All procedures were performed on coded slides to avoid any bias.

Chemicals

Imiquimod [R-837, 1-(2-Methylpropyl)-1H-imidazole[4,5-c]quinoline-4-amine]; Enzo Life Sciences, Farmingdale, NY, USA] was dissolved in 0.5 % hydroxypropylmethyl cellulose (HPMC; Sigma-Aldrich, Saint Louis, MO, USA). Carbachol (CCh, Sigma-Aldrich) was dissolved in distilled water as stock solutions of 10^{-1} M (CCh), further dilutions were performed in distilled water. Dimethyl sulfoxide (DMSO) was obtained from Panreac (Barcelona, Spain). Fluorescein isothiocyanate (FITC)-labeled dextran (FD)

with a mean molecular weight of 4 kDa (FD4; TdB Consultancy AB. Uppsala, Sweden) was stored at 5ºC and was dissolved with Krebs solution (20 mg/ml) at the time of use.

Statistical analysis

All data are expressed as mean ± SEM. A robust analysis (one interaction) was used to obtain mean ± SEM for RT-qPCR data. Comparisons between two groups were performed using Student's unpaired t test. Comparisons between multiple groups were performed using a one-way or a two-way ANOVA, as appropriate; followed, when necessary, by a Newman-Keuls multiple comparisons test. In all cases, results were considered statistically significant when $P < 0.05$. GraphPad Prism 4 (GraphPad Software, La Jolla, California, USA) was used to perform all statistical analysis.

Results

Acute addition of imiquimod to the Ussing chamber did not affect colonic electrical parameters or permeability to macromolecules

Neither apical nor basolateral addition of imiquimod (125 µM) to the Ussing chamber affected basal electrical parameters. Similarly, passage of FD4 was not affected by IMQ (125 µM, either basolateral or apical).

Over-stimulation of colonic TLR7 with imiquimod does not trigger an inflammatory-like response within the colon

Regardless the experimental group considered, no signs of colonic inflammation were observed upon examination of the colon at the time

of necropsy. Similarly, no changes in colonic relative weight or the colonic expression of inflammatory markers (IL-6 and IFNα1) was observed among groups (data not shown). Likewise, regardless the treatments applied, no changes in TLR7 expression were detected (data not shown).

Pretreatment with imiquimod altered colonic epithelial conductance and reduced paracellular permeability in vitro

In colonic samples obtained from animals exposed to imiquimod (300 μg/rat, intracolonic) tissue conductance was slightly decreased, although statistical significant was not reached (P=0.087 vs. vehicle-treated tissues). A lower dose (100 μg/rat, intracolonic) was without effect. Other epithelial electrical parameters were not affected by the pre-treatment with imiquimod (Fig. 1 A-C).

In imiquimod pre-exposed tissues, the apical-to-basolateral flux of FD4 was reduced in a dose-related manner. At the end of the experimental time (1 h) the passage of FD4 was reduced by 28.5 % ($P>0.05$) and 62.2 % ($P<0.05$) for the doses of 100 μg and 300 μg, respectively, when compared with vehicle pre-exposed tissues (fig. 1D).

In general, an increase in I_{sc} in responses to a single concentration of CCh (100 μM), added at the end of the experimental protocol, was observed regardless the treatments applied. Only occasionally (less than 10% of the preparations) tissues were discarded because of lack of responses to CCh, as indicative of tissue damage.

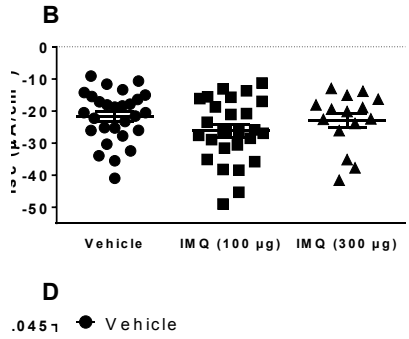

Figure 1. Effects of pre-exposure to imiquimod (IMQ) (intracolonic) on colonic epithelial electrical parameters and permeability to macromolecules. A-C: Epithelial electrical parameters: conductance (G, panel A), short circuit current (I_{SC}, panel B) and potential difference (PD, panel C). Each point represents a colonic sheet, and the line with errors the mean ± SEM (n = 15-28 colonic sheets from 9-16 animals). D: Effects of imiquimod on epithelial permeability to macromolecules. The graph shows mucosal to basolateral passage (as percentage of the amount added to the mucosal reservoir) of fluorescein isothiocyanate-dextran 4kD (FD4) in control conditions (vehicle pre-treatment) and in tissues pre-exposed to imiquimod. Data are mean ± SEM, n = 6-12 colonic sheets from 3-6 animals per group. *: $p<0.05$ vs. Vehicle.

Imiquimod enhances colonic permeability after disruption of the epithelial barrier in in vivo conditions

In basal conditions (animals treated intracolonically with vehicle), passage of FD4 to blood and urine, as well as accumulation in the colonic wall, was detected in a reproducible manner. Pre-treatment with intracolonic imiquimod (300 µg/rat) did not affect the passage of FD4 (Fig. 2).

Intracolonic imiquimod followed by a challenge of the colonic mucosa with DMSO resulted in an enhanced accumulation of FD4 in the colonic wall, with a 2-fold increase in plasma levels ($P<0.05$ vs. animals without DMSO) and an 8.5-fold increase in the FD4 concentration in urine ($P<0.05$ vs. animals without DMSO) (Fig. 2). DMSO, *per se*, resulted in a slight, non-significant, increase in the passage of FD4 to blood and urine (Fig. 2).

Figure 2. Effects of imiquimod (IMQ) on colonic permeability to FD4 in *in vivo* conditions. Data show the accumulation of FD4 on the colonic wall (A) and the passage of FD4 from colon to blood (μg of FD4/mL serum, B) and urine (μg of FD4/mL urine, corrected by its dilution factor; C) during a 30-min period in control conditions and after treatment with imiquimod with or without DMSO. Data are mean ± SEM, n = 9-11 animals per group. **: $p<0.01$ vs. other groups (ANOVA). Veh: Vehicle. SSF: Saline. DMSO: Dimethyl sulfoxide.

Effects of imiquimod on tight junctions-related proteins and barrier-modulators

Expression of the main TJ-related proteins (occludin, tricellulin, claudin-2, claudin-3, JAM-1 and ZO-1) was detected in all colonic samples. Intracolonic imiquimod induced a dose-related down-regulation of ZO-1, achieving a 35.4% reduction at the 300 μg/rat dose ($P<0.05$ vs. vehicle; Fig. 3A). Similar trend was observed for the pore-forming protein claudin-2, but statistical significance was not achieved. Other TJ-related

proteins were not affected by imiquimod (Fig. 3A). Similar relative changes were observed when imiquimod was combined with the epithelial irritation with DMSO (Fig. 3B), without consistent treatment-related changes in protein expression.

All TJ-related proteins assessed were also detected using immunohistochemistry. In control conditions, distribution along the epithelium was protein-specific. In control conditions, claudin-2 expression was restricted to the lower-mid part of the colonic crypts (Fig. 4). No treatment-associated changes were observed in claudin-2 expression (intensity of staining) or distribution, without differences in the percentage of crypt length with immunoreactivity (data not shown). Claudin-3 immunoreactivity was distributed homogeneously in the colonic crypt, while forming intracellular granules located in the supranuclear cytoplasm in the superficial epithelial cells (Fig. 4). Again, no treatment-associated changes in distribution or intensity of staining were observed (Fig. 5). ZO-1 was expressed along the whole crypt, forming a connecting net among epithelial cells; with no detectable changes associated to treatments (Figs. 4 and 5). In all cases, immunoreactivity disappeared when the primary antibody was omitted, thus confirming the specificity of the staining (Fig. 5).

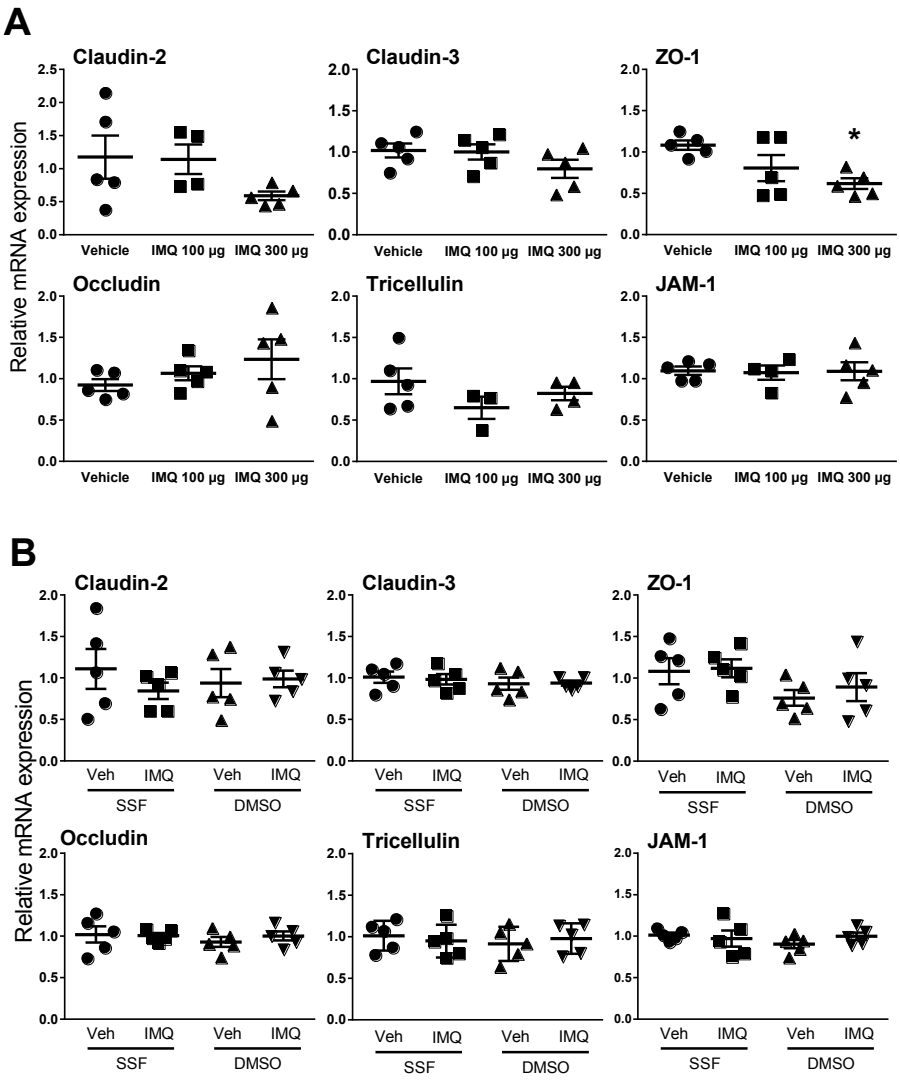

Figure 3. Effects of imiquimod (IMQ) on gene expression of colonic tight junction proteins (claudin-2, claudin-3, ZO-1, occludin, tricellulin, and JAM-1). A: Gene expression in tissues 3h post-intracolonic treatment with imiquimod. B: Gene expression in animals treated with imiquimod combined with an epithelial challenge with DMSO. Each symbol represents an individual animal, the lines with errors correspond to the robust mean ± SEM. *: $p<0.05$ vs. Vehicle. Veh: Vehicle. SSF: Saline. DMSO: Dimethyl sulfoxide.

Expression of proglucagon, precursor of the barrier-enhancer factor GLP-2, was detected in all colonic samples. No treatment-related changes were detected among groups (data not shown).

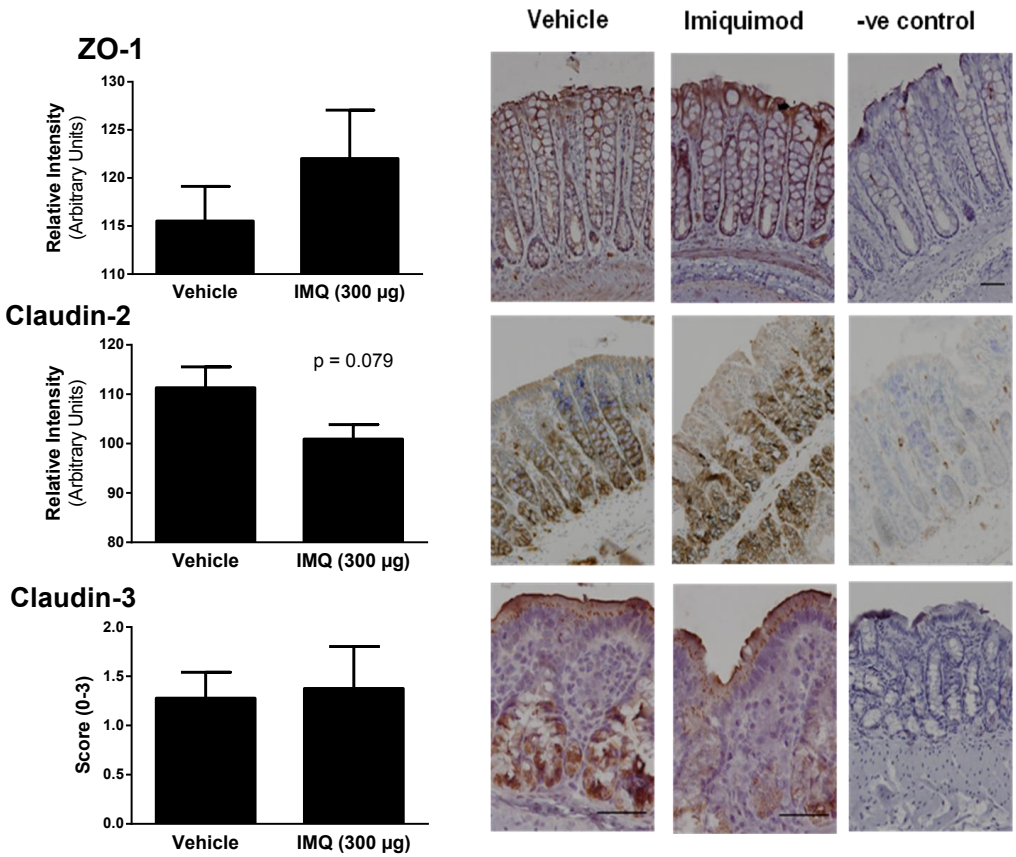

Figure 4. Immunohistochemistry for tight junction-related proteins: ZO-1, claudin-2 and claudin-3. Left column: quantification of immunostaining in control and imiquimod (IMQ)-treated rats (300 µg/rat, intracolonic). See methods for details of quantification. Data are mean ± SEM, n=8-13. Microphotographs: Representative images of a control animal (vehicle), an imiquimod (300 µg, intracolonic)-treated animal and a negative control (-ve control) for ZO-1, claudin-2 and claudin-3. Scale bar: 50 µm.

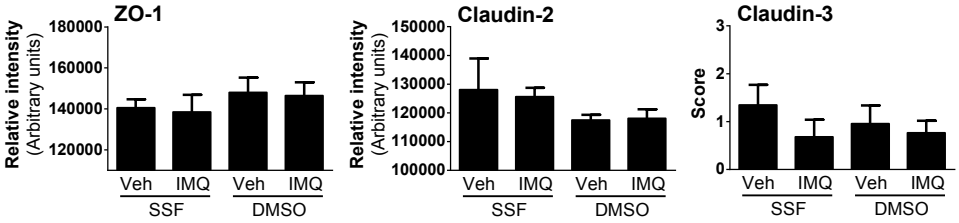

Figure 5. Quantification of immunostaining for tight junction-related proteins (ZO-1, claudin-2 and claudin-3) in control and imiquimod-treated rats with or without DMSO. See methods for details of quantification. Data are mean ± SEM, n=3-5. Veh: Vehicle. SSF: Saline. DMSO: Dimethyl sulfoxide.

Discussion

In this study we show that the local, colonic, over-stimulation of TLR7 with the selective agonist imiquimod modulates colonic EBF in rats. *In vivo* over-stimulation of TLR7 lead to an enhancement of colonic epithelial barrier function, while acute, *in vitro*, stimulation was without effects. In order to evidence these TLR7-dependent pro-barrier effects, over-stimulation of TLR7 was combined with the intracolonic administration of DMSO as a stimulus to alter epithelial permeability. In these conditions, opposite effects were observed, with an increase in epithelial permeability upon TLR7 stimulation. Effects of TLR7 on EBF seem to be independent of the modulation of the expression or the distribution of the main tight-junction-related proteins or the modulation of the expression levels of the barrier-enhancer factor GLP-2.

Alterations of colonic EBF are a component of several gastrointestinal diseases, and are a common finding in IBD and IBS.[1–3] Although the pathophysiological mechanisms underlying these alterations are not

completely understood, compelling evidences suggest the presence of a sustained enhancement of epithelial paracellular permeability facilitating the exposure to luminal antigens, triggering innate mucosal immune system responses, thus perpetuating a state of persistent, and abnormal, immune activation leading to the development of intestinal inflammation. Several studies suggest that gut commensal microbiota and host-microbial interactions might be important pathogenic factors in this process.[5,29–35] Indeed, epithelial interactions with the microbiota and microbial-derived products, as well as increased entrance in the intestinal wall, are altered in states of dysbiosis, likely contributing to the process of immune activation.[3,29,36,37] Nevertheless, the microbial contribution and the exact mechanisms involved are still unclear. In this context, in the present report, we explored the possibility that TLR7-dependent host-bacterial interactions might be part of the mechanisms through which the microbiota modulates epithelial barrier function. For this, we simulated a state of dysbiosis with over-stimulation of TLR7-dependent host-bacterial interactions through direct stimulation of TLR7 with a selective agonist, imiquimod, administered locally (intracolonically). We have previously used this approach to study TLR-dependent host-bacterial interactions in rats and mice.[24,25]

Results obtained show that local (colonic) over-stimulation of TLR7 with imiquimod leads to a reduction in epithelial conductance and paracellular permeability to macromolecules in *in vitro* conditions (Ussing chamber), thus indicating an improvement of EBF. These observations suggest that over-stimulation of TLR7 associated to a dysbiotic state might have a defensive function, increasing tightness of the epithelium and therefore, preventing an increased passage of luminal, microbial-

related, antigens during dysbiosis. This protective role of TLR7 agrees with the postulated role for other TLRs, mainly TLR2. Indeed, data obtained in murine models of dysbiosis indicates that TLR2 is a receptor that, upon activation by luminal microbial products, enhances EBF as a protective response to dysbiosis, preventing excessive passage of luminal antigens and an aberrant activation of the local immune system.[21,38,39] Altogether, these observations suggest that several TLRs, including at least TLR2 and TLR7, might share a similar protective role in states of over-stimulation (as likely occurring during dysbiosis). Taking into account that several TLRs have been largely associated to the activation of intestinal immune responses and the induction of inflammation and secretomotor alterations,[20,40,41] these observations support a dual role for TLRs, mediating both pathogenic and protective responses in a TLR-specific manner. Balance between protective and damaging signals will lead to a final state in which homeostasis is maintained or functional alterations appear.

Interestingly, pro-barrier effects of TLR7 were not observed when the receptor was acutely stimulated *in vitro* (direct addition of imiquimod in the Ussing chamber). This apparent discrepancy *vs.* the observation after the *in vivo* stimulation might suggest that activation of TLR7 triggers a signaling cascade that requires a relatively long time-frame to generate functional changes. In this respect, technical limitations of the technique used, since the viability of the mucosal sheets in the Ussing chamber is limited, might not allow the development of the full response observed after the *in vivo* stimulation. In addition, recruitment of extra-intestinal neuroendocrine mechanisms, not preserved in *in vitro* conditions, might be necessary to elicit the full effects associated to TLR7 stimulation,

which, in consequence, cannot be manifested during the acute *in vitro* stimulation.

Taking into account the potential protective role of TLR7, we hypothesized that its effects would manifest in states of barrier alteration in which epithelial entrance of luminal factors might be facilitated. In these conditions, a more effective activation of TLR-dependent signaling mechanisms, including TLR7-mediated pro-barrier responses, might occur. To test this hypothesis, we assessed the effects of TLR7 over-stimulation during the simulation of a state of favored epithelial permeability, such as during DMSO exposure. In this sense, DMSO is an aprotic solvent that permeabilizes the cell membrane acting as a non-selective penetration enhancer.[27] In the presence of DMSO, we should expect an increased entrance of luminally-administered imiquimod and, therefore, an enhanced stimulation of intracellular TLR7 and the consequent activation of barrier protective mechanisms. However, against this hypothesis, the combination imiquimod-DMSO resulted in a deterioration of EBF, with an increased passage of luminal macromolecules to blood and urine, as assessed *in vivo*. These effects are likely to be TLR7-dependent and not secondary to the permeabilization of the epithelium, since DMSO, *per se*, did not increase epithelial permeability to macromolecules. At the moment, we cannot explain the differences observed between the stimulation of TLR7 by imiquimod alone or with the combination imiquimod-DMSO (TLR7-mediated enhancement of the barrier vs. a TLR7-mediated worsening of the barrier, respectively). We can speculate that after DMSO administration the passage of luminal factors leads to the simultaneous activation of multiple mechanisms, including barrier protective and damaging

pathways, with a final outcome depending upon de balance between both effects.

In any case, results obtained suggest that TLR7-mediated effects on EBF are mainly associated to a modulation of the permeability to macromolecules, which predominantly occurs through paracellular pathways. Since paracellular permeability to macromolecules depends largely on the TJ organized among epithelial cells we also assessed if TLR7 over-stimulation could modify the expression of the main TJ-related proteins (occludin, tricellulin, claudin-2, claudin-3, JAM-1 and ZO-1). Overall, no consistent changes in expression of these proteins were observed following imiquimod administration, either alone or combined with DMSO. In spite of these negative findings, we cannot exclude conformational changes of the already available proteins that could lead to a reorganization of the TJ at a molecular level, thus explaining our functional findings. For instance, Cario *et al*. showed that the stimulation of TLR2 enhances transepithelial resistance of the colonic epithelial barrier through the apical redistribution of ZO-1.[39] Therefore, similar mechanisms cannot be discarded for TLR7. However, when assessing the epithelial distribution of ZO-1, claudin-2 and claudin-3 using immunohistochemistry no consistent treatment-related changes in distribution or intensity of staining were observed during the stimulation TLR7 (with or without DMSO). Overall, these observations indicate that TLR7-dependent modulation of colonic EBF is independent of changes in TJ-related proteins, at least as it relates to occludin, tricellulin, claudin-2, claudin-3, JAM-1 and ZO-1. Nevertheless, further ultrastructural studies addressing the organization of TJ are necessary to further address the potential implication of TJ in TLR7-mediated modulation of EBF.

GLP-2 has been postulated as a harrier-enhancer factor. Indeed, a positive correlation between intestinal proglucagon levels, the precursor of GLP-2, and the expression of TJ proteins (ZO-1 and occludin) has been observed.[26] Similarly, a reduction in proglucagon expression has been described in states of increased epithelial permeability associated to down-regulation of TJ-related proteins.[9] Although these evidences, in our studies, no changes in proglucagon gene expression were detected upon activation of TLR7, despite the presence of changes in EBF. This suggests that GLP-2 is not implicated in the barrier modulatory effects of TLR7.

In previous studies, we observed that intracolonic imiquimod had minor effects on immune activation in rats, with a moderate up-regulation of pro-inflammatory cytokines, observed only after repeated treatment.[24,25] In agreement with these observations, a single treatment with imiquimod neither up-regulated pro-inflammatory cytokines nor induced inflammatory like-changes within the colon, at the macroscopical or microscopical levels (data not shown). This indicates that TLR7-mediated modulation of EBF is not secondary to a local immune activation within the colon.

Overall, we show that the colonic activation of TLR7 might be associated to and enhancement of EBF in normal conditions. On the other hand, a deterioration of barrier function was observed in states of epithelial permeabilization, likely associated to an increased stimulation of the receptor and/or a negative balance between protective-detrimental factors in states of epithelial dysfunction. The mechanisms underlying these effects remain unclear. Results obtained suggest that neither a modulation of the expression and/or organization of the main tight junction-related proteins, nor changes in the expression of

proglucagon, as precursor of the barrier-enhancer GLP-2, mediate the observed changes in EBF. Overall, these observations, together with previous data, suggest that TLRs-mediated host-bacterial interactions might elicit protective or detrimental responses on epithelial barrier function. Modulatory effects of TLRs on EBF might contribute to the pathophysiological alterations observed in gastrointestinal diseases having dysbiosis and barrier alterations as common components, such as IBD or IBS.

Acknowledgement

We thank A. Acosta and E. Martínez for their technical assistance. This work was supported by grants 2014SGR789 from the Generalitat de Catalunya and BFU2009-08229 from Ministerio de Ciencia e Innovación (Spain). JE personal support from the Ministerio de Ciencia e Innovación (FPU program) (Spain).

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Chapter 2

Local over-stimulation of toll-like receptor 7 attenuates epithelial barrier dysfunction during colitis in mice

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Abstract

Introduction/Aim: Altered epithelial barrier function (EBF) is common in intestinal inflammatory disorders. Toll-Like Receptors (TLRs) mediate neuro-immune responses within the gut and contribute to the control of EBF. In this work, we assessed if TLR7 participates in the modulation of EBF during states of intestinal inflammation.

Methods: Colitis was induced in mice by exposure to dextran sodium sulfate (DSS). EBF was assessed *in vitro* (Ussing chambers) and *in vivo* (passage of intracolonic FD4 to blood and urine). Potential modulatory role of colonic TLR7 on EBF was assessed by local over-stimulation with the selective agonist imiquimod in *in vitro* (direct addition to Ussing chambers; 300 μg) and *in vivo* (300 μg/mouse, intracolonic, single or 3-day repeated administration). Changes in gene expression of tight junction-related proteins (occludin, claudin-2, claudin-3, Zona Occludens-1), barrier-modulating factors (proglucagon and myosin light-chain kinase), and IL-12p40 were assessed by RT-qPCR.

Results: DSS led to a state of colitis, as evidenced macro and microscopically. In animals with colitis, basal epithelial electrical parameters were altered, and permeability to macromolecules (FD4) increased, indicating an altered EBF. Over-stimulation of TLR7 restored the epithelial permeability to FD4 in inflamed tissues in *in vitro* and *in vivo* conditions, indicating a restoration of EBF. Colitis down-regulated tight junction-related proteins and barrier-modulating factors. Imiquimod did not revert these effects.

Conclusion: Over-stimulation of TLR7 leads to an improvement of inflammation-induced altered EBF. The mechanisms are independent of

changes in the expression of the assessed tight junction-related proteins and barrier modulators. Pro-barrier effects of TLR7 may present a defensive function, preventing the passage of luminal antigens and the perpetuation of inflammatory responses during states of dysbiosis.

Keywords: Colitis; Epithelial Barrier Function; Imiquimod; Intestinal permeability; Tight junctions; TLR7.

Introduction

Impairment of the intestinal epithelial barrier function is a common finding in functional and inflammatory gastrointestinal disorders, such as Inflammatory Bowel Disease (IBD) and Irritable Bowel Syndrome (IBS).[1] Indeed, increased intestinal permeability, generating a state known as "leaky gut", has been proposed as both initiating and perpetuating factor in the pathogenesis of these pathologies.[2,3]

Epithelial integrity is essential to maintain normal colonic barrier function, preventing the uncontrolled passage of luminal antigens to the internal milieu and regulating the absorption of molecules and electrolytes.[4,5] In particular, tight junctions (TJs) are complex structures that span the apicolateral border of the plasma membrane allowing the epithelium to constitute this physical barrier. TJs maintain the epithelial polarity and function as a gate in paracellular permeability. Structurally, TJs are composed by four types of integral membrane proteins with different functions: claudins, occludin, tricellulin, and junctional adhesion molecule (JAM), and several TJ-associated peripheral membrane proteins, with Zonula Occludens (ZO) 1, 2 and 3 as main players. Furthermore, these protein components are structurally and functionally regulated by several regulatory factors, such as immune-related mediators or gut regulatory peptides.[5–7]

Exogenous factors can also affect intestinal epithelial barrier function. For example, several evidences indicate that gut commensal microbiota (GCM), interacting with the host, is able to modulate epithelial permeability through mechanisms implicating the modulation of tight junctions.[8,9] Host-bacterial interactions are largely dependent on host-mediated bacterial recognition systems. These include a series of

Pattern Recognition Receptors (PRR), being Toll-like receptors (TLRs) the best characterized PRR. Within the gut, microbial products activate TLRs, leading to local neuro-immune responses that affect intestinal functions, including EBF. As it relates to EBF, TLR-mediated effects seem to be receptor-specific and can be either beneficial, enhancing EBF and preventing the passage of luminal factors, or detrimental, opening the epithelial barrier and favouring the passage of luminal antigens. For instance, TLR2 has been shown to enhance transepithelial resistance through TJs-related mechanisms, in particular altering the epithelial distribution of ZO-1.[10] Similarly, the stimulation of TLR9 or TLR7 elicits protective effects during colitis in mice.[11,12] Moreover, we previously showed that over-stimulation of TLR7 alters epithelial function in rats, eliciting both protective and damaging effects depending upon experimental conditions considered.[13]

In the present study, and to gain insight into TLRs-mediated modulation of epithelial barrier function, we explored potential barrier protective effects of TLR7 during a state of intestinal inflammation (colitis) in mice. Furthermore, to understand the potential mechanisms involved we also determined changes in TJs (gene expression of TJ-related proteins) and barrier modulators, namely myosin light chain kinase (MLCK) and glucagon-like peptide 2 (GLP-2), assessed through the expression of its precursor, proglucagon.

Materials and Methods

Animals

Adult male CD1 Swiss mice (6 weeks of age on arrival; Charles-River Laboratories, Lyon, France) were used. On arrival, animals were housed in standard plastic cages under conventional controlled environmental conditions (20–22ºC, 40–70% humidity and 12 h light/dark cycle) for 1 week before any treatment. Mice were fed with a standard pellet diet (Panlab SL, Barcelona, Spain) and tap water *ad libitum* if not otherwise stated. Animals were 7-8 week old at the time of the experiments. All experimental procedures were approved by the Ethical Committee of the Universitat Autònoma de Barcelona and the Generalitat de Catalunya (protocols 3039 and 8823, respectively).

Induction of colitis

Colitis was induced in mice with dextran sodium sulfate (DSS; 35-55 kDa; TDB Consultancy, Uppsala, Sweden) added to the drinking water (5 % final concentration) during a 5-day period, followed by a 2-day washout period with normal drinking water. DSS solutions were prepared daily and animals had *ad libitum* access. The control group received tap water during the same period. Mice were supervised daily for clinical signs of inflammation, including general state, changes in body weight, and presence of rectal bleeding and diarrhea, as previously described by us.[14] Experiments were performed on day 7 (5-day DSS treatment + 2-day washout), corresponding to the acute phase of inflammation.[14]

Experimental protocols and samples collection

In vitro epithelial barrier function - Ussing chamber

At the end of the colitis-induction process, mice were euthanized by cervical dislocation and colonic sheets were collected to be mounted in a Ussing chamber system for the assessment of epithelial barrier function *in vitro.* Four colonic sheets were obtained from every animal and were randomly distributed, in a balanced manner, in four experimental groups for either inflamed or healthy tissues: i) apical imiquimod (IMQ; 300 µg, 25 µL); ii) basolateral IMQ (300 µg, 25 µL); iii) apical vehicle (hydroxypropylmethyl cellulose, HPMC; 5mg/mL, 25 µL); and iv) basolateral vehicle (5mg/mL, 25 µL). Colonic sheets were mounted in the Ussing Chamber, after a 20 min of stabilization, treatments were applied and electrical parameters were assessed for a 30 min period. Thereafter, electrical parameters were recorded for an additional 60 min period.

In vivo permeability studies

Effects of TLR7 stimulation on colonic epithelial permeability to macromolecules (4 kDa fluorescein isothiocyanate–dextran, FD4) *in vivo* were assessed with a single dose or multiple administration (dairy dose every 24 h for 3 days) of IMQ, 90 min before the euthanasia and the collection of samples, respectively. For this, animals were deeply anesthetized with isoflurane (Isoflo®, Esteve Veterinaria, Barcelona, Spain) and treatments were applied intracolonically with a plastic cannula (4 cm from the anus, corresponding to the mid-distal colon).

Mice were randomly distributed in the experimental groups, according to treatments: Control-Vehicle [normal tap water and hydroxypropylmethyl cellulose (HPMC; 5mg/mL; 50 µL, intracolonic)],

Control-IMQ [Normal tap water and Imiquimod (300 µg/mouse; 50 µL, intracolonic)], Colitis-Vehicle (DSS 5% in tap water and HPMC) and Colitis-IMQ [DSS; 5% in tap water and Imiquimod (300 µg/mouse)]. 30 min after the intracolonic administration of FD4, animals were deeply anesthetized with isoflurane. Blood (cardiac puncture), urine tissue samples were collected to asses FD4 passage (see below for detailed description on the procedures followed). At the same time, colonic inflammation was assessed and tissue samples collected for histopathology (4% paraformaldehyde) or gene expression (frozen in liquid nitrogen and maintained at -80ºC until analysis).

Measurement of electrophysiological parameters

After collecting colonic samples, these were immediately flushed with cold Krebs buffer and placed in ice cold oxygenated (95% O_2; 5% CO_2) Krebs buffer containing glucose. Once mounted in the Ussing chambers, colonic epithelial electrical parameters (short circuit current, potential difference and conductance) were assessed *in vitro* in Ussing chambers as a measure of EBF. Short-circuit current (I_{sc}), defined as the charge flow per time when the tissue is short-circuited or clamped to 0 mV, represents a summation of all ionic currents across the epithelium. Potential difference (PD) is defined as the transepithelial voltage generated by the epithelial electrogenic active ionic transport. Conductance (G) is a useful measure of the integrity of the intestinal tissue, and it is regarded as an indicative of the state of the ionic paracellular pathway across the intestinal epithelium.[15,16]

For electrophysiological measurements, colonic segments were stripped of the outer muscle layers and myenteric plexus, opened along the mesenteric border and divided into 0.5 cm^2 flat segments, excluding

colonic patches. Epithelial sheets were mounted in Ussing chambers (World Precision Instruments, Aston, UK) with an exposed window surface area of 0.28 cm^2. Tissues were bathed bilaterally with 5 mL of oxygenated and warmed (37ºC) Krebs buffer (in mmol/L: 115.48 NaCl, 21.90 $NaHCO_3$, 4.61 KCl, 1.14 NaH_2PO_4, 2.50 $CaCl_2$ and 1.16 $MgSO_4$; pH: 7.3–7.4). The buffer also contained 10 mmol/L glucose as energy source. At the end of the experiments, a single concentration of CCh (100 µM) was added to the basolateral side of the chamber to assess the viability of the tissues. The electric equipment of the Ussing chambers consists of two voltage-sensitive electrodes (EKV; World Precision Instruments) to monitor the PD across the tissue and two Ag-AgCl current passing electrodes (EKC; World Precision Instruments) to inject the required I_{sc} to maintain a zero potential difference, as registered via an automated voltage/current clamp (DVC-1000; World Precision Instruments). Ohm's law was used to calculate tissue G, using the change in I_{sc} when a voltage step of 1 mV was applied every 5 min. Tissues were allowed to stabilize for 20 min before baseline values for PD, I_{sc} and G were recorded. Data were digitized with an analog-to-digital converter (MP150; Biopac Systems, Goleta, USA) and measurements were recorded and analyzed with Acqknowledge computer software (version 3.8.1; Biopac Systems). I_{sc} and G were normalized for the mucosal surface area.

Epithelial permeability to macromolecules in vitro

Permeability to macromolecules was evaluated measuring mucosal-to-basolateral fluxes of fluorescein isothiocyanate (FITC)-labeled dextran (FD) with a mean molecular weight of 4 kDa (FD4; TdB Consultancy AB, Uppsala, Sweden) in colonic epithelial sheets from mice.

After stabilization (20 min) and basal electrical parameters measuring period (30 min), FD4 was added to the mucosal reservoir to a final concentration of 2.5 x 10^{-4} M. Basolateral samples (250 µL, replaced by 250 µL of buffer solution with glucose) were taken at 15-min intervals during the following 60 min for measurement of D4. Concentration of fluorescein in the samples was determined by fluorometry (Infinite F200; Tecan, Crailsheim, Germany) with an excitation wavelength of 485 nm (20 nm band width) and an emission wavelength of 535 nm (25 nm band width), against a standard curve. Readings are expressed as a percentage (%) of the total amount of FD4 added to the mucosal reservoir.

In vivo colonic permeability to macromolecules

Blood, urine and colon samples were collected 30 minutes after the intracolonic administration of fluorescein isothiocyanate (FITC)-labeled dextran (FD) with a mean molecular weight of 4 kDa (FD4, 50 mg/mL, 50 µl; TdB Consultancy AB, Uppsala, Sweden). Blood was centrifuged (10000 G, 10 min, 4 ºC) and serum was collected. Urine was obtained intravesically and diluted (1:50). Colonic samples were flushed with saline (0.1 mL/mg) and stored for fluorescence determinations. Afterwards, these were sonicated for 5 min in saline (10 µL/µg) and this liquid was stored for determining its fluorescence. The colon segments were homogenized with GentleMacs™ Dissociator (Miltenyi Biotec, Madrid, Spain) and centrifuged (3000 G, 10 min, 4 ºC). The supernatant was recovered and stored in ice in dark conditions, as the other samples. Concentration of fluorescein in the samples was determined by fluorometry (Infinite F200; Tecan, Crailsheim, Germany) with an excitation wavelength of 485 nm (20 nm band width) and an emission

wavelength of 535 nm (25 nm band width), against a standard curve. Readings of colon are expressed as a percentage (%) of the total amount of FD4 recovered from the colonic tissue and the flushing and sonicating mediums. The measures of serum and urine are expressed in concentration of FD4 (μg/mL).

Quantitative Real Time reverse transcription polymerase chain reaction (RT-qPCR)

Extractions of RNA were performed from colonic samples using Ribopure RNA Isolation Kit (Applied Biosystems, CA, USA) and quantified by Nanodrop (Nanodrop Technologies, Rockland, DE, USA). For cDNA synthesis, 1 μg of RNA was reverse-transcribed in a 20 μl reaction volume using a high capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA). Expression of tight-junction related proteins (occludin, claudin-2, claudin-3, ZO-1), barrier markers (MLCK and proglucagon) and IL-12p40 was determined by quantitative real-time PCR performed with specific Taqman probes (Applied Biosystems; occludin: Mm00500912_m1, claudin-2: Mm00516703_s1, claudin-3: Mm00515499_s1, ZO-1: Mm00493699_m1, MLCK: Mm00653039_m1, proglucagon: Mm01269055_m1, IL-12p40: Mm00434174_m1) mixed with Taqman Universal Master Mix II for 40 cycles (95ºC for 15 s, 60ºC for 1 min) on a 7500 real-time PCR system (Applied Biosystems). β2-microglobulin expression (Mm00437762_m1) was used as an endogenous control for normalizing the mRNA levels of the target gens. Expression levels were analyzed by the $2^{-\Delta\Delta CT}$ method.[17]

Histopathology

Colonic samples fixed in 4% paraformaldehyde were processed routinely for paraffin embedding. 5 μm sections were obtained for Hematoxylin-eosin staining. A histopathological score (ranging from 0, normal, to 12, maximal alterations) was performed evaluating the following parameters: epithelial structure (0: normal; 1: mild alterations of the villi; 2: local villi destruction and/or fusion; 3: generalized villi destruction and/or fusion), structure of the crypts (0: normal; 1: mild alterations of the crypts; 2: local destruction of the crypts; 3: generalized destruction of the crypts), presence of edema (0: normal; 1: mild local edema in submucosa and/or lamina propria; 2: moderate diffuse edema in submucosa and/or lamina propria; 3: severe generalized edema in submucosa and/or lamina propria), and presence of inflammatory infiltrate (0: normal; 1: mild localized infiltrate; 2: mild generalized infiltrate; 3: severe generalized infiltrate). Scoring was performed on coded slides by 2 independent researchers.

Chemicals

Imiquimod [R-837, 1-(2-Methylpropyl)-1H-imidazole[4,5-c]quinoline-4-amine] was acquired from Enzo Life Sciences (Farmingdale, NY, USA). The vehicle was Hydroxypropylmethyl cellulose (HPMC), obtained from Sigma-Aldrich (Saint Louis, MO, USA). Imiquimod was dissolved in HPMC (0.5% w/v) at 12 μg/μl and 6 μg/μl. Dextran Sulphate Sodium (DSS; 35-55 kDa) was purchased from TdB 5 %; (Consultancy, Uppsala, Sweden) and diluted in water (5% w/v) every day of treatment. CCh was purchased from Sigma-Aldrich and stock solutions of CCh (10^{-1} M) in distilled water were stored at -30ºC. The fluorescein isothiocyanate (FITC)-labeled dextran (FD) with mean molecular weight of 4 kDa (FD4; TdB Consultancy

AB. Uppsala, Sweden) was dissolved with Krebs solution (20 mg/mL) or serum (50 mg/mL) at the time of its use.

Statistical analysis

All data are expressed as mean ± SEM and analyzed by a two-way ANOVA test. A robust analysis (one interaction) was used to obtain mean ± SEM for RT-qPCR data. In all cases, results were considered statistically significant when $P<0.05$. GraphPad Prism 5 (GraphPad Software, La Jolla, California, USA) was used to perform all statistical analysis.

Results

DSS-induced colitis in mice and effects of TLR7 over-stimulation on inflammation

Mice treated with DSS (5 %) during a 5-day period showed clinical signs and changes in body weight consistent with the development of colitis and similar to those previously described.[14,18] At necropsy, a shortening of the colon and an increased colonic relative weight were also observed. Similarly, at the histopathological level, DSS-treated mice showed structural alterations (epithelial damage, submucosal edema and inflammatory infiltrate) consistent with a state of acute inflammation. Treatment with IMQ had no effects on colitis development, as indicated by the clinical signs, macroscopical changes in the colon and the presence of histological alterations at the microscopic level (Fig. 1).

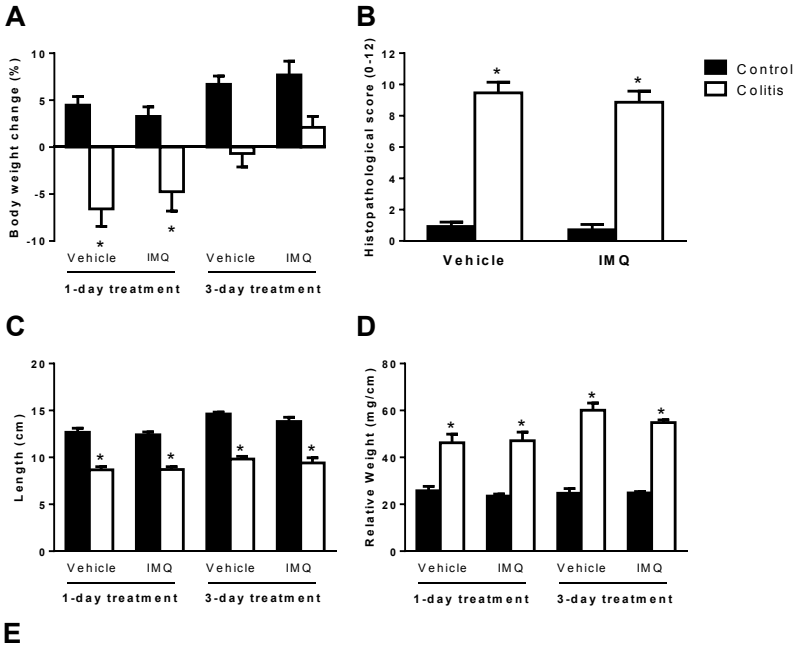

Figure 1. DSS-induced colitis and effects of imiquimod. A: Body weight change at day 7 in healthy (control) and DSS-treated animals (colitis), treated with either vehicle or imiquimod (single or 3-day repeated treatment. *: P<0.05 vs. respective control). B: Histopathological scores in healthy (control) or DSS-treated mice (colitis) and effects of a 3-day repeated treatment with imiquimod or vehicle. See methods for details on the scoring system. *: P<0.05 vs. respective control. C: Length of the colon in healthy (control) or DSS-treated mice (colitis) and effects of a single or 3-day repeated treatment with imiquimod or vehicle. *: P<0.05 vs. respective control. D: Colonic relative weight in healthy (control) or DSS-treated mice (colitis) and effects of a single or 3-day repeated treatment with imiquimod or vehicle. *: P<0.05 vs. respective control. E: Gene expression of IL12p40 in healthy (control) or DSS-treated mice (colitis) and effects of a single or 3-day repeated treatment with imiquimod. Data are mean ± SEM, n= 7-12 animals per group. IMQ: Imiquimod.

During colitis, colonic expression of the cytokine IL12p40 was increased by 2-fold (although statistical significance was not achieved probably because of the relatively large variability in the data). In healthy animals IMQ had no effects on IL12p40, but normalized the relative increase observed during colitis (Fig. 1).

Effects of colitis and over-stimulation of TLR7 on epithelial electrical parameters as assessed in Ussing chambers

Inflammation altered basal epithelial electrical parameters, as assessed *in vitro* in a Ussing chamber setup. Colonic sheets from DSS-treated mice showed reduced PD and I_{sc} (both $P<0.05$ vs. non-inflamed control tissues; Fig. 2) and an increased ionic conductance (Fig. 2).

Apical addition of IMQ (300 µg) had minor effects on electrical parameters, either on healthy or inflamed tissues. Basolateral addition of IMQ (300 µg) increased potential difference inflamed tissues (both $P< 0.05$ vs. basal conditions or responses to vehicle treatment; Fig. 2), with minor effects on healthy tissues. Apical addition tended to reduce conductance ($P = 0.096$ vs. responses to vehicle; Fig. 2), being normalized to control levels.

In general, at the end of the experiments, addition of CCh elicited a clear change in I_{sc}, lack of responses was indicative of tissue damage. Less than 10% of the tissues assessed were discarded because of lack of responses to CCh.

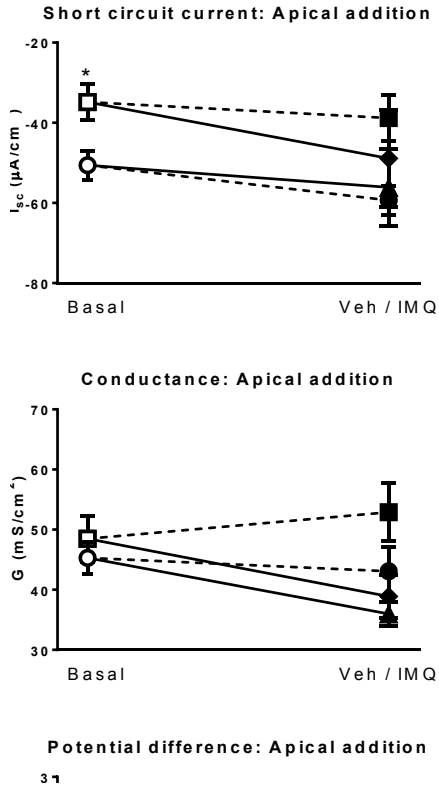

Figure 2. Effects of colitis and imiquimod on basal colonic epithelial electrical parameters. Data show basal values of short circuit current (I_{sc}), conductance (G), and potential difference (PD) and changes 30 min after the addition, to the apical or the basolateral side, of vehicle or imiquimod. Data are mean ± SEM of 5-20 epithelial sheets obtained from 7-10 animals. In each group (control or colitis), after obtaining a basal measurement sheets were treated with either vehicle or imiquimod (n=5-10 for each treatment and condition). *: $p<0.05$ Colitis vs. Control; #: IMQ vs. respective control (Veh). Veh: Vehicle; IMQ: Imiquimod.

Effects of TLR7 over-stimulation on epithelial permeability to macromolecules during DSS-induced colitis, as assessed in Ussing chambers

In colonic sheets from DSS-treated animals mounted in Ussing chambers the transepithelial flux of FD4 was increased by 2-fold vs. non-inflamed tissues (P<0.05; Fig. 3). In these conditions, over-stimulation of TLR7 by the apical direct addition of IMQ (300 µg) restored epithelial permeability to FD4 to basal levels (Fig. 3). Basolateral addition of IMQ (300 µg) tended to reduce the flux of FD4 (by 24 % reduction vs. vehicle in inflamed tissues, P>0.05). In healthy tissues, IMQ, either apical or basolateral, showed a clear trend to reduce the passage of FD4, although statistical significance was not achieved (44% and 41% reduction vs. vehicle in healthy tissues for apical and basolateral IMQ, respectively) (Fig. 3).

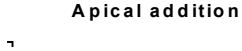

Figure 3. Effects of colitis and imiquimod on colonic permeability to macromolecules in *in vitro* conditions (Ussing chamber). Data show flux of FD4 (% from total amount added to the mucosal side) during a 60 min period, and the effects of imiquimod added to the apical or the basolateral side. Data are mean ± SEM, n = 6-10 colonic sheets, from 7-10 animals, per group. *: p<0.05; ***: p<0.001 vs. vehicle. **: p<0.01 vs. DSS-Veh. Veh: Vehicle; IMQ: Imiquimod.

Effects of TLR7 over-stimulation on DSS-induced colonic barrier dysfunction in in vivo conditions

In healthy animals treated with vehicle, passage of intracolonic FD4 to plasma and urine was relatively low and within a similar range in all cases, regardless the experimental protocols followed (single, n=10, or repeated treatment, n=7-9), therefore, for the sake of clarity, all data has been combined in a single control group.

In animals with DSS-induced colitis plasma and urine levels of FD4 were increased by 6- and 13-fold, respectively, compared with the levels in animals without inflammation (both $P<0.05$ vs. non-inflamed controls; Fig. 4). In these conditions, IMQ attenuated in a dose-related manner (single treatment or repeated treatment) the increase in permeability (Fig. 4). Overall, plasma FD4 levels were reduced by 34 % ($P>0.05$) and 69 % ($P=0.077$) during the single and repeated treatment with IMQ, respectively. Likewise, urine FD4 levels were reduced by 11% and 51% during the single and repeated treatment with IMQ. However, due to the relatively high variability in the data, statistical significance was not reached.

Effects of colitis and TLR7 over-stimulation on gene expression of tight junction-related proteins and barrier modulating factors

Gene expression levels were similar in all vehicle-treated healthy animals, therefore, as for the permeability experiments, data from the single and repeated treatment has been pooled as a single control group. In animals with DSS-induced colitis, gene expression of TJ-related proteins showed a general down-regulation (by 50 % to 80 % depending

upon the protein considered; Fig. 5). In healthy animals, IMQ had no effects on gene expression of TJ-related proteins, except for a slight, but significant, reduction of claudin-3. Similarly, in animals with colitis, IMQ (single or 3-day repeated treatment) did not affect gene expression.

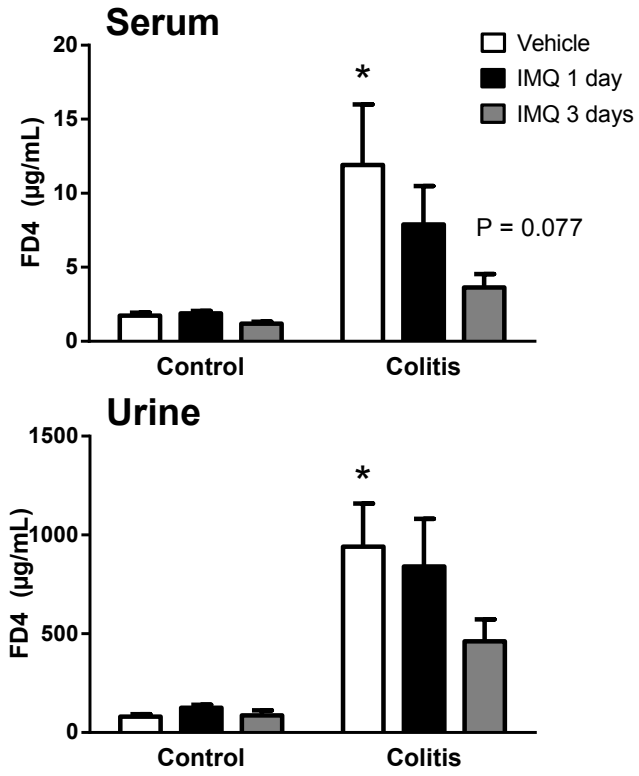

Figure 4. Effects of colitis and imiquimod on colonic permeability to macromolecules in *in vivo* conditions. Data show passage of intracolonic FD4 to serum and urine in healthy animals (control) and during DSS-induced colitis (colitis) and the effects of a single or a 3-day repeated treatment with imiquimod. Data are mean ± SEM, n = 7-10 animals per group. *: $p<0.05$ vs. control-vehicle; P = 0.077 vs. Colitis-Vehicle.

As it relates to the barrier modulating factors MLCK and proglucagon (precursor of GLP-2), both were significantly down-regulated during colitis (50-60 % down-regulation, both P<0.05 vs. expression in healthy tissues). Treatment with IMQ, either single or repeated, was without effect (Fig. 6).

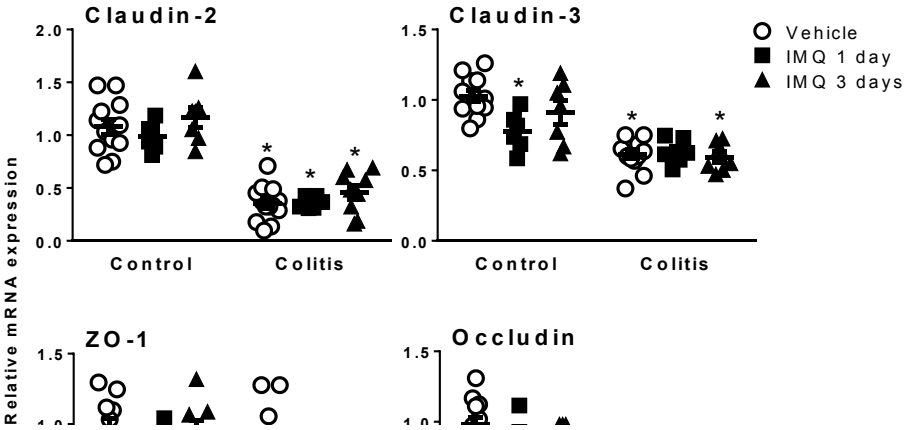

Figure 5. Effects of colitis and imiquimod on gene expression of tight junction proteins (claudin-2, claudin-3, ZO-1 and occludin). Each symbol represents an individual animal, the lines with errors correspond to the mean ± SEM. *: p<0.05 vs. respective control-vehicle group.

Figure 6. Effects of colitis and imiquimod on gene expression of the barrier modulators MLCK and proglucagon. Each symbol represents an individual animal, the lines with errors correspond to the mean ± SEM. *: $p<0.05$ vs. respective control-vehicle group.

Discussion

In this study we show that the local, colonic, selective stimulation of TLR7 with imiquimod improves colonic EBF during colitis in mice. Pro-barrier effects of TLR7 were observed both *in vitro* (modulation of epithelial electrical parameters and permeability to macromolecules) and *in vivo* (modulation of permeability to macromolecules). The mechanisms mediating these effects remain elusive; and seem to be independent of the modulation of the main tight junction proteins or the barrier modulators MLCK and GLP-2 (proglucagon).

Defects in epithelial barrier function are common in several gastrointestinal disorders. In particular, barrier alterations leading to a state of "leaky gut" are considered key components in the pathophysiology of inflammatory and functional gastrointestinal disorders, such as IBD and IBS.[2,3] Nevertheless, the causal relationship remains uncertain, and it is not clear if the barrier alterations should be

regarded as cause or as consequence of these pathologies.[3] In any case, a sustained enhancement of epithelial paracellular permeability could facilitate the exposure to luminal antigens, triggering innate mucosal immune system responses and the development of inflammation and subsequent secretomotor and sensory alterations.[19] Although the pathophysiological mechanisms are not completely understood, several studies suggest that gut commensal microbiota and host-bacterial interactions might be important in this process.[19-21] For instance, we have shown than in states of dysbiosis, TLR-mediated host-bacterial interactions are altered, leading to local neuro-immune changes.[14,23–26] Similarly, several evidences indicate that TLRs are involved in the control of barrier function.[27]

Although assessing the role of TLR7 on inflammation was not the primary objective of the present work, results obtained suggest that the over-stimulation of TLR7 does not modify colitis development in the DSS model, at least as it relates to clinical signs and colonic macroscopical and histopathological alterations. Interestingly, over-stimulation of TLR7 was able to attenuate the changes in IL-12p40, indicating some TRL7-dependent immunomodulatory activity; in agreement with previous observations from our group in rats.[25,26,28] These observations are rather limited (only expression of IL-12p40 was determined) but warrant further studies assessing in a more systematic manner a potential role for TLR7 modulating local immune responses during intestinal inflammation. In the context of the present study, the potential modulation of IL-12p40 by TLR7 is of particular interest because IL-12 has been shown to disrupt TJs leading to a deterioration of epithelial barrier function.[29] Therefore, a

down-regulation of IL-12 might represent also a potential mechanism promoting a pro-barrier effect.

Colonic epithelial barrier function was altered during DSS-induced colitis, as shown *in vitro* and *in vivo*. Indeed, both electrical parameters, related to hydro-electrolytic transport, and macromolecular fluxes were altered in colonic epithelial sheets, as assessed *in vitro*, in a manner suggesting an increase of epithelial permeability. Similarly, in *in vivo* conditions, the passage of intracolonic macromolecules (FD4) to the internal milieu (blood and urine) was also increased. All together these observations clearly show a situation reminiscent of the "leaky gut" state that characterize inflammatory and functional bowel disorders[3,30,31] and similar to that described in other models of intestinal inflammation.[32,33]

Over-stimulation of TLR7 with the selective agonist IMQ partially restored epithelial barrier function during colitis. In *in vitro* conditions, direct addition of IMQ (300 μg) to the reservoir of the Ussing chamber improved epithelial electrical properties (increased potential difference and reduced conductance) and reduced permeability to macromolecules, thus suggesting an enhancement of the barrier. Interestingly, macromolecular permeability was particularly affected after the apical addition of IMQ, while basolateral addition showed only residual effects. This might suggest a predominant modulatory role for TLR7 located in epithelial cells.[34,35] Therefore, it is possible to speculate that, in physiological conditions, TLR7 receptors might be able to respond rapidly to luminal stimuli (such as dysbiosis with production of TLR7 ligands). Within the epithelium, this will initiate responses directed towards an enhancement of the barrier, to prevent the excessive passage of antigens and bacterial products, likely produced in abnormal proportions during a

dysbiotic state. This is also consistent with the effects observed *in vivo*. In *in vivo* conditions, over-stimulation of TLR7 leads to dose-related (a single treatment vs. a 3-day repeated treatment) attenuation of inflammation-related barrier alterations, reducing the passage of macromolecules to blood and also their accumulation in urine and the colonic wall (data not shown).

Other TLRs, in particular TLR2, TLR4, and TLR9 have been implicated in the regulation of EBF. Modulatory effects of TLRs on barrier function seem to be receptor subtype-specific since evidences indicate that TLR4 mediates barrier damaging responses,[36,37] while TLR2, TLR9 and, according to the present observations, also TLR7 mediate barrier-protective actions.[10,12,38,39]. Overall, these observations indicate that TLR-dependent modulation of EBF is likely to depend upon de balance of multiple modulatory effects, detrimental and beneficial, mediated by the simultaneous stimulation of different TLRs within the gut.

Passage of macromolecules across the intestinal epithelium takes place, in most cases, through the paracellular pathway. Numerous evidences demonstrate that paracellular permeability is largely dependent upon the integrity of tight junctions.[4,40] Moreover, changes in the expression and distribution of the TJ proteins are present in the intestinal epithelium of IBD patients presenting a state of “leaky gut”.[41,42] Therefore, and taking into consideration the consistent improvement observed in the permeability to macromolecules upon TLR7 stimulation, we assessed potential effects of TLR7 over-stimulation on the expression of tight junction-related proteins. We assessed the expression of the main sealing (claudin-3, ZO-1 and occluding) and pore-forming proteins (claudin-2) of the tight junction. During colitis, gene expression of

claudin-3, ZO-1 and occludin was down-regulated, in agreement with previous results in other models of intestinal inflammation[32] and consistent with the increased epithelial permeability observed in animals the same animals. Interestingly, we observed a down-regulation of the pore-forming protein claudin-2, which contrast with findings in other models of intestinal inflammation or with patients with IBD, where this protein presents a higher expression and has been related with epithelial barrier dysfunction.[32,43] In our conditions, down-regulation of claudin-2 could represent a possible compensatory mechanism (against the existing barrier dysfunction) within the complex net of factors modulating TJs. Over-stimulation of TLR7 failed to affect gene expression of TJ-related proteins, thus suggesting that the pro-barrier effects observed are independent of the modulation of TJs, at least as it relates to claudin-2, claudin-3, ZO-1 and occludin. These observations contrast with the pro-barrier effects of TLR2, that acts through ZO-1-dependent mechanisms[39] and suggest that TLRs might act on a receptor-specific manner to modulate EBF.

It is necessary to mention some limitations of the present study. First, we only studied the expression of limited number of TJ-related proteins, although representing the basis of the structure and function of TJ.[44] Secondly, we assessed changes in gene expression, but not in protein distribution within the epithelial cells. Therefore, a cellular redistribution, without changes in gene expression, determining the functional activity of TJs; as described for TLR2-mediated pro-barrier actions.[39] cannot be discarded. Nevertheless, we previously described that over-stimulation of colonic TLR7 in rats was not associated to changes in the epithelial distribution of TJ-related proteins.[13]

Finally, we also assessed the expression of two well-known barrier-modulating factors, namely MLCK and proglucagon (as precursor of the bioactive molecule GLP-2).[45–47] Consistent with previous reports, and with the functional data shown here, gene expression of these factors was down-regulated during colitis.[32] IMQ was without effects, thus indicating that the pro-barrier effects associated to the over-stimulation of TLR7 are independent of these barrier-modulating factors. It is worthy to mention that in the case of MLCK, not only the presence of the protein is important but also its activation state.[48] However, in trials performed by us in which MLCK was inhibited by ML-7, we obtained no differences (data not shown). Therefore, future studies should address not only potential changes in the levels of MLCK, but also the level of activation of the enzyme. As mentioned above, IL-12 can be regarded also as a barrier modulating factor, since it has been shown to disrupt TJs leading to a deterioration of epithelial barrier function.[29] In this sense, the IMQ-mediated down-regulation of IL-12 might represent a potential mechanism promoting a pro-barrier effect.

In summary, the present study shows a potential role for TLR7 modulating EBF in states of intestinal inflammation. Selective stimulation of TLR7 resulted in an improvement in epithelial electrical parameters associated to the hydroelectrolitic transport as well as in the permability to macromolecules. The mechanisms underlying these pro-barrier effects remain elusive but seem to be indpendent of the modulation of the main TJ-related proteins (claudin-2, claudin-3, occludin and ZO-1) or the barrier enhancing factors MLCK and GLP-2 (progucagon). TLR7-mediated immunomodulatory mechanisms should be further characterized as potential pathways modulating EBF. Overall, these observations suggest

that stimulation of TLR7 might represent a protective mechanism in states of dysbiosis. TLR7-mediated enhancement of barrier function might limit the entrance of luminal antigens and bacterial products and the consequent inmmune activation, preventing the development of abmornal innmune responses and the generation of overt inflammatory states.

Acknowledgement

We thank A. Acosta and E. Martínez for their technical assistance. This work was supported by grants 2014SGR789 from the Generalitat de Catalunya and BFU2009-08229 from Ministerio de Ciencia e Innovación (Spain). JE personal support from the Ministerio de Ciencia e Innovación (FPU program) (Spain).

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Chapter 3

Local over-stimulation of TLR5 aggravates epithelial barrier dysfunction during colitis in mice

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Abstract

Background/Aims: Dysbiosis, altered epithelial barrier function (EBF), and deregulated immune responses are common findings in intestinal inflammatory disorders. Toll-Like receptors- (TLR) mediated host-bacterial interactions modulate immune responses and EBF within the gut. We assessed the implication of TLR5 in the modulation of EBF during colitis in mice.

Methods: Colitis was induced in mice by administration of dextran sodium sulfate (DSS). Potential modulatory role of colonic TLR5 on EBF was determined assessing epithelial electrical parameters and permeability to macromolecules *in vitro* (Ussing chambers) and colonic permeability *in vivo*, following receptor over-stimulation with the natural ligand, flagellin. Changes in gene expression of tight junction-related proteins (occludin, claudin-2, claudin-3, zonula occludens-1), barrier-modulating factors (proglucagon and myosin light-chain kinase) and inflammatory markers (IL-6, IL-12p40, IL-1β and IFNγ) were assessed by RT-qPCR.

Results: In DSS-treated animals epithelial permeability to macromolecules was increased. In *in vivo* conditions, flagellin further enhanced colitis-altered epithelial permeability, thus indicating a deterioration of EBF. No effects were observed after the *in vitro* stimulation of TLR5. In DSS-treated animals, expression of tight junction-related proteins and barrier related factors was down-regulated. Local over-stimulation of TLR5 did not alter these effects. However, flagellin tended to enhance the expression of inflammatory markers. In healthy animals, stimulation of TLR5 with flagellin did not affect EBF, either *in vivo* or *in vitro*.

Conclusions: Over-stimulation of TLR5 leads to a deterioration in EBF only in states of colitis-induced barrier dysfunction. This suggests that dysbiosis-associated enhanced TLR5-mediated signalling might act as an aggravating factor during intestinal inflammation, contributing to the perpetuation of inflammation and abnormal immune responses that characterize gut inflammatory disorders.

Keywords: Colitis; Epithelial Barrier Function; Flagellin; Intestinal permeability; Tight junctions; TLR5.

Introduction

Within the gut, the intestinal epithelium constitutes a physical and functional barrier preventing the passage of luminal factors and facilitating mechanisms of tolerance. Alterations of this barrier promote excessive antigen exposure and the development of abnormal immune responses that might lead to the development of states of persistent inflammation. For instance, intestinal barrier dysfunction with enhanced antigen exposure and altered immune responses are a common feature in functional and inflammatory gastrointestinal disorders, such are Inflammatory Bowel Disease (IBD) and Irritable Bowel Syndrome (IBS)[1]. Moreover, when initiated, factors altering barrier function act in a self-maintaining cycle leading to a chronic state of immune activation and/or overt inflammation.[2,3]

Among the components of the epithelial barrier, tight junctions (TJs) among epithelial cells are considered a key component, determining epithelial polarity and paracellular permeability, preventing the uncontrolled passage of luminal antigens to the internal milieu.[2,4] TJs are complex protein structures including both sealing proteins and pore-forming proteins whose interactions reduce or enhance epithelial permeability, respectively. Main sealing proteins include claudin-3, zonula occudens 1 (ZO-1) and occludin; on the other hand, claudin-2 constitutes the main pore-forming protein. TJs are structurally and functionally regulated by several endogenous factors, such are cytokines and barrier modulating factors (mainly myosin light chain kinase, MLCK, and glucagon-like peptide 2, GLP-2), as well as exogenous factor, represented mainly by the gut commensal microbiota (GCM).[4–7]

During the last years, GCM has emerged as an important factor modulating gastrointestinal homeostasis. Host-bacterial interactions influence physiological and pathophysiological responses within the gut affecting immune mechanisms, secretomotor and sensory functions.[8–10] Host-bacterial interactions are mediated by multiple bacterial recognition systems. Among these, Toll-like receptors (TLRs) are a key component mediating host-bacterial interactions within the gut. Stimulation of intestinal TLRs mediates a series of neuro-immune responses within the gut that modulate motor, sensory and secretory functions, including epithelial barrier function (EBF). In this sense, several TLRs, mainly TLR2, TLR3, TLR4, TLR7 and TLR9 have been related to EBF, showing receptor-specific effects that can be either protective or detrimental.[11–16] For instance TLR2 and TLR3 enhance barrier functionality,[11,12] while TLR7, and probably also TLR5, leads to dual protective or detrimental responses depending upon de conditions assessed.[14,17,18]

TLR5 is highly expressed in intestinal epithelial cells and is selectively activated by flagellin,[19,20] a bacterial protein expressed by flagellated bacteria. Interestingly, flagellin-expressing bacteria include a significant proportion of human pathogenic bacteria,[21,22] suggesting that activation of TLR5 largely mediates the pathophysiology of intestinal infections and/or dysbiotic states.[17,23] Supporting this view, dysbiosis and increased levels of anti-flagellin antibodies are common findings in IBD and IBS patients.[24–29] However, observations in different animal models, including TLR5 knock-out mice, also suggest that TLR5 might exert protective actions within the gut.[18,30,31]

Based on this background, in the present work we assessed the potential role of TLR5 modulating colonic EBF in healthy conditions and during a state of colitis. To further understand the mechanisms involved in the potential barrier activity of TLR5 we also assessed the implication of TJs and barrier-modulating factors (MLCK and GLP-2). Furthermore, a potential immune modulatory role of TLR5 during inflammation was also assessed.

Materials and Methods

Animals

Adult male CD1 Swiss mice (6 weeks/25 g at arrival; Charles-River Laboratories, Lyon, France) were used. On arrival, animals were housed in standard plastic cages under conventional controlled environmental conditions (20–22ºC, 40–70% humidity and 12 h light/dark cycle) for 1 week before any treatment. Mice were fed with a standard pellet diet (Panlab SL, Barcelona, Spain) and tap water *ad libitum* during the acclimatization period. Animals were 7-8 week old at the time of the experiments. All experimental procedures were approved by the Ethical Committee of the Universitat Autònoma de Barcelona and the Generalitat de Catalunya (protocols 3039 and 8823, respectively).

Induction of colitis

Colitis was induced in mice with dextran sodium sulfate (DSS; 5 %; (35-55 kDa; TDB Consultancy, Uppsala, Sweden) in water *ad libitum* over a 5-day period followed by 2 days with normal drinking water. DSS solutions were prepared daily for the inflamed group whereas the control group of mice received regular tap water. Mice were supervised

daily for their clinical symptoms monitoring for rectal bleeding, diarrhea, and general signs of morbidity.

Experimental protocol and samples collection

In vitro epithelial barrier function - Ussing chambers

At day 7 from having started the treatment with DSS, mice were euthanized by cervical dislocation and colonic sheets were collected to be mounted in the Ussing chamber to assess the epithelial barrier function *in vitro.* Four colonic sheets were obtained from each animal and were randomly distributed in four experimental groups according to treatments: Flagellin (5 µL, 1 µg/µL) or vehicle (sterile distillate water; 5 µL) added directly to the apical or the basolateral side. Colonic sheets were mounted in the Ussing Chamber, after a 20 min of stabilization, treatments were applied and electrical parameters were assessed for a 30 min period. Thereafter, electrical parameters were recorded for an additional 60 min period.

In vivo permeability studies

The effects of the TLR5 stimulation on colonic epithelial permeability to macromolecules (4 kDa fluorescein isothiocyanate–dextran, FD4) *in vivo* were assessed with multiple administration (dairy dose of 3 µg every 24 h for 3 days) of flagellin, administering the last dose 90 min before the euthanasia and the collection of samples. For this, animals were deeply anesthetized with isoflurane (Isoflo®, Esteve Veterinaria, Barcelona, Spain) and treatments were applied intracolonically with a plastic cannula (4 cm from the anus, corresponding to the mid-distal colon).

Mice were randomly distributed in the experimental groups, according to treatments: Control-Vehicle [normal tap water and sterile distillate water (50 µL, intracolonic)], Control-Flagellin [Normal tap water and flagellin (3 µg/mouse; 50 µL, intracolonic)], Colitis-vehicle (DSS 5% in tap water and sterile distillate water) and Colitis-Flagellin (DSS 5% in tap water and flagellin). 30 min after the intracolonic administration of FD4 animals were deeply anesthetized with isoflurane. Blood (cardiac puncture), urine tissue samples were collected to asses FD4 passage (see below for detailed description on the procedures followed). At the same time, colonic inflammation was assessed and tissue samples collected for gene expression (frozen in liquid nitrogen and maintained at -80ºC until analysis).

Measurement of electrophysiological parameters

After collecting the colonic samples, these were immediately flushed with cold Krebs buffer and placed in ice cold oxygenated (95% O_2; 5% CO_2) Krebs buffer containing glucose. Once mounted in the Ussing chambers, colonic epithelial electrical parameters (short circuit current, potential difference and conductance) were assessed *in vitro* in an Ussing chamber system as a measure of EBF. Short-circuit current (I_{sc}), defined as the charge flow per time when the tissue is short-circuited or clamped to 0 mV, represents a summation of all ionic currents across the epithelium. Potential difference (PD) is defined as the transepithelial voltage generated by the epithelial electrogenic active ionic transport. Conductance (G) is a useful measure of the integrity of the intestinal tissue, and it is regarded as an indicative of the state of the paracellular pathway across the intestinal epithelium.[32,33]

For electrophysiological measurements, colonic segments were stripped of the outer muscle layers and myenteric plexus, opened along the mesenteric border and divided into 0.5 cm^2 flat segments, excluding colonic patches. Epithelial sheets were mounted in Ussing chambers (World Precision Instruments, Aston, UK) with an exposed window surface area of 0.28 cm^2. Tissues were bathed bilaterally with 5 mL of oxygenated and warmed (37ºC) Krebs buffer (in mmol/L: 115.48 NaCl, 21.90 $NaHCO_3$, 4.61 KCl, 1.14 NaH_2PO_4, 2.50 $CaCl_2$ and 1.16 $MgSO_4$; pH: 7.3–7.4). The buffer also contained 10 mmol/L glucose as energy source. At the end of the experiments, a single concentration of CCh (100 μM) was added to the basolateral side of the chamber to assess the viability of the tissues. The electric equipment of the Ussing chambers consists of two voltage-sensitive electrodes (EKV; World Precision Instruments) to monitor the PD across the tissue and two Ag-AgCl current passing electrodes (EKC; World Precision Instruments) to inject the required I_{sc} to maintain a zero potential difference, as registered via an automated voltage/current clamp (DVC-1000; World Precision Instruments). Ohm's law was used to calculate tissue G, using the change in I_{sc} when a voltage step of 1 mV was applied every 5 min. Tissues were allowed to stabilize for 15–25 min before baseline values for PD, I_{sc} and G were recorded. Data were digitized with an analog-to-digital converter (MP150; Biopac Systems, Goleta, USA) and measurements were recorded and analyzed with Acqknowledge computer software (version 3.8.1; Biopac Systems). I_{sc} and G were normalized for the mucosal surface area.

Epithelial permeability to macromolecules in vitro

Permeability to macromolecules was evaluated measuring mucosal to basolateral fluxes of fluorescein isothiocyanate (FITC)-labeled dextran

(FD) with a mean molecular weight of 4 kDa (FD4; TdB Consultancy AB, Uppsala, Sweden) in colonic epithelial sheets from mice.

After stabilization (20 min), baseline electrophysiological parameters were assessed and 30 min later FD4 were added to the mucosal reservoir to a final concentration of 2.5 x 10^{-4} M. Basolateral samples (250 µL, replaced by 250 µL of buffer solution with glucose) were taken at 15-min intervals during the following 60 min for measurement of FD4. Concentration of fluorescein in the samples was determined by fluorometry (Infinite F200; Tecan, Crailsheim, Germany) with an excitation wavelength of 485 nm (20 nm band width) and an emission wavelength of 535 nm (25 nm band width), against a standard curve. Readings are expressed as a percentage (%) of the total amount of FD4 added to the mucosal reservoir.

In vivo colonic permeability to macromolecules

Blood, urine and colon samples were collected 30 minutes after the intracolonic administration of fluorescein isothiocyanate (FITC)-labeled dextran (FD) with a mean molecular weight of 4 kDa (FD4, 50 mg/mL, 50 µl; TdB Consultancy AB, Uppsala, Sweden). Blood was centrifuged (10000 G, 10 min, 4 ºC) and serum was collected. Urine was obtained intravesically and diluted (1:50). The colonic samples were flushed with saline (0.1 mL/mg) and stored for fluorescence determinations. The colonic samples were sonicated for 5 min in saline (10 µL/µg) and this liquid was stored for determining its fluorescence. The colon was homogenized with GentleMacs™ Dissociator (Miltenyi Biotec, Madrid, Spain) and centrifuged (3000 G, 10 min, 4 ºC). The supernatant was recovered and stored in ice in dark conditions, as the other samples.

Concentration of fluorescein in the samples was determined by fluorometry (Infinite F200; Tecan, Crailsheim, Germany) with an excitation wavelength of 485 nm (20 nm band width) and an emission wavelength of 535 nm (25 nm band width), against a standard curve. The measures of serum and urine are expressed in concentration of FD4 (µg/mL).

Quantitative Real Time reverse transcription polymerase chain reaction (RT-qPCR)

Extractions of RNA were performed from colonic samples using Ribopure RNA Isolation Kit (Applied Biosystems, CA, USA) and quantified by Nanodrop (Nanodrop Technologies, Rockland, DE, USA). For cDNA synthesis, 1 µg of RNA was reverse-transcribed in a 20 µl reaction volume using a high capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA). Expression of tight-junction related proteins (occludin, claudin-2, claudin-3, ZO-1), barrier markers (MLCK and proglucagon), and inflammatory markers (IL-12p40, IL-6, IFNγ and IL1β) was determined by quantitative real-time PCR performed with specific Taqman probes (Applied Biosystems; occludin: Mm00500912_m1, claudin-2: Mm00516703_s1, claudin-3: Mm00515499_s1, ZO-1: Mm00493699_m1, MLCK: Mm00653039_m1, proglucagon: Mm01269055_m1, IL-12p40: Mm00434174_m1, IL-6: Mm00446190_m1, IFNγ: Mm01168134_m1, and IL1β: Mm00434228_m1.) mixed with Taqman Universal Master Mix II for 40 cycles (95ºC for 15 s, 60ºC for 1 min) on a 7500 real-time PCR system (Applied Biosystems). β2-microglobulin expression (Mm00437762_m1) was used as an endogenous control for normalizing the mRNA levels of the target gens. Expression levels were analyzed by the $2^{-\Delta\Delta CT}$ method.[34]

Chemicals

Flagellin was acquired from Adipogen AG (Liestal, Switzerland). Flagellin was dissolved in sterile distillate water at 1 µg/µl, diluted if necessary, and stored at -30ºC. Dextran Sulphate Sodium (DSS; 35-55 kDa) was purchased from TdB (Consultancy, Uppsala, Sweden) and diluted in water (5% w/v) every day of treatment. CCh was purchased from Sigma-Aldrich and stock solutions of CCh (10^{-1} M) in distilled water were stored at -30ºC. The fluorescein isothiocyanate (FITC)-labeled dextran (FD) with mean molecular weight of 4 kDa (FD4; TdB Consultancy AB. Uppsala, Sweden) was dissolved with Krebs solution (20 mg/mL) or saline (50 mg/mL) at the time of its use.

Statistical analysis

All data are expressed as mean ± SEM and analyzed by a two-way ANOVA. In all cases, results were considered statistically significant when $P<0.05$. GraphPad Prism 6 (GraphPad Software, La Jolla, California, USA) was used to perform all statistical analysis.

Results

DSS induced colitis in mice and effects of TLR5 stimulation on inflammation

Mice treated with DSS (5 %) during 5 days developed an acute colitis showing clinical signs and loss of body weight, consistent with that previously described (Fig. 1).[35] Moreover, at necropsy, DSS-treated animals showed a shortening of the colon and an increase in colonic relative weight, consistent with a state of colitis (Fig. 1).[35]

Figure 1. DSS-induced colitis and effects of flagellin (3-day repeated treatment) on the body weight change at day 7, length of the colon, and the colonic relative weight. Data are mean ± SEM, n=3-7. *: P<0.05 vs. Control-Vehicle.

The pro-inflammatory cytokines assessed (IL12p40, IL-6, IFNγ and IL-1β) were detected in all samples analyzed, although a relatively high inter-animal variability was observed in some cases (Fig. 2). Basal expression levels were relatively low and stable in healthy animals; with minor effects of flagellin. Colitis tended to increase cytokines expression, and treatment with flagellin showed a trend to further enhance expression levels. However as mentioned, due to variability in the data (and the limited number of samples in some cases) statistical significance was not achieved (Fig. 2).

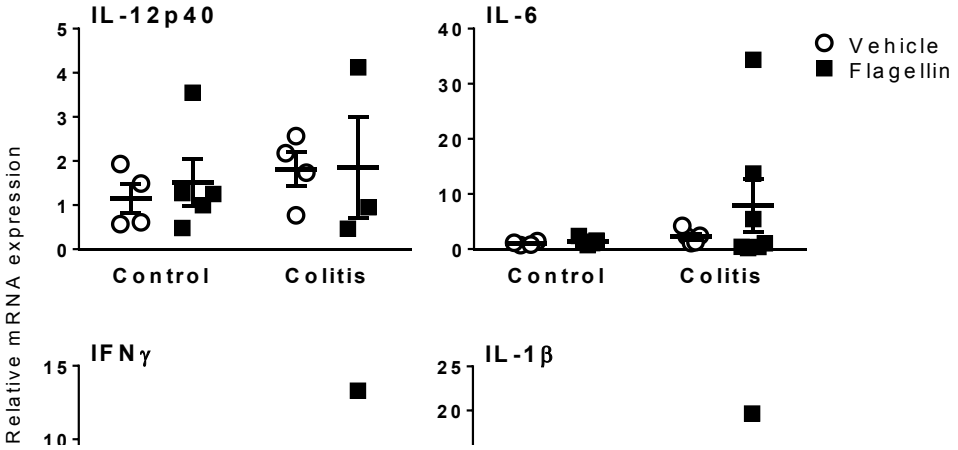

Figure 2. Effects of colitis and flagellin on colonic gene expression of inflammatory markers (IL-12p40, IL-6, IFNγ, and IL-1β). Data are mean ± SEM, n=3-7.

Effects of the direct stimulation of TLR5 with flagellin on epithelial electrical parameters and permeability to macromolecules in vitro (Ussing chambers)

In epithelial sheets from animals with DSS-induced colitis there were minor changes in basal epithelial electrical parameters, affecting mainly I_{sc}, which was slightly reduced (Fig. 3). Neither the apical nor the basolateral addition of flagellin induced consistent changes in electrophysiological parameters, with time-related changes similar to those observed in vehicle-treated tissues (Fig. 3).

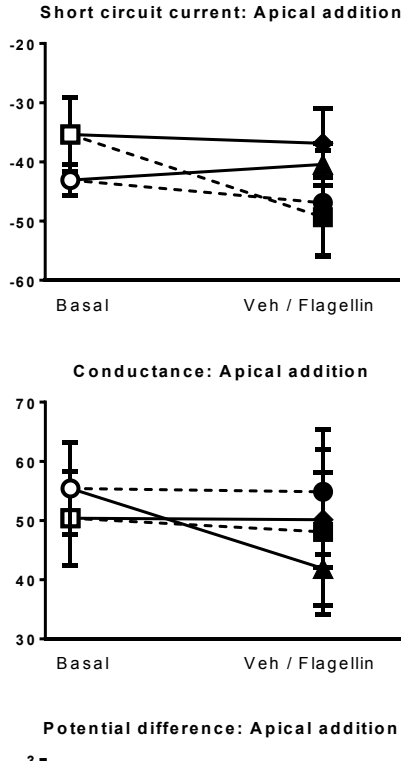

Figure 3. Effects of colitis and flagellin on basal colonic epithelial electrical parameters. Data show basal values of short circuit current (I_{sc}), conductance (G) and potential difference (PD), and changes 30 min after the addition, to the apical or the basolateral side, of vehicle or flagellin. Data are mean ± SEM of 4-6 epithelial sheets obtained from 4-6 animals. In each group (control or colitis), after obtaining a basal measurement sheets were treated with either vehicle or flagellin (n=4-6 for each treatment and condition). Veh: Vehicle.

Permeability to macromolecules was increased in inflamed tissues; passage of FD4 was numerically increased between 65% and a 73% at min 60 vs. non inflamed tissues (Fig. 4). Addition of flagellin, either to the apical or the basolateral side, was without effects on the fluxes of FD4.

In general, at the end of the experiments, addition of CCh elicited a clear change in I_{sc}, lack of responses was indicative of tissue damage. Less than 10% of the tissues assessed were discarded because of lack of responses to CCh.

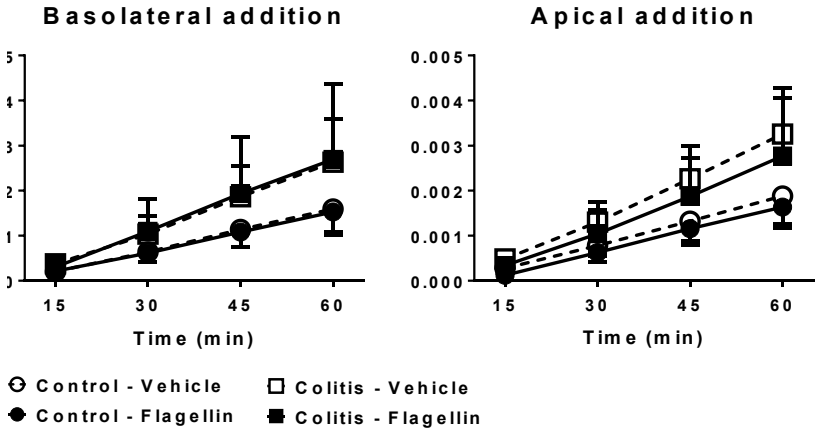

Figure 4. Effects of colitis and flagellin on colonic permeability to macromolecules in *in vitro* conditions (Ussing chamber). Data show flux of FD4 (% from total amount added to the mucosal side) during a 60 min period, and the effects of flagellin added to the apical or the basolateral side. Data are mean ± SEM, n = 4-5 colonic sheets, from 4-6 animals, per group.

Effects of stimulation of TLR5 with flagellin on colonic permeability to macromolecules in vivo

In healthy animals, the passage of intracolonic FD4 to blood and urine was low, with small inter-animal variability. In animals with colitis, FD4 passage to blood and urine was increased by 2.7-folds ($P<0.05$ vs. control conditions; Fig. 5) and 5.3-folds ($P<0.05$ vs. control conditions; Fig. 5), respectively.

In animals with colitis, flagellin increased the passage of FD4 to blood ($P<0.05$ vs. colitis-vehicle group; Fig. 5). FD4 concentrations in urine were also augmented but, probably due to variability in the data, statistical significance was not reached.

Figure 5. Effects of colitis and flagellin on colonic permeability to macromolecules in *in vivo* conditions. Data show passage of intracolonic FD4 to plasma and urine in healthy animals (control) and during DSS-induced colitis (colitis) and the effects of a single or a 3-day repeated treatment with flagellin. Data are mean ± SEM, n=4-7. *: $P<0.05$ vs. Control-Vehicle. #: $P<0.05$ vs. Colitis-Vehicle.

Effects of stimulation of TLR5 with flagellin on the expression of tight junction-related proteins and barrier modulating factors

Expression of TJ-related proteins, namely claudin-2, claudin-3, ZO-1 and occludin, was detected in all colonic samples analyzed. In all cases, gene expression was down-regulated during inflammation (50-80% reduction; $P<0.05$ in all cases; Fig. 6). Addition of flagellin was without effect on protein expression, either in healthy or inflamed tissues (Fig. 6).

Similar pattern was observed for the barrier modulating factors MLCK and proglucagon (GLP-2). Both factors were down-regulated during inflammation with no effects associated to flagellin treatment (Fig. 7).

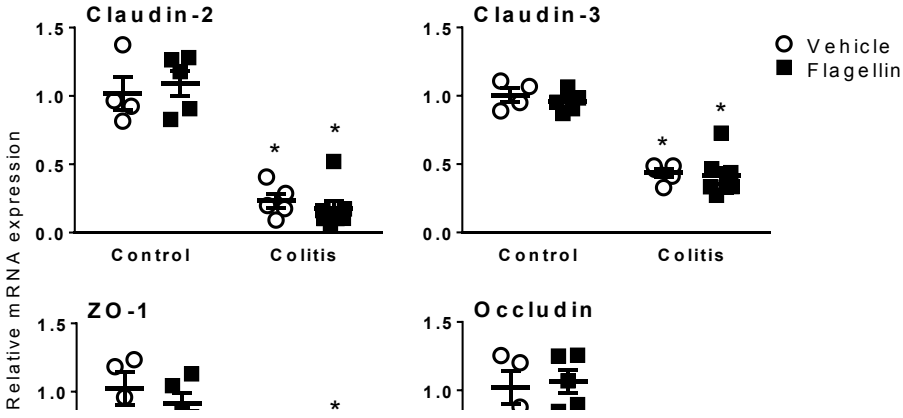

Figure 6. Effects of colitis and flagellin on gene expression of tight junction proteins (claudin-2, claudin-3, ZO-1 and occludin). Data are mean ± SEM, n=4-7. *: $P<0.05$ vs. Control-Vehicle

Figure 7. Effects of colitis and flagellin on the colonic gene expression of barrier-modulating factors MLCK and proglucagon. Data are mean ± SEM, n=4-7. *: P<0.05 vs. Control-vehicle

Discussion

Results obtained show that local over-stimulation of colonic TLR5 with the selective agonist flagellin deteriorates colonic barrier function in states of colitis in mice, causing an increase in colonic permeability to macromolecules in *in vivo* conditions. Interestingly detrimental effects of TLR5 over-stimulation were observed in states of barrier disruption, but not in physiological conditions.

Within the gut, EBF represents a key mechanism regulating the passage of antigenic molecules from the lumen to the internal milieu. Therefore, the presence of barrier alterations is usually associated to an increased passage of luminal factor and, in consequence, an over-stimulation of the local immune system. In some cases, this might lead to an aberrant immune response that perpetuates barrier changes generating an estate known as “leaky gut”. IBD and IBS represent pathophysiological conditions in which barrier alterations with enhanced

penetration of luminal antigens and exacerbated immune responses are present.[36,37] Evidences indicate that dysbiosis of the commensal microbiota, with altered host-bacterial interactions, are a contributing factor to these alterations. In this sense, TLR-dependent signalling has been directly implicated in host-bacterial interactions affecting gut homeostasis, including EBF.[26,29,38,39]

TLR-dependent effects on EBF seem to be receptor subtype- specific. As it relates to TLR5, there are contradictory studies showing both beneficial and detrimental effects. Our observations in *in vivo* conditions show that over-stimulation of colonic TLR5 is associated to an aggravation of colitis-associated barrier dysfunction in mice, as indicated by the increased passage of macromolecules to blood and urine. However, flagellin had no effects on epithelial electrical parameters assessed *in vitro*, neither in healthy nor in inflamed tissues. Observations in *in vivo* conditions are in agreement with previous studies showing that flagellin aggravated colitis in mice but had no effect in animals without inflammation.[17,40] Rhee *et al.* (2005) showed also that flagellin had no effect on transepithelial resistance of naïve human colonic tissue in *in vitro* conditions (Ussing chamber)[18]. Again, this observation is also consistent with our findings in tissues from healthy animals, showing no effects of flagellin, either *in vivo* or *in vitro*. All together, these observations suggest that TLR5-mediated effects can only be elicited when the epithelial lining is somehow altered and that bacterial flagellin, *per se*, is not able to alter EBF. This might be related to the predominant basolateral distribution of TLR5 in the colon,[19,41] limiting the access to luminal flagellin in normal conditions.

TLR5-mediated enhanced permeability to macromolecules observed *in vivo* was not reproduced in *in vitro* conditions. This apparent discrepancy might be related to the intrinsic limitation of the *in vitro* procedures, mainly the absence of all extrinsic neuro-humoral mechanisms contributing to EBF and the potential effects of TLR5. The different experimental protocols should also be taken into account. In the case of *in vitro* experiments only acute effects of flagellin (30 min incubation) were tested, while in *in vivo* conditions a 3-day repeated retreatment with flagellin was performed. These differences might indicate that flagellin-mediated effects are slow and involve changes that require a long time and/or the persistent over-stimulation of TLR5. Further studies addressing these differences are necessary to solve the apparent contradictions observed in the effects of flagellin.

Flagellin-TLR5-mediated enhanced colonic permeability seems to be unrelated to changes in the expression of the main TJ-related proteins (claudin-2, claudin-3, ZO-1 and occludin). Inflammation significantly down-regulated the sealing proteins claudin-3, ZO-1 and occludin, potentially explaining the associated increase in epithelial permeability. Interestingly, the pore-forming protein claudin-2 was also reduced during inflammation, suggesting a compensatory mechanism to the down-regulation of sealing proteins, to avoid excessive opening of the epithelium. Taking into account this hypothesis, one can speculate that activation of the flagellin-TLR5 pathway might reverse this effect, rising claudin-2 expression and, therefore, increasing epithelial permeability, as shown by the functional data. However, claudin-2 expression remained down-regulated during TLR5 over-stimulation, thus indicating that

alternative mechanisms, yet to be characterized, should mediate the detrimental effects observed in EBF.

Barrier modulating factors, namely MLCK and proglucagon (GLP-2 precursor), were consistently down-regulated during inflammation, being consistent with previous studies in the same model and in other inflammatory conditions of the gut.[14,42] Flagellin did not affect expression in healthy tissues or the inflammation-induced down-regulation. These observations indicate that these modulatory factors are not implicated in flagellin-TRL5 effects on EBF.

Within the gut, TLRs exhibit immunomodulatory actions.[43] In particular, several reports indicate that TLR5 modulates the expression of inflammatory cytokines and the development of intestinal inflammation; [17,44] although with conflicting results indicating in some cases a protective role for TLR5 and in other a pro-inflammatory action.[18,30,31] In the present work, over-stimulation of TLR5 with flagellin did not modify the course of DSS-induced colitis, according to the clinical and macroscopical signs of inflammation assessed. Although colitis development was not affected, flagellin tended to up-regulate pro-inflammatory cytokines during colitis, with high variability, thus suggesting a potential immunomodulatory activity. These results are difficult to discuss given the discrepancies found in the literature. Overall, these differences might suggest that additional experimental factors might be important determining the effects of TLR5. Among these we propose that the microbiota composition and the activity of other TLRs (and in general other host-bacterial interaction systems) should be taken into consideration. Final effects on gut homeostasis, facilitating or interfering with inflammation

are likely to depend upon a balance between all signalling mechanisms activated by a particular microbiota.

Pro-inflammatory cytokines, such as IL-12 or IFNγ, have been shown to act as barrier modulating factors contributing to the disruption of the epithelial barrier and thus increasing permeability.[45] In this work, and as mentioned above, proinflammatory cytokines were moderately up-regulated in animals with colitis treated with flagellin. Although the data presented is very limited, it is tenting to speculate that these changes in cytokines expression might be a factor contributing to the enhanced epithelial permeability to macromolecules observed in the same animals. These preliminary data warrant further studies assessing in deep the flagellin-TLR5-pro-inflammatory cytokines as a potential mechanism explaining the modulatory role of TLR5 on EBF in certain experimental conditions.

In summary, data presented here show that over-stimulation of TLR5 with its natural ligand, flagellin, deteriorates EBF in states of colitis, without having any modulatory effects on healthy conditions. The underlying mechanisms are yet to be elucidated. However, changes observed seem to be independent of the modulation of the main TJ-related proteins (claudin-2, claudin-3, ZO-1 and occludin) or the barrier-modulating factors MLCK and GLP-2 (proglucagon). Preliminary observation warrants further studies assessing the importance of the pathway flagellin-TLR5-pro-inflammatory cytokines. These studies illustrate the importance of TLR-mediated host-bacterial interactions in the maintenance of EBF and the role that microbial dysbiosis might have in the development of barrier-related intestinal pathologies, such as IBS or IBD.

Acknowledgement

We thank A. Acosta and E. Martínez for their technical assistance. This work was supported by grants 2014SGR789 from the Generalitat de Catalunya and BFU2009-08229 from Ministerio de Ciencia e Innovación (Spain). JE personal support from the Ministerio de Ciencia e Innovación (FPU program) (Spain).

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General Discussion

Intestinal epithelial barrier dysfunctions are common pathophysiological findings in functional and inflammatory gastrointestinal disorders, like IBD and IBS.[32,33,48,78,84] However, the cause-effect relationships are not clear and barrier dysfunctions can be regarded as either consequence or cause of these alterations.

Currently, IBD and IBS are regarded as the result of the interaction between an altered gut commensal microbiota (dysbiosis) and a genetic predisposition to manifest altered immune responses against luminal antigens.[42,147] An altered control in the passage of antigens through the epithelium, due to an epithelial barrier dysfunction, will induce an over-activation of the immune system,[42] promoting inflammatory responses, which will cause the characteristic symptoms of IBD and IBS. Accordingly, host-bacterial interactions might represent an important mechanism regulating epithelial functions, including permeability to luminal antigens, and the development of local immune responses and inflammation.

Host-bacterial interactions depend upon multiple mechanisms, including different PRRs such are TLRs. In this work, to gain insight in the implication of host-bacterial interactions in the control of epithelial barrier function, we assessed the role of TLR5 and TLR7 as potential modulators of colonic epithelial barrier function.

TLR7 improves colonic epithelial barrier function in rodents

Results obtained in this work indicate that the activation of TLR7, particularly in *in vivo* conditions, triggers responses related with the improvement of the epithelial barrier function both in rats and mice.

Nevertheless, the responses observed were different in both species. In rats, the acute over-stimulation of TLR7 *in vivo* resulted in an improvement of EBF in normal conditions. However, in mice, no effects on normal barrier function were observed when TLR7 was over-stimulated in basal conditions. This supports the existence of specie-related differences in the involvement of TLR7 in the control of EBF in normal, physiological, conditions.

Moreover, the fact that effects of TLR7 in rats were only observed when the receptor was over-stimulated in *in vivo* conditions, but not during the acute stimulation *in vitro* (Ussing chamber), suggest that pro-barrier effects of TLR7 require mechanisms not present in the *in vitro* set up[87]. This suggests that neuro-immuno-endocrine mechanisms involving extra-intestinal components might be required for TLR7 to elicit its protective effects on barrier function. Further systematic studies addressing this possibility are required to fully understand the pro-barrier effects of TLR7.

The protective role of TLR7 on colonic EBF was also evidenced in states of colitis in mice. In colonic sheets obtained from animals with DSS-induced colitis the over-stimulation of TLR7, by the apical addition of imiquimod into the Ussing chamber, normalized the passage of macromolecules, thus indicating a restoration of EBF. This, compared with the lack of effects on EBF observed upon direct over-stimulation of TLR7 in normal tissues (as discussed above), suggest that multiple mechanisms mediate the barrier effects of TLR7, with differences between normal and inflamed tissues. The cellular location of TLR7 may be also of importance mediating these differences. TLR7 is an intracellular receptor expressed in endosomal vesicles.[198] In this sense,

receptor availability might be increased in states of inflammation, in which IEC are affected and cellular permeability likely increased. Therefore, the manifested pro-barrier effects of TLR7 during DSS-induced colitis in mice might also reflect increased receptor stimulation vs. that achieved in normal tissues. However, this contrasts with the results obtained in rats when the colonic epithelium was challenged with DMSO. In this case, availability of intraepithelial TLR7 is likely to be also increased (as during colitis), but barrier function was deteriorated as a result of this enhanced TLR7 stimulation. These discrepancies between rats and mice might be due to experimental differences (related to the presence or not of active inflammation) but might also reflect specie-related differences in the role of TLR7 modulating barrier function, as discussed above. Moreover, epithelial permeabilization with DMSO might also favor the uptake of luminal factor eliciting barrier changes that compensate the beneficial effects associated to the selective stimulation of TLR7. Further studies should investigate these apparent specie-related differences, particularly as it relates to the presence of inflammation as a factor unrevealing the pro-barrier actions of TLR7.

TLR5 aggravates the colonic epithelial barrier dysfunction induced by colitis in mice

Repeated intracolonic over-stimulation of TLR5 with its natural ligand, flagellin, deteriorates colonic EBF in mice with DSS-induced colitis. However, TLR5 over-stimulation in non-inflamed tissues was without effects on colonic EBF.

Lack of effects of TLR5 in normal, non-inflamed murine tissues, agrees with previous data showing that the intact intestinal mucosa does

not respond to flagellin.[195] Moreover, this agrees with the absence of modulatory effects observed during the over-stimulation of TLR7, thus suggesting that these receptors might play a minor role regulating barrier function in normal, physiological, conditions in mice.

As previously shown, EBF was altered during DSS-induced colitis.[199,200] In this conditions, over-stimulation of TLR5 leads to a further deterioration of the epithelial barrier, manifested as an increase in the accumulation of macromolecules in colonic tissues as well as to an increased passage to blood and urine. This agrees with previous reports showing a harmful effect of TLR5 on the epithelial barrier when a pre-existing damage was present.[195] Given the cellular location of colonic TLR5, predominantly expressed in the basolateral membrane,[160] a pre-existing damage in the epithelium, such as during colitis, will facilitate the passage of luminal ligands and, therefore, the stimulation of the receptor. To date the only known natural ligand for TLR5 is flagellin, a bacterial protein present on nearly all flagellated bacteria,[201–203] including many important human enteric pathogens (i.e. *Salmonella*, enteropathogenic *Escherichia coli*, *Campylobacter*).[204,205] Moreover, anti-flagellin antibodies are frequently detected in post-infectious IBS patients and Crohn's Disease.[206,207] In summary, during states of dysbiosis with the presence of flagellated bacteria, disruption of the epithelial barrier will facilitate the entrance of flagellin[205], the stimulation of TLR5 and therefore, as observed in the present work, a further damage of the barrier, leading to a vicious-cycle in which immune reactions, barrier alterations and, probably, dysbiosis are auto-perpetuated.

TLR5- and TLR7-mediated modulation of barrier function is independent of changes in tight-junction-related proteins

In an effort to characterize the mechanisms underlying the barrier effects associated to the over-stimulation of TLR5 and TLR7, and given the importance of TJ in epithelial paracellular permeability, we assessed changes in gene expression and cellular distribution of the main TJ-related proteins. Overall, neither the over-stimulation of TLR5 nor TLR7 leads to variations in protein expression consistent with the functional changes observed in barrier function. This does not seem to reflect a methodological problem since in the colon of animals with DSS-induced colitis a general down-regulation was observed for the main TJ-related proteins, a change consistent with the increase in epithelial permeability observed in the same animals. Similarly, no consistent treatment-related changes were observed when assessing the cellular distribution of the main TJ-related proteins. Nevertheless, these negative results do not exclude TLR5/7-mediated effects on TJs as underlying mechanism for the changes observed. First, we only assessed changes in the main TJ-related proteins but given the complexity of the TJ (with over 50 proteins involved[45]), effects in other components cannot be discarded. Second, we did not assess the phosphorylation state of some components of the TJ and, while expression can be unchanged, the rate of phosphorylation of some proteins can induce structural alterations leading to functional changes.[14,52,69,208–211] This can be of particular importance taking into account that the signaling cascades of TLRs implicate modulation of different kinases.[179,183,186,195,212] Therefore, future studies at a molecular level should be performed to gain insight in these aspects.

Effects of TLR5 and TLR7 on colonic epithelial barrier function are independent of MLCK and GLP-2

MLCK and GLP-2 are considered as key barrier-modulating factors, affecting TJ dynamics (phosphorylation processes and protein assembling and protein expression, respectively).[34,71–73] Therefore, alterations of barrier function have been associated to expression changes of these molecules.[74,82,213,214] In our conditions, neither TLR5 nor TLR7 over-stimulation was associated to changes in the expression of MLCK or proglucagon, the precursor of GLP-2. This is consistent with the lack of changes observed in TJs (gene expression and protein distribution), as discussed above. Interestingly, both factors were down-regulated in colonic samples from mice with colitis. This is consistent with the increased colonic permeability detected in these animals and agrees with previous observations in other models in which altered intestinal permeability was associated to a down-regulation of both MLCK and proglucagon expression.[48] MLCK-mediated modulation of TJs not only depends upon its gene expression, but also upon its enzymatic activity,[213,215,216] therefore both expression and activity levels should be considered to completely discard the implication of MLCK in the responses observed.

Over-stimulation of colonic TLR5 and TLR7 leads to a local immunomodulatory response

Activation of both TLR5 and TLR7 has been associated to local immune responses, with an up-regulation of proinflammatory mediators. Here, we assessed the local (colonic) expression of different cytokines to assess the possibility of a TLR-mediated immune response. In physiological conditions neither the over-stimulation of TLR5 nor that of

TLR7 was associated to changes in cytokines expression, in agreement with previous reports.[188,189,195] On the other hand, under states of inflammation (DSS-induced colitis) divergent immunomodulatory effects were observed during the over-stimulation of TLR5 or TLR7.

In mice with DSS-induced colitis, over-stimulation of TLR7 leads to a normalization in the expression of the pro-inflammatory cytokine IL12-p40. However, during over-stimulation of TLR5 pro-inflammatory cytokines (IL12-p40, IL-1β, IL-6 and IFNγ) showed a clear trend to be up-regulated. Overall, these results are in line with the barrier effects observed under over-stimulation of TLR7 and TLR5, exhibiting, pro-barrier and barrier-damaging effects, respectively. Although the number of immune mediators assessed in our studies is limited the results obtained clearly suggest a local TLR-mediated immune modulation, in line with the changes in barrier functionality. These observations further support an involvement of TLR-dependent mechanisms in local immune responses and the development of intestinal inflammation.

In summary, the results obtained in this work provide evidence for a modulatory role of TLR5 and TLR7 in the control of colonic epithelial barrier function. All together, results obtained indicate that while TLR5 should be regarded as a barrier-damaging factor, TLR7 should be considered a barrier-protective factor. Overall, barrier changes will depend upon the balance between the action of barrier-protective and barrier-damaging mechanisms (including TLR5, TLR7, other TLRs and non-TLR-mediated effects). In normal conditions, a predominance of barrier-protective actions is likely to be expected and this will be associated to the maintenance of a correct function or to a restoration of the normal barrier functionally (in states of dysfunction) and the normalization on

the local immune responses (and likely a restoration of a state of eubiosis). However, as discussed along this work in several points, if barrier-damaging mechanisms predominate, for instance during a state of persistent dysbiosis with over-stimulation of TLR5, a self-perpetuation of the alterations can occur, contributing to the development of pathologies such are IBD or IBD. The present observations further support the importance of host-bacterial interactions in the homeostasis of the EBF and the development of pathophysiological alterations. Furthermore, these results suggest that TLR5 and TLR7 should be regarded as potential therapeutic targets in the control of EBF and local immune responses in functional and inflammatory gastrointestinal disorders, such as IBS and IBS.

Conclusions

1. In rats, *in vivo* over-stimulation of colonic TLR7 with the selective agonist imiquimod improves colonic epithelial barrier function in normal tissues, while causing barrier dysfunction, leading to an increase in permeability, under states of epithelial disruption.

2. In rats, the underlying mechanisms mediating the effects of TLR7 on epithelial barrier function are independent of the regulation of the expression and epithelial distribution of tight junction related proteins, at least as it relates to zonula occludens 1, occludin, claudin-2, claudin-3, junctional adhesion molecule 1, and tricellulin. Likewise, TLR7 actions are independent of expression changes of the barrier-enhancer factor glucagon-like peptide 2.

3. In mice, over-stimulation of colonic TLR7 with the selective agonist imiquimod improves epithelial barrier function in a model of DDS-induced colitis, while having no effects in normal conditions.

4. In mice, TLR7-mediated effects in epithelial barrier function are independent of the regulation of the expression of tight junction related proteins, at least as it relates to zonula occludens 1, occludin, claudin-2, and claudin-3. Likewise, TLR7 actions are independent of expression changes of barrier-modulating factors, namely myosin light chain kinase and glucagon-like peptide 2.

5. In mice, over-stimulation of colonic TLR5 with its natural ligand, flagellin, does not affect epithelial barrier function in normal conditions. On the other hand, TLR5 over-stimulation during DSS-

induced colitis aggravates inflammation-induced barrier dysfunction, leading to an increase in epithelial permeability.

6. In mice, TLR5-mediated effects on epithelial barrier function are independent of the regulation of the expression of the tight junction related proteins zonula occludens 1, occludin, claudin-2, and claudin-3. Similarly, modulatory effects of TLR5 are independent of changes in the expression of barrier-modulating factors, namely myosin light chain kinase and glucagon-like peptide 2.

Conclusiones

1. En la rata, la sobre-estimulación del receptor TLR7 del colon con el agonista selectivo imiquimod en condiciones *in vivo* mejora la función barrera epitelial en tejidos normales, pero altera su actividad, aumentando la permeabilidad del epitelio, en estados de disrupción epitelial.

2. En la rata, los mecanismos que median los efectos del receptor TLR7 sobre la función barrera epitelial no dependen ni de la regulación de la expresión génica ni de la distribución epitelial de las proteínas relacionadas con las uniones estrechas *zonula occludens*-1, ocludina, claudina-2, claudina-3, molécula de adhesión de la unión de tipo 1 o tricelulina. Igualmente, los efectos del receptor TLR7 son independientes de cambios en la expresión génica del factor promotor de la barrera péptido similar al glucagón tipo 2.

3. En el ratón, la sobre-estimulación del receptor TLR7 del colon con el agonista selectivo imiquimod mejora la función barrera epitelial en un modelo de colitis inducida por sodio sulfato de dextrano, mientras que, en condiciones normales, no modifica la actividad de la misma.

4. En el ratón, los efectos mediados por el receptor TLR7 sobre la función barrera epitelial no dependen de la regulación de la expresión génica de las proteínas de uniones estrechas *zonula occludens*-1, ocludina, claudina-2 o claudina-3. Del mismo modo, las acciones del receptor TLR7 son independientes de cambios en la expresión génica de los factores moduladores de la función barrera epitelial quinasa de la cadena ligera de la miosina y péptido similar al glucagón tipo 2.

5. En el ratón, la sobre-estimulación del receptor TLR5 del colon con flagelina, su ligando natural, no altera la función barrera epitelial en condiciones normales. Sin embargo, en un estado de colitis inducida por sodio sulfato de dextrano, la sobre-estimulación del receptor TLR5 agrava la disfunción de la barrera inducida por la inflamación, aumentando la permeabilidad epitelial.

6. En ratones, los efectos inducidos por la sobre-estimulación del receptor TLR5 del colon no dependen de la regulación de la expresión génica de las proteínas relacionadas con las uniones estrechas *zonula occludens*-1, ocludina, claudina-2 o claudina-3. Asimismo, los efectos del receptor TLR5 sobre la barrera epitelial no dependen de cambios en la expresión génica de los factores moduladores de la función barrera epitelial quinasa de la cadena ligera de la miosina y péptido similar al glucagón tipo 2.

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Appendices

Publications derived from this Thesis

Scientific papers

Estévez J, Martinez V. *Local activation of Toll-like Receptor 7 (TLR7) modulates colonic epithelial barrier function in rats.* (*Scandinavian Journal of Gastroenterology*, submitted).

Estévez J, Martínez V. *Local over-stimulation of Toll-Like Receptor 7 (TLR7) attenuates epithelial barrier dysfunction during colitis in mice.* (manuscript).

Estévez J, Martinez V. *Local over-stimulation of Toll-Like Receptor (TLR5) aggravates epithelial barrier dysfunction during colitis in mice.* (manuscript).

Abstracts

Estévez J., Martínez V. Stimulation of TLR5 aggravates epithelial barrier dysfunction during colitis in mice. Gastroenterology 150 (Sppl 1): S-594. 117th Annual Meeting of the American Gastroenterological Association – Digestive Disease Week. San Diego, California USA. May 21st-24th, 2016.

Estévez J., Martínez V. Stimulation of Toll-like receptor 7 attenuates epithelial barrier dysfunction during colitis in mice. United European Gastroenterology Journal 3 (Sppl 1): A443.
United European Gastroenterology Week 2015. Barcelona, October 24-28, 2015. Selected abstract for travel grant.

Estévez J., Martínez V. Stimulation of Toll-Like Receptor 7 normalizes colonic epithelial barrier function during colitis in mice. Journal of Crohn's and Colitis (Vol. 9, Issue suppl 1): S81.

10th Congress of ECCO – Inflammatory Bowel Diseases. European Crohn's and Colitis Organisation. February 18th-21st, 2015.

Estévez J., Martínez V. Toll-Like Receptor 7- dependent modulation of colonic permeability depends upon the integrity of the epithelial barrier. United European Gastroenterology Journal 2: A408.
United European Gastroenterology Week. Viena. October 18th-22nd, 2014.

Estévez J., Martínez V. Toll-Like Receptor 7 modulates colonic barrier function without changes in the gene expression of tight junction-related proteins. Acta Physiologica 212 (Sppl. 698), p. 45.
XXXVII *Congreso de la Sociedad Española de Ciencias Fisiológicas (SECF)*. Oral communication. Granada, September 24th-26th, 2014.

Estévez J., Martínez V. Toll-Like Receptor 7 increases colonic epithelial permeability in states of epithelial disruption in rats. Acta Physiologica 212 (Sppl. 698), p. 45.
XXXVII Congreso de la Sociedad Española de Ciencias Fisiológicas (SECF). Comunicación Oral. Granada, September 24th-26th, 2014.

Estévez J., Aguilera M., Paricio R., Martínez M. Activation of Colonic Toll-Like Receptor 7 (TLR7) Enhances Epithelial Barrier Function in Rats. Gastroenterology 144 (Sppl 1): S-933.
114th Annual Meeting of the American Gastroenterological Association – Digestive Disease Week. Orlando, Florida, USA. May 18th-21st, 2013.

Estevez J., Aguilera M., Paricio R., Martinez V. Toll-Like Receptor 7 modulates colonic epitelial barrier function in rats. Gut 61(S3): A273.
United European Gastroenterology Week. Amsterdam. October, 2012.

Estévez J., Aguilera M., Paricio R., Fernández-Blanco J.A., Vergara P., Martinez V. Local activation of Toll-like receptor 7 enhances colonic epithelial barrier function in rats. Neurogastroenterol Motil. 2012, 24 (Sppl s2): 77. International Symposium on Neurogastroenterology and Motility. Bolonia. September 7th-9th, 2012.

Estévez J., Aguilera M., Fernández-Blanco J.A., Vergara P., Martinez V. Local Activation of Toll-like Receptor 7 Alters Colonic Epithelial Barrier Function in Rats. Gastroenterology 142 (Sppl 1): S-67.
113th Annual Meeting of the American Gastroenterological Association – Digestive Disease Week. San Diego, California USA. May 19th-22nd, 2012. Oral communication.

Curriculum vitae

Javier Estévez Medina

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Education

- Master in Neurosciences. Universitat Autònoma de Barcelona. September, 2012.
- Doctor in Veterinary Medicine (DVM). Universitat Autònoma de Barcelona. September, 2010.
- Other courses:
    - Advanced Certificate of English (C1 level of the Council of Europe). Official Language School (Barcelona). June, 2014.
    - Training course for the use of laboratory animals (FELASA). Level C – Scientist responsible for directing animal experiments. Universitat Autònoma de Barcelona. September, 2011.

Grants and awards

- PhD grant: FPU from the Spanish Government ("Ministerio de Educación, Cultura y Deporte"; reference: FPU12/00620). January, 2012.
- PhD grant; Generalitat de Catalunya. Declined acceptance. 2012.
- Travel award for young scientists. 24th United European Gastroenterology Week. October, 2015.
- Research support grant from the Department of Cell Biology, Physiology and Immunology (Universitat Autònoma de Barcelona). February, 2011.
- Best student award, promotion 2005-2010. Universitat Autònoma de Barcelona.

Teaching experience

- Training course for the use of laboratory animals. Universitat Autònoma de Barcelona (UAB). 2012-2015.
- Co-tutor in *Argó* program (approaching science to high school students). 2012-2015
- Master of Science and Technology of the Laboratory Animal. Practical classes. UAB. 2014.
- Co-tutor in the practical internship of the Biomedical Sciences Degree (UAB).
- Laboratory tutor. System's Physiology. Biomedical Sciences Degree (UAB).
- Laboratory tutor. Animal physiology. Degree in Genetics (UAB).

Present position

- Technical Support and R&D&i management at Industrial Técnica Pecuaria, S.A. (ITPSA). Since November, 2015.

Publications

- **Estévez J**, Martinez V. Local activation of Toll-like Receptor 7 (TLR7) modulates colonic epithelial barrier function in rats. Scand J Gastroenterol. (Submitted).
- **Estévez J**, Martinez V. Local over-stimulation of Toll-Like Receptor 7 (TLR7) attenuates epithelial barrier dysfunction during colitis in mice. (Manuscript in preparation).
- **Estévez J**, Martinez V. Local over-stimulation of Toll-Like Receptor (TLR5) aggravates epithelial barrier dysfunction during colitis in mice. (Manuscript in preparation).
- Fernández-Blanco JA, **Estévez J**, Shea-Donohue T, Martinez V, Vergara P. Changes in epithelial barrier function in response to parasitic infection: implications for IBD pathogenesis. J Crohns Colitis. 2015.

Participations in congresses and meetings

16 abstracts since 2010

2 national meeting
11 international meetings