Universitat Autònoma de Barcelona

Understanding the role of Toll-like receptors in the lower gastrointestinal tract

By

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A dissertation submitted in partial fulfilment of the requirements

for the degree of Doctor of Philosophy

Immunology Doctoral Programme

Department of Cell Biology, Physiology and Immunology

Universitat Autònoma de Barcelona

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2014

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I certify that the dissertation entitled: “Understanding the role of Toll-like receptors in the lower gastrointestinal tract” submitted by JUAN FRANCISCO BURGUEÑO BANÚS in partial fulfilment of the requirements for the degree of Doctor of Philosophy was carried out under my supervision and I authorise the submission to undertake its oral defence.

In witness whereof, I hereby sign this document,

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This work was funded by the Ministerio de Salud and the Ministerio de Educación y Ciencia of the Spanish Government (Grant support: PS09/01127 and BFU2006-15063-C03-01/BFI).

Juan Francisco Burgueño was supported by the Ministerio de Educación y Ciencia of the Spanish Government (Grant support: AP2005-1160).

The author would like to acknowledge such financial support.

Acknowledgements

Parece que no, pero todo llega, y después de tantos años pensando en por qué y a quiénes debería incluir en este capítulo, se me hace un tanto frustrante el darme cuenta de que mi corta memoria no abarca a recordar tantas experiencias vividas durante este inusualmente largo camino. Mirando hacia atrás, uno se da cuenta de que este recorrido carecería de valor si no fuera por todas las personas que me han ayudado a completarlo, y por eso quería dedicaros unas líneas a aquellos que habéis hecho de esta tesis un cúmulo de recuerdos que, llegados a este punto, sólo pueden arrancarme sonrisas. Pese a que he intentado recordar a todas estas personas, es más que probable que haya olvidado mencionar a alguien, por lo que espero que pueda disculparme.

En primer lloc voldria agrair a la meva directora, l'Ester, tantes coses que costa saber per on començar. Gràcies per donar-me l'oportunitat de descobrir la ciència, per creure en mi des del començament i no haver perdut aquesta fe a mida que avançaven els anys. Gràcies per comptar sempre amb mi, per haver compartit les meves alegries i haver-te bolcat en els mals moments que he passat. Gràcies per tot el recolzament que m'has donat, per tenir la porta del despatx sempre oberta i haver suportat el meu assetjament quan els resultats han estat bons. Gràcies pel "si sabéssim el que els experiments han de donar, no els faríem", i el "l'estadística ha de donar respostes a les teves preguntes concretes". Gràcies per haver-me ajudat a créixer com a investigador, i en definitiva, gràcies per com t'has portat amb mi tot aquest temps. Gràcies Ester.

Voldria agrair també al Dr. Miquel Àngel Gassull el fet d'haver-me acollit en el seu grup de recerca i haver-me facilitat l'accés al fantàstic centre de recerca que ha aconseguit muntar amb el seu esforç. Així mateix, vull donar les gràcies a tots els investigadors sènior que han invertit part del seu temps en el meu creixement personal i a potenciar les meves motivacions científiques. Gràcies a l'Adolfo per aportar-me el seu perfeccionisme i meticulositat en la feina, així com per tot el temps que va passar amb mi buscant un TLR2 que hom es va arribar a plantejar que no existís. Gràcies al Josep per compartir amb mi la seva passió per la ciència en forma de petites tertúlies, així com per posar sempre al meu abast tots els recursos materials i humans que he necessitat. Gràcies a la Sílvia pels seus consells i el seu enfoc experimental pràctic que he intentat fer meu. Gràcies al Miquel i al José Carlos per demostrar-me que es pot ser un gran investigador sense renunciar a l'humor i la qualitat humana. I'd also like to thank Dr. Michel Neunlist for accepting me in his research group, providing me of all resources to develop my experiments and teaching me fundamental lessons concerning questions and focusing affairs. Thanks Dr. Bernard Lardeux for sharing with me his unconditional love for science, while always keeping a smile on his face. Gràcies al Marcel per haver posat al meu abast de forma desinteressada qualsevol

fàrmac que he necessitat, així com per haver-me aconsellat amb diversos plantejaments experimentals. Gracias a Patri por haberme tenido siempre en buena consideración y porque me consta que siempre has intentando allanar mi camino cuando he necesitado cualquier cosa. Gràcies a la Maite pel teu humor satíric i per haver-te mogut per mi quan m'ha fet servei. A l'Eva, per facilitar-me l'accés a la PCR i aconsellar-me en temes d'amplificació de DNA genòmic. Gràcies també a la Dra. Anna Bassols per la seva disposició a facilitar el meu treball i per l'interès que hi ha mostrat. Gràcies a tots / Gracias a todos / Thank you all.

Gracias a todos los doctorandos y residentes que habéis compartido las alegrías y miserias de este tan mal recompensado trabajo conmigo. Gracias a mi amiga Cri por tantos momentos buenos que hemos pasado: de congreso, ligando en alemán, en las cabinas de flujo laminar, midiendo colons, mangando trabajos ajenos, hablando de aviones de guerra y de tus inmunos quemadas… Gracias por tu ayuda y por haber ilustrado este trabajo con tus maravillosas figuras. Espero que la vida te sonría como mereces. Gràcies al meu estimat Ferran per haver estat sempre tan efusiu i friendly amb mi, per les innumerables vegades que hem rigut junts, per haver estat un dels meus pilars de recolzament en els pitjors moments, per escoltar-me i ensenyar-me lliçons de la vida i per motivar el meu coneixement de l'estadística. Seguirem ballant la Kylie allà on anem. A la Fani, per la seva simpatia... i aquí m'hauria de quedar. Gràcies per haver-me escollit com a "home dels encàrrecs", per les hores que hem passat xerrant i rient mentre tornàvem a casa junts de mil llocs i per haver suportat amb gran esportivitat el meu cinisme. Si ens tornem a trobar a una cantonada, suposo que ens saludarem. A la Helens, gràcies per haver estat sempre al peu del canó per ajudar-me, i perdona perquè he trigat massa temps a adonar-me que vals molt més del que inicialment pensava. Ànims amb aquesta tesi que ja veuràs com la traurem! A l'Esther, per haver-me aportat punts de vista de les coses que altrament no hauria estat capaç d'apreciar, i per ensenyar-me que es pot avançar contracorrent. Segur que poques coses se't resisteixen. A Diana, por destilar bondad y hacer el ambiente más agradable y acogedor allá donde va. Al JAF, per les grans lliçons que m'has ensenyat: que les coses és millor prendre-les amb humor (evocant el teu "alegria a la feina") i que la clau de la investigació és... la que tots sabem. Al Víctor, per la seva professionalitat i solidaritat extrema amb els companys que l'hem envoltat, així com pel detall que et vas marcar amb mi als agraïments de la teva tesi. A la Míriam, per haver suportat les meves bromes sobre el valencià i per mostrar-me que lluitant tot és possible. Passaràs corrent per sobre del que et proposis com ho vas fer per davant del Cédric i meu. Al Chechi, por su glamour, por haber aprobado puntualmente mi vestimenta y por hacerme creer que he llegado a dominar alguna técnica laboratorial. Me gustaría dejar aquí constancia de tu triple chasquido de dedos, pero... A la Mònix, pel seu assessorament científico-tècnic il·limitat, el seu punt de vista pragmàtic, per intentar contenir els meus brots d'histèria post-anàlisi dels resultats i per descobrir-me que els bons investigadors poden sortir fins i tot de la Catalunya més

profunda. És cert, això dels macròfags tampoc era tan greu... A na the Marin Frost, pel seu somriure etern, rain or shine, sheep or ship. Continua contagiant la teva alegria, perquè a més d'un li fa veritablement falta. Al Javier, A.K.A. "sabotage boy", pel seu suport i la infundada bona imatge que té de mi, així com per ensenyar-me que es pot ser intel·ligent i espavilat, tot en un. M'hauries de fer unes transfusions de picardia. A la Sepi, por tu paciencia con Ferrán y conmigo, y por poner al mal tiempo buena cara, y al soso Facebook, tus mejores fotos. ¡Hazle caso a Asun y no vayas a la tuya, que te veo! A la Noe, pel teu caràcter tranquil sempre acompanyat d'un somriure. Thanks Jakub for discussing my ideas during our train trips, tying to offer me new approaches to puzzling results. A l'Anna, per tenir sempre una estona per escoltar-me i estar sempre disposada a ajudar-me amb els westerns. Tot i això, espero que aquesta samarreta d'aquest equip de futbol teu se t'embruti de manera irreversible. A l'Albert, per tota l'ajuda i confiança que em vas donar. Espero que passessis tan bona estada com jo mentre hi vas ser. A Lina, por su amabilidad conmigo y por enseñarme que se puede conseguir lo que sea que uno se propone. No limits, Lina. A la Yoli, per tractar-me sempre amb un somriure i haver-te molestat en fer una addenda per agrair-me la meva nul·la col·laboració a la teva tesi. A Bego, por ser siempre tan dulce, por transmitirme todo tu ánimo y fuerza en este final de tesis, y por haber tenido bonitos detalles conmigo. A la Mariona, per la teva ajuda i consells durant el temps que vam passar junts al laboratori. Al Ricard, pel tracte exquisit que em vas oferir a Leuven. A Álvaro, por la tranquilidad que me transmitiste en los pocos momentos que hemos coincidido. A Hernán, por compartir tus gustos musicales conmigo y ofrecerme un remanso conversacional en un departamento dominado por el sexo femenino. A la Mònica Porras, per suportar la meva xerrameca en determinats moments. A l'Anna Domènech, per ser sempre tan dolça i agradable amb mi, i pel temps que vas dedicar a ensenyar-me a dissecar i preparar whole mounts. A Javier Benito, por ser un superclase, por tu humor, tu carácter integrador, por pasar tus horas de la madrugada preparando presentaciones para que pudiéramos mejorar la toma experimental de muestras, y por tu compañía transatlántica cuando estuve de stage. A Alicia, gracias por tu entusiasmo, tu calidez, tu sonrisa eterna y tu vitalidad. Seguro que allá donde vayas, triunfarás. A Paco, por ofrecerme siempre una visión en clave de humor de las miserias a las que todos nos hemos ido viendo sometidos durante todos estos años. A Elena, por molestarse en que yo no quedara como un dejado en alguno de mis cumpleaños. A Gloria, por el marujeo constante y reinante cada vez que nos reunimos. A Sandra, porque nunca me perdió nada… o al menos nada que haya echado en falta. A l'Asun, per fer-me sentir a la meva salsa quan organitzem lab meetings improvisats al despatx. A la Mònica Comalada, per ser tan diligent i resolutiva. Hauria d'haver après moltes més coses de tu. A l'Ester Rodríguez y a Sandra Ocampo por vuestra agradable compañía bajo tierra en la época en que todo eran virus o siRNAs. A Roma i a l'Àngels,

por vuestro apoyo y el ánimo que me habéis transmitido tanto cuando estabais por la facultad como cuando no. Muchas gracias, os deseo lo mejor a todos.

Muchas gracias a todas aquellas personas repartidas por la UAB que han contribuido al desarrollo de mi trabajo desde el punto de vista técnico y que me han enseñado a llevar a cabo tareas diversas. Gracias a mi queridísima Emma Martins Peters por todo el cariño que me ha brindado, por haber aguantado más de una impertinencia mía, por venir a la cabina a preguntarme qué hacía y marcharse sigilosamente mientras yo quedaba explicándole al incubador el experimento en ciernes, y por tantas veces que me ha hecho reír con sus comentarios y preguntas embarazosas. Si no existieras, habría que inventarte, Peters. Gracias a Antonio por su impagable labor en secretaría, por su predisposición habitual a resolver mis problemas y por no enfadarse al recibir constantemente mails corrigiendo los mandados 5 minutos antes. A Pepe, por haberte mostrado siempre con buena disposición para echarme una mano con la burocracia. Qué peligro tienes con el long… A David, por tu disponibilidad constante para que los pedidos hechos a última hora llegaran lo antes posible. A la Clàudia, per haver-me facilitat dades i protocols quan te'ls he demanat. A la Paqui del Servei de Cultius, per haver-te mostrat sempre favorable a proporcionar-nos tota mena de material quan veníem d'urgències. A mi amigo Fran, por todo el tiempo que pasaste entrenándome en el trabajo con cultivos celulares, por todas las líneas con las que hemos traficado y por estar siempre dispuesto a facilitarme cualquier tipo de material o ayuda personal. A la Núria, per totes les hores que hem passat davant el confocal intentant que les fluorescències sortissin el millor possible, i per abordar els meus problemes tècnics sempre des d'un punt de vista positiu, constructiu i optimista. A Blanca y Aída, por trabajar a destajo para que yo pudiera tener mis preparaciones un día antes de la fecha habitual, así como por vuestros ánimos y conversación cada vez que he bajado a veros. Gracias.

Igualment vull agrair tot el personal de l'Institut Germans Trias i Pujol que ha contribuït a fer les meves estades en aquest centre més agradables. Gràcies a l'Eli per la teva paciència amb els meus dubtes de PCR, per posar a la meva disposició tot el material i temps que he necessitat, per ensenyar-me a treballar amb la tècnica més exquisida i pulcra, així com per aconsellar-me en molts aspectes. A la Vio, per rebre'm sempre amb bona disposició i imbuir-me del sentiment d'ésser benvingut, així com per mostrar-me també el seu rigor tècnic i facilitar-me la feina. A l'Aida, per tota l'ajuda que m'has donat amb cèl·lules i cultius diversos, així com amb els WB, i per tenir sempre un somriure a la cara. A l'Alba, Anna, Jovita, Laura, Maribel, Sònia, Vanessa, Sara, Marian, Ramon i Gemma per compartir la vostra experiència professional amb mi i per amenitzar els dinars. Gràcies per la vostra aportació.

Je voudrais remercier aussi à tous ces bons collègues que j'ai connu dans mon stage à l'U913 de l'Institut National de la Santé et de la Recherche Médical, à Nantes. À mon presque frère Sébastien, pour toutes ces expériences qu'on a vécu : tes visites dans mon bureau, nôtres carrières dans la campagne, ce voyage en bateau, chaque visite au Leclerc… Pour partager avec moi tes connaissances du WB, pour me faire sentir chez moi partout et pour m'aider dans tous ces affaires que t'as pu. T'es un grand professionnel et une meilleure personne. À Maddalena, pour partager aussi avec Séb et moi ce très bon séjour qu'on a passé ensemble, pour essayer de m'intégrer depuis la première journée, pour m'aider avec tous mes affaires, pour tes visites aussi dans mon bureau (les tiennes étaient pires), nôtre voyage à Begur, et tous ces repas chez toi et chez Séb. J'espère que tu seras heureuse avec Jul, le petit Sohan et ton récent titre de PhD. À Maxime, pour ton dévouement à m'apprendre Français et quelques trucs pour la PCR. T'es un chercheur admirable. À Mandy, pour te rigoler avec mes blagues, et pour toute cette aide à la préparation de la culture primaire. À Julien et Margarida, pour bien m'apprendre aussi à la préparation des cultures primaires. À Cédric, pour ton amitié et toutes ces conversations scientifiques et pas du tout scientifiques qu'on a eu en courant, au sport, au fut et même dans la boîte. Bon courage quand tu rentreras en France. À Mario, parce que t'es tellement incroyable comment fantastique ! A Omar, por ser mi vía de escape hispano-parlante entre tanto francés, y por esa comida mejicana que compartimos. À tous ces autres personnes qui ont fait de mon stage une expérience inoubliable : Tanguy, Charlotte, Rodolphe, Raphael, Thibaud, Hind, Philipe et Pascal. Merci à tous.

Finalmente, quería dar las gracias a mi familia y por su apoyo incondicional durante todos estos años. A Maite, por haber hecho parte de este camino conmigo, siempre sin perder el entusiasmo, el optimismo y la sonrisa; por tu cariño, tu apoyo y todos los detalles que has tenido conmigo a pesar de la distancia. A Kitty y Javi, por tratarme como a un hijo y por tener la fe que mi tesis acabaría al día siguiente de cada vez que hemos estado juntos. Al Padrí, per creure en mi sobre totes les coses, i perquè tenir supporters gairebé radicals com tu pot resultar molt moralitzant en els pitjors moments. A Asun y al Rospo, por toda la fuerza y los ánimos que me han transmitido en los momentos más duros, por escucharme, aconsejarme, aceptar mis errores y compartir mi alegría en los buenos momentos. A la Burguer, per haver estat al meu costat quan realment has vist que ho necessitava. A mi Papi, por tu amor, por haberme dado la mejor educación posible, por enseñarme que todo requiere un esfuerzo y que las personas son lo primero, por recogerme cuando he caído y por haber sido siempre un sólido pilar sobre el que apoyar mi crecimiento personal y profesional. A la Mama, pel teu amor, la teva comprensió, el teu continu recolzament moral i psicològic, per haver-me ensenyat que hom sempre es pot exigir més, per creure en mi i recolzar-me, escoltar-me, aconsellar-me i per haver estat l'altra cama sobre la que alçar la meva esquena. Os quiero / Us estimo.

Para acabar, quiero hacer un agradecimiento especial a Ariadna por haber recorrido conmigo la parte más complicada de esta travesía. Gracias por todas las veces que me has acompañado al trabajo y que has colaborado activa y desinteresadamente en mis experimentos. Gracias por apoyarme pese a todo el sufrimiento que esta tesis te ha causado, y perdona por haberte hecho cargar con un peso que deberíamos haber llevado juntos, y por todo el desgaste que ello te ha provocado. Gracias por haber compartido tu tiempo conmigo y por, pese a las dificultades, querer seguir a mi lado, dándome tu amor. Gracias. Lo siento. Te quiero.

Joan Burgueño Banús, Barcelona, gener 2014

“Science never solves a problem without creating ten more”

George Bernard Shaw

Irish Dramatist

Publications and participation in scientific meetings

Published or submitted articles and book chapters:

- J.F. Burgueño, A. Barba, E. Eyre, C. Romero, M. Neunlist, E. Fernández. Role of macrophages and enteroglia in the enteric nervous system responses to bacterial motifs via Toll-like receptors. Glia (In submission).

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Participation in scientific meetings:

- J.F. Burgueño, A. Río, A. Raventós, C. Romero, E. Fernández. Distribution of TLR receptors in the mouse colon. Effects of intrarectal Zymosan on TLR2 expression. Journal of Crohn's and Colitis Supplements, Volume 2, Issue 1, February 2008, Page 85. 3rd Congress of the European Crohn's and Colitis Organisation (ECCO), 28 February - 01 March, 2008, Lyon, France.

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- J.F. Burgueño, A. Barba, E. Eyre, M. Neunlist, E. Fernández. Activation of Toll-like receptors expressed in the enteric nervous system promotes chemoattraction and induces tolerance through nitric oxide-derived compounds. Gut October 2012, Vol. 61, Supplement III, Page A60-61. 20th United European Gastroenterology Week, October 20-24, 2012, Amsterdam, The Netherlands. Oral communication.

Acronyms

αIL-10	Anti-IL-10 neutralising antibody
ACh	Acetylcholine
Akt	Protein kinase B
AP-1	Activating protein-1
APC	Antigen presenting cell
AU	Arbitrary units
AUC	Area under the curve
CD	Crohn's disease
CDx	Cluster of differentiation (where "x" is a number)
CMC	Carboxymethyl cellulose
CNS	Central nervous system
COX-2	Cyclooxygenase-2
Ct	Cycle threshold
DAI	Disease activity index
DAMP	Damage-associated molecular pattern
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle's medium
DSS	Dextran sulphate sodium
EGC	Enteric glial cell
ELISA	Enzyme-linked immunosorbent assay
ENS	Enteric nervous system
Et	Ethanol

EtOH	Ethanol
FBS	Foetal bovine serum
GFAP	Glial fibrillary acidic protein
GI	Gastrointestinal
GSNO	S-nitrosoglutathione
HRP	Horseradish peroxidase
IBA-1	Ionised calcium binding adapter molecule-1
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IκB	Inhibitor of kappa-light-chain-enhancer of activated B cells
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRF	Interferon regulatory factor
LNNA	Nω-nitro-L-arginine
LPS	Lipopolysaccharide
LRR	Leucin-rich repeat
MAMP	Microbial-associated molecular pattern
MAPK	Mitogen-associated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MHC	Major histocompatibility complex
ML	Muscular layers
MLN	Mesenteric lymph nodes

MPO	Myeloperoxidase
MyD88	Myeloid differentiation primary response protein 88
nAChR	Nicotinic acetylcholine receptor
NAF	Nuclear area factor
NANC	Non-adrenergic, non-cholinergic
NE	Norepinephrine
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Neurokinin
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOD	Nucleotide Oligomerisation Domain receptors
NOS	Nitric oxide synthase
NSE	Neuron specific enolase
ODN	Oligodeoxynucleotide
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PG	Prostaglandin
PI3K	Phosphoinositide 3-kinase
PRR	Pattern recognition receptor
RT-PCR	Reverse-transcriptase polymerase chain reaction
S100β	S100 calcium binding protein β
s3T6	Stimulated 3T6 fibroblast (culture)
SAA	Serum amyloid A
SBL	Mucosal and submucosal layers
SDHA	Succinate dehydrogenase complex subunit A

SEM	Standard error of the mean
sENS	Stimulated enteric nervous system (culture)
sJUG2	Stimulated JUG2 (culture)
SP	Substance P
TBS	Tris buffered saline
TBST	Tris buffered saline + Tween 20
TGF-β	Transforming growth factor-β
Th	T helper lymphocyte
TIR	Toll/IL-1 receptor
TJ	Tight junction
TLR	Toll-like receptor
TNBS	Trinitrobenzenesulfonic acid
TNF	Tumour necrosis factor
Treg	T regulatory lymphocyte
TRIF	TIR-domain-containing adapter-inducing interferon-β
u3T6	Unstimulated 3T6 fibroblast (culture)
UC	Ulcerative colitis
uENS	Unstimulated enteric nervous system (culture)
uJUG2	Unstimulated JUG2 (culture)
VIP	Vasoactive intestinal peptide
WB	Western blot
wENS	Washed enteric nervous system (culture)
Wnt	Wingless-Int
Zym	Zymosan

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Summary

Summary

The mucosa of the gastrointestinal (GI) tract is the widest surface of the organism exposed to the external milieu. The epithelial barrier keeps trillions of microorganisms self contained within the GI lumen and separated from the immune cells. In this system, preservation of tolerance to resident microbiota is essential to maintain homeostasis; indeed, any event causing a dysregulation of these relationships might trigger pro-inflammatory responses such as those observed in inflammatory bowel diseases (IBDs). As the main receptors mediating the interplay between the host and the microbiota, Toll-like receptors (TLR) have been associated with the pathogenesis of IBD. Although initially described in immunocytes, knowledge regarding TLR expression and function has rapidly evolved in recent years, and it is currently accepted that their functions depend thoroughly on the cell type they are expressed in.

The aim of this work was to approach some aspects of the function of TLRs in the GI tract, in an attempt to offer an integrated view on their role in different cell types in particular conditions. Specifically, our work has focused on how stimulation of TLR2/4/9 in different cell types populating the lower GI tract might influence their responses during homeostasis and inflammation. In order to achieve our objectives, we studied TLR expression and distribution in the context of the dextran sulphate sodium (DSS)-induced murine model of colitis, as well as the effects of intracolonic administration of different doses of TLR2/4 ligands. In addition, we assessed the putative roles of TLR2/4/9 in the enteric nervous system (ENS) and enteroglial cell (EGC) cultures in terms of cytokine release, chemoattraction and subsequent priming of TLR-induced cytokine expression in a macrophage-like cell line.

Our results show that TLR2/4 display a wide expression thorough the lower GI tract in physiological conditions, and are up-regulated during inflammation, especially in colonocytes and immunocytes. Intracolonic administration of their ligands in physiological conditions had no apparent effects in the classical parameters used in assessment of colitis severity. Contrastingly, instillation of lipopolysaccharide (LPS) during inflammation in the described specific regime attenuated colitis severity and reduced expression of deregulated TLR2/4. The mechanism driving such effects seems to rely on increased epithelial preservation through induction of a proliferative

response in epithelial cells, since higher epithelial preservation index was associated to increased crypt length and to enhanced expression of proliferation markers in colonocytes of DSS+LPS-treated animals. On the other hand, our findings additionally demonstrate that EGCs express functional TLR4 that activates the NF-κB signalling pathway after LPS challenge, inducing the release of cytokines and chemokines, and increasing chemoattraction of immunocytes. Similar responses were observed in ENS cultures, but the presence of resident macrophages in such cultures makes it difficult to quantify the participation of each cell type. ENS cultures had also functional TLR2/9, but no responses were observed to their ligands unless they were added in combination with LPS. Interestingly, upon TLR4/9 stimulation, synergistic responses were obtained in secretion of soluble molecules that subsequently primed the responses of macrophage-like cells, reducing their production of pro-inflammatory cytokines.

The findings summarised in this manuscript contribute to improve the understanding of the functions that TLRs develop in the lower GI tract during homeostasis and inflammation. Overall, TLR roles may vary depending on the challenged cell type and its environmental situation. Some of the responses driven by TLRs can be used to modulate inflammation, such as those observed in epithelial cells, whereas some others must be avoided to prevent exacerbation of these processes (those in EGCs, for instance). Selectivity is the key, and might be achieved through accurate dosage and precise administration regimes.

Resum

La mucosa del tracte gastrointestinal (TGI) constitueix la superfície de contacte més àmplia de l'organisme amb el medi extern. A la llum del TGI, la barrera epitelial permet la separació entre els bilions de microorganismes residents i els immunòcits. En aquest context, el manteniment de la tolerància a la microbiota resident és essencial per a preservar l'homeòstasi; qualsevol esdeveniment que causi la desregulació d'aquestes interaccions pot desencadenar respostes pro-inflamatòries com les observades a la malaltia inflamatòria intestinal (MII). Els receptors Toll-like (TLR) regulen el diàleg entre l'hoste i la microbiota, i s'han associat a la patogènesi de la MII. Tot i que inicialment foren descrits en immunòcits, la seva caracterització ha evolucionat ràpidament en els últims anys, i actualment s'accepta que els seus rols depenen àmpliament de les cèl·lules que els expressen.

Aquest treball tracta diferents aspectes de la funció dels TLRs en el TGI, oferint una visió integrada del seu paper en diversos tipus cel·lulars i condicions particulars. La nostra investigació s'ha centrat en com l'estimulació dels TLR2/4/9 influencia les respostes de diferents tipus cel·lulars del TGI inferior en condicions fisiològiques i d'inflamació. Amb aquesta finalitat, hem estudiat l'expressió i distribució dels TLRs en un model murí de colitis induïda per dextrasulfonat sòdic (DSS), així com els efectes de l'administració intracolònica de diferents dosis de lligands dels TLR2/4. També hem avaluat el paper potencial dels TLR2/4/9 en cultius de sistema nerviós entèric (SNE) i cèl·lules enteroglials (CEG) en termes de producció de citocines, quimiotaxi i sensibilització de la producció de citocines en macròfags.

Els nostres resultats demostren que els TLR2/4 tenen una àmplia expressió en condicions fisiològiques al TGI, i es troben incrementats durant la inflamació, especialment en colonòcits i immunòcits. L'administració intracolònica dels seus lligands en condicions fisiològiques no alterà els paràmetres clàssicament avaluats per al seguiment de la colitis. Per contra, la instil·lació de lipopolisacàrid (LPS) durant la inflamació amb un protocol específic atenuà els símptomes de colitis i reduí l'expressió dels TLR2/4 desregulats. El mecanisme efector sembla basat en afavorir la preservació epitelial promovent la proliferació de colonòcits. De fet, s'observà un increment de l'índex de preservació epitelial associat a augments en l'alçada de les criptes i en

l'expressió de marcadors de proliferació en colonòcits de ratolins tractats amb DSS+LPS. Per altra banda, els nostres experiments també demostren que les CEGs expressen TLR4 funcional que activa la via de senyalització NF-κB post-estimulació amb LPS, induint l'alliberament de citocines i quimiocines, i incrementant la quimiotaxi d'immunòcits. Als cultius de SNE s'observaren respostes similars, malgrat que la presència de macròfags residents fa difícil quantificar la contribució de cada tipus cel·lular. Aquests cultius també expressen TLR2/9 funcionals, però no s'observaren respostes als seus lligands si no s'afegeixen en combinació amb LPS. De fet, l'estimulació dels TLR4/9 donà lloc a respostes sinèrgiques en la secreció de molècules solubles que després sensibilitzaren les respostes de macròfags, disminuint la seva producció de citocines pro-inflamatòries.

Els resultats presentats en aquesta memòria contribueixen a millorar la comprensió de les funcions dels TLRs en el TGI inferior durant l'homeòstasi i la inflamació. Com a conclusió, el paper dels TLRs varia en funció del tipus cel·lular estimulat i el seu ambient. Algunes de les respostes dirigides pels TLRs, com les observades en cèl·lules epitelials, poden ser utilitzades per a modular la inflamació, però d'altres, com les de les CEGs, han de ser evitades per a prevenir l'exacerbació d'aquests processos. En aquest aspecte, la selectivitat és clau, i podria ser aconseguida a través d'una dosificació i uns protocols d'administració acurats.

Introduction

Inflammatory bowel disease

Toll-like receptors

Enteric nervous system

1) Inflammatory bowel disease

1.1) General aspects

The designation "Inflammatory bowel disease" (IBD) refers to a group of pathologies of unknown etiology that involve inflammation of the gut. The two major types of IBD in the clinical practice are Crohn's disease (CD) and ulcerative colitis (UC). Their study has generated and increasing interest in the last 20 years due to different epidemiologic, medical and economic features that make these diseases the most important chronic pathologies affecting the human gastrointestinal (GI) tract.

Their epidemiological importance lies in the fact that both CD and UC have increasing or sustained incidence rates worldwide as reported by temporal trend analyses [1]. Incidence is higher in westernised nations, where it has stabilised in 3-15 cases/100,000 person-years for CD and 3-15 cases/100,000 person-years for UC, but it is rising in developing countries as they become industrialised [2]. Thus, IBD is emerging as a global disease.

Medically, IBD is challenging because it is a chronic and relapsing pathology, with unknown course, unpredictable presentations, extraintestinal manifestations and severe complications such as colorectal cancer [3]. In addition, available therapeutic options are only palliative, involving prolonged medical and surgical interventions. All these aspects result in an impaired quality of life in patients suffering from this disease. Finally, the economic impact of IBD is derived in part from medical, surgical and hospitalisation costs, and in part from productivity decreases, as the peak of incidence for these diseases is from 20 to 40 years [1]. A systematic literature review study estimated a total economic burden for CD of $ 10.9-15.5 billion in the United States and € 2.1-16.7 billion in Europe [4]. In summary, IBD is a disease with an increasing incidence disease, severe medical symptoms and important long-term costs to patients, health care system and society.

Even though they are included in the same clinical entity and share common signs like diarrhoea and abdominal pain, CD and UC show multiple differences in localisation, symptoms and microscopic features (summarised in Table 1). CD is a relapsing transmural inflammatory disease of the mucosa that can affect the entire GI tract from

the mouth to the anus. Typical presentations include the discontinuous involvement of different portions of the GI tract, and the development of complications like abscesses or fistulas. On the other hand, UC is a relapsing diffuse mucosal inflammation that extends proximally from the rectum to a varying length. Patients usually present bloody diarrhoea with pus, mucus or both. Extraintestinal manifestations are frequent in both diseases, affecting 25% of patients, and neoplastic complications seem to be correlated with duration of colitis [3].

	CD	UC
Localisation		
Segment	Any (mouth to rectum)	Rectum ± colon
Distribution	Focal, discontinuous and asymmetric	Continuous and symmetric
Clinical features		
Haematochezia	Rare	Common
Passage of mucus or pus	Rare	Common
Abdominal mass	Sometimes	Rare
Small-bowel obstruction	Common	Rare
Colonic obstruction	Common	Rare
Fistulas and perianal disease	Common	No
Extraint. manifestations	Common	Common
Pathological features		
Inflammation	Transmural	Mucosal
Ulceration	Deep	Superficial
Distribution	Discontinuous	Continuous
Distorted crypt architecture	Uncommon	Yes
Cryptitis and crypt abscesses	Yes	Yes
Granulomas	Yes	No
Fibrosis	Yes	No

Table 1 - Differential diagnosis of CD and UC. Based on their specific immune response, CD and UC display different phenotypes in terms of localization, symptoms, macroscopic and microscopic lesions.

Adapted from Baumgart, D. C., and W. J. Sandborn. 2007. Inflammatory bowel disease: clinical aspects and established and evolving therapies. Lancet 369: 1641-1657.

Classic therapeutic management of IBD includes symptomatic treatment with anti-inflammatory and immunosuppressive drugs, and surgery to induce remission or to treat complications. New emerging therapeutic approaches such as the use of antibiotics or probiotics to manipulate the gut flora, or the use of antibodies or other biologic preparations to block or neutralise cytokines, receptors or signalling molecules, etc., are based in advances in the pathogenesis knowledge of IBD. However, these

strategies are far from being optimal since a clear picture of the mechanisms involved in developing CD or UC is still lacking.

1.2) Pathogenesis

The GI tract constitutes the highest surface of the human body in contact with the external milieu. It harbours an estimated quantity of 10-100 trillion microorganisms and, in parallel, bears the largest amount of immune cells in the organism. Equilibrium between the resident microbiota and the host immune system is essential to maintain their symbiotic relationships, and it is dependent on limiting bacterial penetration of host tissues [5]. Several cell types, immune and non-immune, are involved in conferring the intestinal mucosal surface a tolerogenic phenotype towards commensal flora while retaining the capability of mounting an inflammatory response to pathogens [6]. In this context, any event interfering with the normal function of these cells could alter the relative anergy of the immune system to the resident microbiota, resulting in an uncontrolled inflammation that would perpetuate in time due to persistence of the antigen in the milieu. This is mechanistically what seems to happen in IBD, where the presence of an initiating factor in genetically predisposed individuals [7, 8] would cause a chronic and inappropriate inflammation towards the resident microbiota due to a loss of tolerance [9].

Several studies have tried to establish cause-effect relationships between environmental and/or genetic factors and the development of IBD. The general conclusion is that there is not a unique factor that could itself explain IBD's phenotype, but the combination and reciprocal influence of several ones predispose to it. Thus, to better understand pathogenesis of IBD it is important to integrate lessons learned from 4 main fields: epidemiology, microbiology, genetics and immunology.

1.2.1) Epidemiological facts and environmental risk factors

Several epidemiological facts suggest that environmental factors participate in development of IBD. Perhaps the most compelling evidences are the low concordance rate of these diseases between monozygotic twins (10–15% in UC and 30–35% in CD) and their worldwide temporal trends in incidence and prevalence. Actually, IBD started

to be diagnosed in North America and Northern Europe, as these countries became industrialised and achieved earlier development. Then its frequency increased in Central and Western Europe, Japan, Australia... and it is currently rising in "developing" countries such as China, India, South Korea, Iran or Thailand [10, 11]. In this regard, it seems that the access to better sanitation conditions, higher quality water and better medical standards is facilitating the emergence of such pathologies. Furthermore, migration studies have reported that, in individuals moving from "developing" to "developed" countries, the incidence of immune-mediated diseases such as IBD does not change amongst adult immigrants, but the susceptibility of their offspring is similar to that of the native population [1, 10]. All these epidemiologic observations strongly suggest the contribution of environmental factors to the increase in IBD's incidence.

Some epidemiological studies have sought for causal mechanisms in IBD. Different risk factors have been described thereof, such as smoking, use of oral contraceptive pills or non-steroidal anti-inflammatory drugs, appendectomy, diet, breastfeeding, antibiotic treatment, stress or socio-economic status. Although none of these factors can itself explain the environmental determinants for IBD, they have been shown to affect, through different mechanisms, the intestinal epithelial barrier function, the composition of the microbiota or the activation state of the innate and acquired immune response [2]. Thus, they might be contributing influences to the disease, but cannot be considered as causatives.

In 1989, Strachan proposed the "hygiene hypothesis" for hay fever [12]. It stated that a lack of exposure to common infections in early life negatively affects the proper development of the immune system, which becomes less "educated" and is less prepared to deal with new challenges later in life, predisposing the individual to immunological diseases. This hypothesis was then adapted to IBD and has undergone some refinements. It is currently hold by some authors that such immunologic deregulation is not dependent on exposure to pathogenic agents, but on a reduced colonization of commensal microorganisms [13, 14].

1.2.2) Microbial involvement

Initial microbiologic research in IBD was directed to identify candidate etiologic agents for these diseases. Perhaps the most relevant results in this respect are those

referent to the adherent-invasive *Escherichia coli*, that was isolated from ileal mucosa of CD patients [15]; however, antibiotherapy against coliforms fails to cure CD patients [11]. Similar approaches have been made for other bacteria, but no improvement has been reported for IBD patients after selective elimination of specific pathogens.

Although these studies have failed to establish a direct cause-effect relationship between pathogenic microorganisms and IBD, they have settled new lines of evidence suggesting the participation of the gut microflora on the onset of these pathologies. Thus, the presence of adherent-invasive *Escherichia coli* in CD [15] and the increases in mucosal adherent bacteria in IBD patients [16] might underlie possible defects in the mechanisms that keep bacteria far from the surface of the intestinal epithelium. In the same vein, several IBD risk genes bear mutations that impair the maintenance of the mucus layers that cover the epithelium, the production of antimicrobial peptides or the immune recognition of microorganisms [17].

Various studies in animal models also support the essential role of microbiota as a cofactor of inflammation. Work from the group of Sartor demonstrates that in murine models of spontaneous colitis, the presence of non-pathogenic bacteria is imperative for the development of inflammation [18, 19]. Similar results have been obtained in other models of inflammation [20], concluding that regardless of the genetic background of the animals and the method used to induce inflammation, all animals raised in germ-free conditions fail to develop experimental inflammation unless they are reconstituted with gut flora.

Despite this role of microflora as a starter of the inflammatory response, it is also fundamental to remark its importance for the correct development of the GI immune system. It is generally accepted that from instants after birth to the first 2-3 years of life, commensal microorganisms from environment and food colonise the GI tract. This flora will remain stable for the rest of the host's life. In these stages, microbiota makes key contributions to the gut maturation in terms of nutrient absorption efficiency, strengthening of the epithelial barrier, angiogenesis and "education" of the immune system [17, 21]. In this scene, as already stated, the IBD hygiene hypothesis proposes that the increased sanitation standards, the westernised lifestyle and the changes in adult diet (which have a direct impact on neonatal microflora) have caused a severe

impairment in the microbial species colonising the GI tract [13, 14]. Helminths exemplify these premises: there is a clear inverse correlation between the frequency of helminth infections and the prevalence of IBD [14]. Studies in animal models conclude that different helminth parasites ameliorate experimental colitis through different mechanisms that end-up in induction of Th2 cytokines such as interleukin (IL)-4 or IL-13, and large quantities of regulatory factors like IL-10 or transforming growth factor-β (TGF-β), which may promote in turn differentiation of T naïve cells in T regulatory cells [14, 22]. Hence, there have been several clinical trials using helminths to treat IBD, and results from these trials suggest that controlled worm infection improves the clinical outcomes of IBD [14, 22]. Similarly, recent studies show that segmented filamentous bacteria can directly modulate the T helper cell repertoire of the gut [23], further confirming the key role of microflora in shaping the host's immune system and, by extension, the inflammatory response.

1.2.3) Genetics

The implication of genetic factors in IBD's pathogenesis was suggested by epidemiologic data. On one hand, incidence and prevalence are different depending on the ethnicity: they are higher in Caucasian people, especially among the Jewish. On the other hand, there is a high familial aggregation and a higher concordance in monozygotic than in dizygotic twins. Actually, the most important risk factor for developing IBD is having a relative that suffers from it, and abnormal intestinal permeability has been observed in IBD patients and in some of their first-degree relatives, even if they do not develop the disease [24].

Genome-wide association studies have identified, up-to-date, 99 non-overlapping genetic risk loci, including 28 that are shared by CD and UC, indicating that these diseases have common pathways [25]. Analyses of the genetic loci implicated in IBD demonstrate that an important part of these genes are implicated in maintenance of homeostasis, mucus production, barrier function, epithelial restitution, microbial defence, innate immune regulation, autophagy, etc. Interestingly, there seems to be ethnicity differences among IBD-associated variants, for example nucleotide oligomerisation domain receptor *NOD2* or autophagy genes, that are only shared by Caucasian and Jewish patients but not Chinese or Japanese [11].

One of the first susceptibility genes that could be associated to IBD is *NOD2*. *NOD2* codifies for a protein bearing the same name that acts like a pattern recognition receptor (PRR). It is involved in recognition of microbial-associated molecular patterns (MAMP), triggering innate immunity upon stimulation. Several polymorphisms have been described in its sequence, resulting in a gain or loss of function that in both cases predispose to suffer from IBD [7, 8]. Other loci encoding for PRRs, such as *TLR2* and *TLR4*, have been also associated with IBD. But perhaps the candidate gene that better illustrates the importance of genetic and microbial factors in IBD is *ATG16L1*. Patients with CD carrying the *ATG16L1* (T300A) mutation show Paneth cell granule abnormalities, and have an impaired degradation of cellular and bacterial products. These deficiencies lead to a reduced antimicrobial activity, due to decreased defensin secretion and defective autophagy [25]. Recent work by Cadwell and colleagues demonstrated that mice expressing hypomorphic ATG16L1 that were additionally infected by a murine norovirus exhibited, under a chemical disruption of the epithelial barrier, multiple hallmarks of human's CD. Such lesions were not observed in uninfected *Atg16l1* hypomorphic mice [26], providing a good example of how microbiota and environmental factors can determine the phenotype of hosts carrying disease susceptibility genes.

1.2.4) Immune and non-immune aspects of inflammation

Most of the cell types that make up the GI tract are implicated in the control of homeostasis. Immune cells, intestinal epithelial cells (IEC), myofibroblasts, endothelial cells, cells of the enteric nervous system (ENS) and even the extracellular matrix are in constant communication through a complex interplay of substances, providing the organ with the capacity to tolerate commensal flora and react against pathogens [27].

The mucus layers that cover the epithelial cell barrier represent the first obstacle to bacterial penetration (Figure 1). Although the outer layer is colonised with bacteria, the inner layer is resistant to bacterial penetration due to its densely packed mucin MUC2 [28], that retains antimicrobial peptides [29] such as defensins and lysozyme secreted by Paneth cells and soluble immunoglobulin (Ig) A produced by B cells. Mice lacking the mucin MUC2 are unable to maintain this inner layer devoid of bacteria and suffer from intestinal inflammation [28].

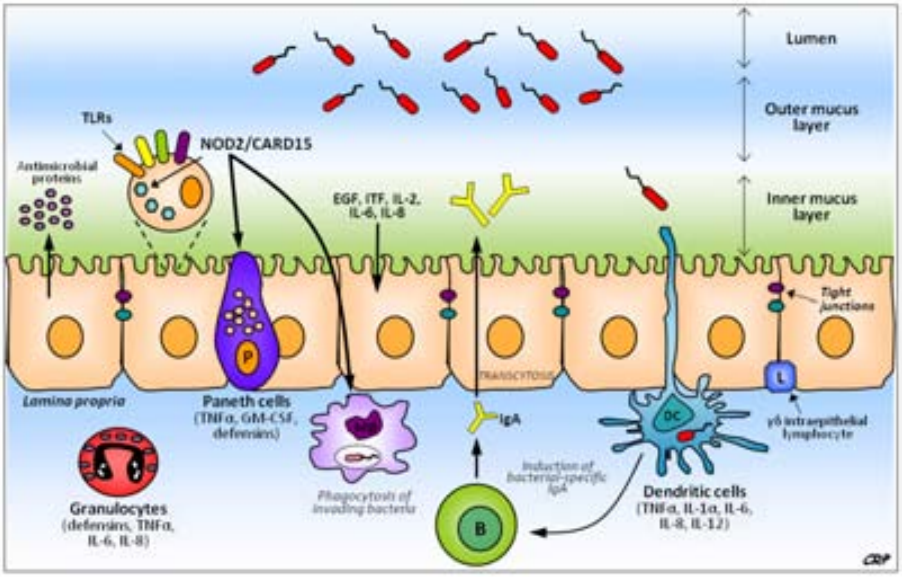

Figure 1 - Mechanisms participating in the barrier function. Immune and non-immune cells cooperate to maintain the inner mucus layer devoid of bacteria. **EGF**, epidermal growth factor; **GM-CSF**, granulocyte-macrophage colony stimulating factor; **IGT**, intestinal trefoil factor.

Adapted by Carolina Romero from Duerkop, B. A., S. Vaishnava, and L. V. Hooper. 2009. Immune responses to the microbiota at the intestinal mucosal surface. Immunity. 31: 368-376.

Just beneath these layers the IECs form a polarized, continuous monolayer that is tightened through the expression of various proteins organising in the tight junctions (TJ) and *zonula adherens*. These cells and TJs separate the internal from the external milieu (Figure 1), what is essential for homeostasis, as abnormal permeability is observed in IBD patients and several IBD-associated loci are involved in maintenance of barrier integrity [25]. But IECs do not only exert a physical action, but also secrete enzymes that modify the lipopolysaccharide (LPS) from gram negative bacteria (reducing its inflammatory effects), and act like non-professional antigen presenting cells (APC) [30]. Moreover, these IECs also express PRRs, what allows them to sample the bacteria penetrating the inner mucus layer and produce cytokines and chemokines in the basolateral pole, thus providing a first signal to underlying immune cells [31]. Additionally, embedded in this layer, goblet cells, Paneth cells and γδ T cell receptor bearing intraepithelial lymphocytes contribute to defensive tasks through mucin,

defensin and antimicrobial protein secretion [17]. Impaired functions of these cells lead to bacterial colonisation of the intestine and subsequent inflammation, as already exposed [25, 26, 28].

Myofibroblasts in the subepithelial layer may also contribute to milieu monitoring through expression of PRRs [32]. The role of endothelial cells in preserving this physiologic equilibrium relies in the modulation they exert on leukocyte migration, and depends on their activation state, synthesis and liberation of cytokines and expression of adhesion ligands. Actually, cultured vascular endothelial cells from chronically inflamed areas of IBD patients display increased leukocyte adhesion compared to uninflamed areas from the same patients or from healthy individuals [33].

Most of the cells of the immune system participate in maintaining a controlled "physiologic" state of inflammation. B cells from the *lamina propria* produce bacteria-specific IgA, which is essential for avoiding penetration of bacteria into the host tissues [17]. Macrophages from the healthy GI tract show a unique tolerogenic phenotype: although retaining phagocytic activities, they display reduced APC function (through low expression of costimulatory molecules) and no secretion of cytokines in response to MAMPs [34, 35]. But perhaps the most important cells in generating tolerance are the dendritic cells (DC), as they shape adaptive immune responses. Immature DCs dwell in mucosal surfaces, where they are able to modify TJs and extend their dendrites between IECs to directly sample the intestinal lumen through their PRRs (Figure 1) [36, 37]. At this stage, DCs do not express functional chemokine and cytokine receptors, but migrate to the draining mesenteric lymph nodes (MLN). There, as they engage naïve T cells, immature DCs promote anergy and T cell differentiation into T regulatory (Treg) cells ($CD4^+CD25^+$, Tr1 or Th3; Figure 2a). Conversely, when they are stimulated through PRRs ligands, they maturate and express costimulatory molecules at the same time that induce transport of major histocompatibility complex (MHC) class II molecules to the cell surface [38]. In these new conditions, trafficking of DCs to MLNs is enhanced, and depending on the costimulatory molecules and the cytokines secreted around the MHC-T cell receptor complex, T naïve lymphocytes differentiate into different subsets of T helper cells: Th1, Th2 or Th17 [33]. These T cells drive the subsequent inflammatory response through secretion of their classic and characteristic cytokines (depending on the subset). After elimination of the pathogen or the infiltrating microorganism, this

effector response is modulated again by apoptosis of T helper cells and predominance of Treg cells, which bring the situation back to basal conditions.

In IBD, several mechanisms have been proposed as initiating factors: deficiencies in epithelial barrier function, mucin or defensin production, impairment of PRR signalling in non-professional or professional APCs, persistence of T lymphocytes that do not respond to apoptosis signals... Any of these mechanisms might lead to a chronic, uncontrolled inflammation that could be further amplified by activation profile changes in neighbouring immune and non-immune cells.

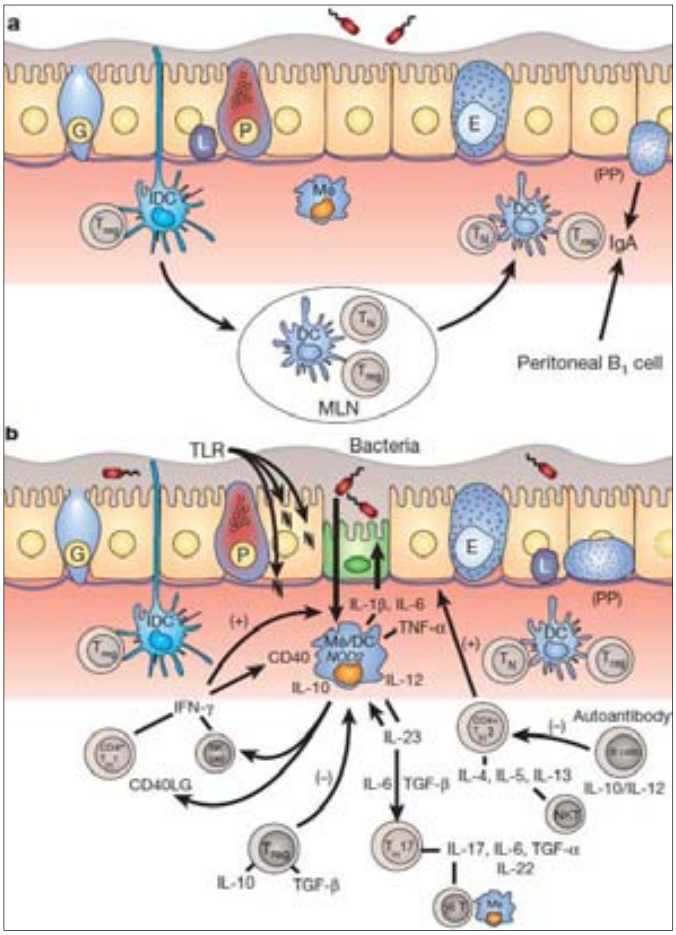

Figure 2 - Mucosal immune response in physiologic (a) and pathologic conditions (b). DCs shape the adaptive immune response depending on the stimuli they get from microbiota. Th1/Th17 or Th2 responses drive IBD outcomes. **E**, enteroendocrine cell; **G**, goblet cell; **IDC**, immature dendritic cell; **Mϕ**, macrophage; **NK**, natural killer cell; **TN**, T naïve lymphocyte; **P**, Paneth cell; **PP**, Peyer's patches.

From Xavier, R. J., and D. K. Podolsky. 2007. Unravelling the pathogenesis of inflammatory bowel disease. Nature 448: 427-434.

The adaptive response predominating in CD and UC explains, to a long extent, their differences in localisation, symptoms and pathologic features. On one hand, CD is a Th1/Th17 disease, characterised by subsets of T cells that produce IL-12, interferon (IFN)-γ and IL-17 [31, 39]. On the other, UC is considered an atypical Th2 response, with secretion of IL-5 and IL-13 by Th cells and natural killer T cells (Figure 2b) [31]. However, both entities share common secondary pathways. Production of inflammatory cytokines induces a weakening of the barrier function (as seen in IBD patients), which might in turn ease the entrance of microorganisms into de host tissues. Engagement of microbiota with PRRs from DCs and macrophages in a pro-inflammatory environment leads to an increased immune response (in fact, DCs and macrophages from IBD patients show activated phenotypes, with increased IL production [11, 33]). And meanwhile, cytokines induce activation of endothelial cells, expression of adhesion molecules and homing of blood immune cells. Finally, the presence of large quantities of inflammatory mediators, antibodies and metabolites promotes important tissue damage, which enhances proliferation of fibroblasts and tissue fibrosis.

1.3) Animal models of IBD

Although they do not represent the complexity of human disease and cannot replace studies with patient material, animal models have greatly contributed to the understanding of the underlying mechanisms causing inflammation in IBD. Currently, more than 20 different models of intestinal inflammation have been described [40]. None of these models recapitulates all pathogenic and clinical features of IBD, but combined interpretation of resulting evidences is contributing to improve our knowledge of such disease.

Animal models can be classified, depending on the pathogenic mechanisms they involve, in models of intestinal inflammation due to barrier integrity disturbance, innate immune cell defects or adaptive immune deficiencies [41]. They can also be classified, depending on the method of induction, in chemical, genetic, immunological or spontaneous models [40]. Each one of these categories implies intrinsic features that must be considered by the researcher to make a good model choice depending on the aims. For instance, genetic models are useful for assessing the role of key molecules in

the pathogenesis of chronic intestinal inflammation, while adoptive transfer models are more interesting to evaluate T cell function in the control of immunity [42]. In parallel, several other considerations must be taken in account when choosing a model: easiness of induction, reproducibility, variability among animals, cost-efficiency advantages, animal species and strains or the disease (CD or UC) to be addressed.

The optimal model should display similar characteristics in aetiology, pathogeny, symptoms and pathology to IBD. The animals of interest should have a well-defined background and a well-characterised immune system, along with well-defined criteria concerning successful management and manipulation, as well as accessible reagents available for experimentation [40]. In this regard, perhaps the best mouse model resembling CD is the SAMP1/YitFc, as these mice develop spontaneous severe transmural inflammation of the terminal ileum without exogenous manipulation. However, the onset and severity of disease in spontaneous and genetic models are highly variable and largely depending on environmental factors, and in some cases like in IL-10$^{-/-}$ mice, it takes several months for manifestation of colitis. This makes these models not suitable for certain purposes [41]. Hence, the use of chemically induced models is widely extended in research. They are easy to perform, display high reproducibility and have several cost-efficiency advantages, as they are relatively inexpensive. In addition, they are very useful for studying biochemical pathways on inflammation, especially those related to epithelial barrier disruption, and provide proof of concept for therapeutic challenges [42]. They also allow for an easy combination of models, as novel transgenic mouse strains can be treated with chemicals to evaluate the effect of the target gene in distinct initiating conditions. Nevertheless, they only resemble human disease in some aspects, and tend to cause acute rather than chronic colitis.

Among the chemically induced murine models of inflammation, the dextran sulphate sodium (DSS) model of colitis is one of the most commonly used. DSS decreases the thickening of the inner mucus layer, facilitating the penetration of bacteria to reach the intestinal epithelial barrier within 12 hours, before any infiltration of inflammatory cells [43]. Supporting this observation, permeability of the barrier is increased just after 24 hours [44]. Regarding the immune response, two phases can be differentiated. The acute phase of inflammation is useful for studying innate immune

responses, as it does not require the participation of T and B cells [45]. Conversely, the chronic phase is characterised by a polarisation of the response to a Th1/Th17 profile, resembling CD [44, 46]. However, the symptoms and histopathologic lesions found are more akin to UC, so it has been classically considered a model of UC [46, 47].

The quality of the inflammation induced is highly dependent on the mouse strain used [46], the DSS molecular weight [48] and its concentration in water. In our studies, we have used the C57BL/6 mouse strain because a single cycle of DSS derives in chronic inflammation due to their strong Th1 response [46]. The DSS molecular weight was 36-50 kDa, as it has been described to induce middle-distal colitis [48], and the concentration added to water was 3%. In these conditions, mice display weight loss and bloody diarrhoea, along with colon shortening, epithelial crypt loss, oedema and mononuclear infiltration of mucosal and submucosal layers. This model has been thoroughly used in our group, is well characterised and has been suitable to test the usefulness of new therapeutic agents like adenoviruses and short interference RNAs in amelioration of inflammation [49, 50]. Furthermore, it has been shown to reproduce the efficacy of different drugs used in IBD management, demonstrating again the utility of this model in the proof of concept of drug use before clinical trials [51].

2) Toll-like receptors

2.1) General aspects

In vertebrates, immune systems are divided in two basic categories: innate and adaptive immunity. The adaptive immunity is mediated by T and B lymphocytes, which expand clonally in response to a specific antigen. The generation of an adaptive response requires a number of days, and is only present in vertebrates. In contrast, the innate immune system allows the host to respond in a fast way towards penetration of pathogenic microorganisms, although its responses are more limited than those of the adaptive immune system. This innate immune system is evolutionarily conserved, represents the first line of defence against invading microbes and acts via two different types of mechanisms, namely the constitutive and the inducible. Constitutive immunity is represented majorly by physical barriers, like the epithelial barrier in the gut, consisting of mucus layers, antimicrobial peptides and IECs. Inducible mechanisms are driven by macrophages, granulocytes and DCs, and require the participation of a number of receptors able to differentiate between self and non-self. In this regard, the PRRs recognise MAMPs, which are molecular structures exclusive from microorganisms and essential for their survival, so that they are highly conserved [52]. Different PRRs have been described, but in the last years the study of two families of these receptors has centred the interest in PRR research: the NOD receptors and the Toll-like receptors (TLR).

Antifungal response in Drosophila adults was shown to be dependent on the Toll receptor, which had been previously demonstrated to be involved in the embryonic dorsoventral patterning [53]. Homology research studies by Medzhitov and colleagues described a human homologue of Toll, which is currently known as TLR4, and showed the ability of this protein to induce production of pro-inflammatory cytokines and costimulatory molecules [54]. Subsequent studies in LPS-hyporesponsive mice reported mutations in the *Tlr4* gene [55], thus defining its ligand, and up to date at least 13 different TLRs have been described in mammals, each one of them featuring unique capabilities to recognise specific MAMPs.

TLRs are germ-line encoded transmembrane proteins consisting of an extracellular leucin-rich repeat (LRR) domain, and a cytoplasmic Toll/IL-1 receptor (TIR) domain [54]. The LRR domain selectively recognises the appropriate ligand (Figure 3a) in the extracellular milieu, and induces the dimerisation of the receptor. This allows the TIR domain to interact with TIR-domain-containing cytosolic adapters like myeloid differentiation primary response protein 88 (MyD88) and others (Figure 3b), triggering different signalling pathways (Figure 3b) that culminate in activation of transcription factors like nuclear factor (NF)-κB and activating protein-1 (AP-1) [56]. These factors translocate to the nucleus and induce the production of pro-inflammatory cytokines like tumour necrosis factor (TNF)-α, IL-1β, IL-6 and IL-12 [54, 56]. In addition, as NF-κB and mitogen-activated protein kinases (MAPK) also target genes involved in cell proliferation, TLRs can promote survival and suppression of apoptosis [57].

An additional role that has been attributed to TLRs too is the recognition and signalling of damage-associated molecular patterns (DAMP), which allows them to signal sterile inflammation too and direct tissue repairing actions [58]. Thus, TLRs modulate the innate immune response towards infection or injury by directly inducing the secretion of antimicrobial peptides, pro-inflammatory cytokines and chemokines and by enhancing the phagocytic activity of macrophages [59]. But their actual importance does not only lie in these actions, but also in the fact that they promote maturation of APCs and expression of costimulatory molecules, providing the second signal necessary to prime T lymphocytes after T-cell receptor recognition of class II MHC molecules [52, 59]. As TLRs occupy such important biologic roles (shaping the onset of the adaptive immunity and further inhibiting apoptosis to expand the response), they are controlled by tight inhibitory regulation mechanisms. Those include soluble forms of the receptors, inhibitors of the signalling pathways, compartmentalisation of receptors or inhibition of their expression, among others [60, 61].

TLRs are expressed in most tissues of the organism, especially in those bearing myelomonocytic cells, like spleen or peripheral blood, and almost all tissues express at least one TLR, indicating their importance [62]. Within the cells, most TLRs are located in the cell surface. However, TLR3, TLR7, TLR8 and TLR9 are usually found in internal compartments like phagosomes because they recognise motifs that need internalisation and degradation of viral and bacterial particles [56].

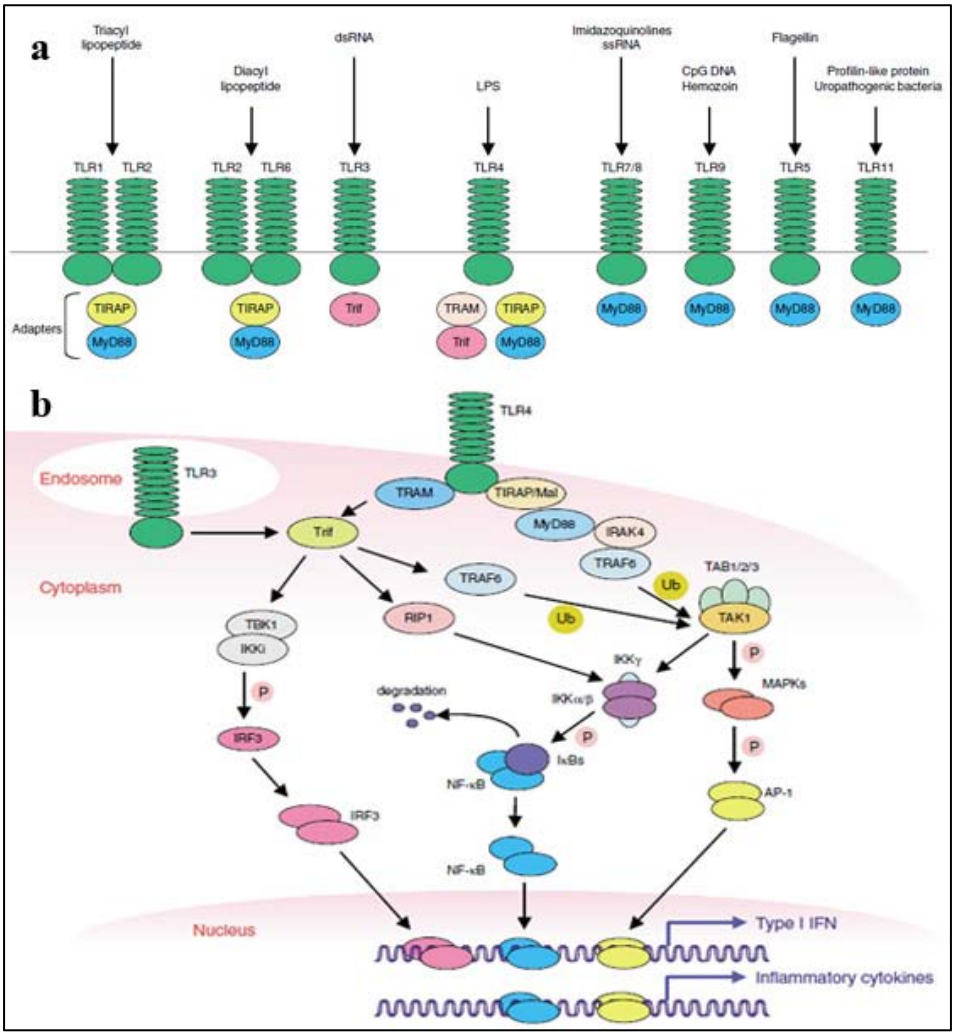

Figure 3 - TLRs and their ligands, adapters (a) and signaling pathways (b). The different TLRs recruit common adapter proteins that subsequently activate either the NF-κB , the MAPK or the IFN regulatory factor (IRF) pathways to produce inflammatory cytokines or type I IFNs. **CpG DNA**, CpG-rich unmethylated DNA; **dsRNA**, double stranded RNA; **P**, phosphorylation; **ssRNA**, single stranded RNA; **Ub**, ubiquitination.

From Kawai, T., and S. Akira. 2006. TLR signaling. Cell Death. Differ. 13: 816-825

2.2) TLR2, TLR4 and TLR9 particularities

TLR2 is involved in recognition of a number of ligands: lipoproteins and lipopeptides, peptidoglycan, lipoteichoic acid, zymosan (MAMPs) and heat shock proteins (DAMPs) [63]. Recently, it has been also reported that TLR2 is involved in signalling of lipoproteins derived from lipid peroxidation products [64], thus amplifying the inflammatory response by linking the reactive oxygen species and the MAMPs activation. Such capability of recognition is achieved through interaction with other receptors, namely TLR1, TLR6 and dectin-1. TLR1-TLR2 heterodimerisation allows for the recognition of triacyl lipopeptides [65], whereas TLR2-TLR6 allows for the recognition of diacyl lipopeptides like the synthetic Pam2CSK4 [66]. Dectin-1 collaborates with TLR2 to signal the fungus cell wall component zymosan [67]. Heterodimerised TLR2 essentially signals through the MyD88-dependent pathway.

TLR4 recognises LPS [55] and some DAMPs like heat shock proteins or hyaluronic acid [63, 68]. To activate TLR4, LPS must be first extracted from the bacterial membrane by the LPS binding protein and then transferred to CD14. CD14 subsequently transfers LPS to the accessory protein MD-2, that allows for TLR4-mediated responses [69]. After homodimerisation of two receptor complexes, TLR4 can trigger both the MyD88-dependent and the TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent signalling pathways [70]. Activation of one or both cascades has been proposed as a unique mechanism to differentiate signals from self and non-self [58].

TLR9 is located on the endosomes in order to recognise viral, fungal and bacterial DNA. It specifically detects unmethylated CpG motifs, as they appear in 20-fold greater frequency in microbial DNA compared to vertebrate DNA [71]. The effect of these DNAs can be mimicked by synthetic oligodeoxynucleotides (ODN) containing the proper CpG motifs. At least two types of TLR9-activating CpG-DNAs have been described depending on the response they elicit: B/K-type and A/D-type. B/K-type CpG ODNs were identified first and are potent inducers of cytokines like TNF-α and IL-12. A/D-type CpG ODNs are structurally different and have a greater ability to induce IFN-α production in plasmacytoid DCs [59]. TLR9 activates only the MyD88-dependent pathway [72].

2.3) TLRs in the GI tract and tolerance

Given that high microbial burdens inhabit the GI tract and that TLRs mediate the immune responses to some of their components, it is reasonable to think that these receptors have important functions in the GI immune system. Indeed, TLRs orchestrate the inflammatory responses towards invading microorganisms once the epithelial barrier is broken. However, their role in the mucosa of the bowel is much more complex, as they have been also involved in regulation of epithelial cell proliferation and regeneration, IgA production, IEC barrier integrity and antimicrobial peptide expression and release [73, 74].

TLRs are expressed in most cell types of the GI epithelium, including the four IEC lineages: absorptive enterocytes [75-77], Paneth cells [78, 79], goblet cells [80] and enteroendocrine cells [81]. In each case, TLRs seem to participate in the functions these cells develop in response to bacterial motifs. In absorptive enterocytes, for instance, ligand-induced TLR2 activation has been shown to increase transepithelial resistance *in vitro* by redistributing the TJ protein zona occludens-1 to the apical side of the barrier [82]. Indeed, MyD88$^{-/-}$ mice suffer impaired epithelial barrier function [83]. Other studies have demonstrated that TLR-stimulated enterocytes secrete chemokines that recruit DCs and B-cells to the subepithelial compartment and increase the frequency of DC projections to the lumen as well as the production of IgA [37, 84]. In Paneth cells, the expression profile of antimicrobial peptides and their role in limiting bacterial translocation to MLNs is entirely dependent on functional MyD88 [78], and direct degranulation has been described after oral administration of TLR3 or TLR9 ligands [79]. On the other hand, TLR2 is implicated in the correct maturation of goblet cells, as administration of a TLR2 agonist increases trefoil factor-3 expression and goblet cell proliferation and size in small intestine and colon [80]. Finally, TLRs present in enteroendocrine cells participate in the liberation of chemokines, defensins and hormones such as cholecystokinin [81], which might be involved in the development of diarrhoea in response to pathogens [73].

TLRs are also expressed in other cells found in the mucosal and submucosal layers, such as subepithelial myofibroblasts [32], macrophages [34, 85] and DCs [86]. In these cells, TLRs carry out their classic functions involving recognition of microbial ligands and

production of pro-inflammatory cytokines. However, the bowel must remain hyporesponsive to resident microflora. To achieve such tolerogenic phenotype, IECs and professional APCs such as resident macrophages and DCs in the bowel have developed different strategies to bring TLRs to a hyporesponsive state towards MAMPs. Two of these tolerogenic strategies are based in reduction of expression of TLRs or accessory signalling molecules and localisation of the receptors in inner parts of the epithelium, which are difficult to reach for microorganisms. Thus, expression of TLR2 and TLR4 in enterocytes constituting the intestinal epithelial barrier of normal human colon specimens has been reported to be low [85, 87, 88], and in studies where more important expression has been described, both proteins seem to be localised in the crypt bottom but not in the surface epithelium [76, 89]. In the same vein, expression of TLR4 transcripts in intestinal lymph DCs is significantly lower than in bone marrow-derived DCs, what makes them unable to respond to LPS [86]. In addition, expression of accessory molecules like MD-2 or CD14 is low in human colonic epithelial cells as well as in resident macrophages, which further contribute to a reduced response to LPS [34, 88].

Aside these constitutive strategies, most tolerogenic mechanisms involve TLR signalling, and are thus inducible. Several lines of evidence show that most of them are based in rearrangements in location of receptors or expression changes, up-regulation of signalling inhibitors and production of immunomodulatory cytokines. First, compartmentalisation of TLR2 and TLR4 from the apical surface of IECs to cytoplasmic compartments near the basolateral membrane is observed in polarised IEC monolayers after MAMP recognition, which may contribute to render cells insensitive [90]. In addition, differential signalling exists for TLR9 depending on the surface stimulated (apical versus basolateral) in IECs [91]. Second, cross-regulation of TLR expression has been described after differential ligand recognition in IECs: TLR2 and TLR4 ligands cause down-regulation of TLR4 and TLR5, attenuating subsequent NF-κB activation [92]. Third, up-regulation of the Toll-interacting protein, which inhibits the TLR signalling cascade, can be observed after prolonged TLR stimulation in IECs, which leads to a state of hyporesponsiveness to subsequent challenges in terms of MAPK activation and chemokine secretion [77]. And fourth, TLR2 stimulation of immature DCs promote strong secretion of IL-10, which is capable of blocking the Th1 cytokine profile elicited by other

TLR ligands [93], and might then bias in vivo the immune response to a Th2 phenotype through differential signalling in DCs [94]. Furthermore, tolerogenic DCs pre-stimulated with LPS show important up-regulation of TLR2, sensitising them to subsequent challenges with TLR2 ligands, and enhancing their IL-10 production [95].

Thus, these inducible tolerogenic mechanisms represent potential therapeutic targets of intervention, as exogenous addition of TLR ligands could modify the behaviour of epithelial and immune cells to render them less responsive to luminal antigens. Such rationale has been extended to inflammatory conditions, where TLRs also play important roles in the onset of inflammation and its resolution.

2.4) TLRs in GI inflammation and repair

As TLRs are implicated in the immune responses to pathogenic microorganisms, it is likely that a deregulated or aberrant response of these receptors towards resident microflora may lead to sustained and chronic inflammation. Actually, some studies have addressed the functional implication of TLR polymorphisms in IBD. Mutations in *TLR2*, *TLR4*, *TLR5* and *TLR9* have been associated with IBD, but these polymorphisms seem to have a more important impact in phenotype severity than in predictive disease risk [74]. In agreement with the term "deregulation", both "gain-of-function" and "loss-of-function" mutations can lead to increased disease severity. For instance, the TLR2-R753Q polymorphism causes a loss of production of trefoil factor-3 in Caco-2-transfected cells, leading to impaired wound healing and increasing the risk of suffering pancolitis in UC patients [80]. In the same regard, $TLR5^{-/-}$ mice develop spontaneous colitis due to a loss of expression of TLR5-derived host defence genes, resulting in increased bacterial burdens and enhanced TLR4-driven hematopoietic responses [96]. Conversely, "gain-of-function" mutations in the *TLR4* gene would lead to increased responses to physiologic concentrations of LPS, which would also prompt exaggerated inflammatory responses [74].

Other evidence supporting the hypothesis of a deregulated response is the increased expression of TLRs and accessory signalling molecules in specimens from IBD patients. TLR2 and TLR4 up-regulation of mRNA and protein has been reported in the submucosal macrophages found in inflamed areas of CD and UC patients [85, 97]. In

addition, TLR4 is also increased in IECs of inflammation-involved areas [87]. In the same vein, TLR9 increased transcripts and protein have been also found increased in biopsies from UC patients [98]. Of note, non-inflamed specimens of IBD patients show similar expression of TLR2 and TLR4 than control specimens, suggesting that these receptors retain their physiologic functions in the uninvolved areas [97]. Similarly, in the murine models of colitis induced by DSS and trinitrobenzenesulfonic acid (TNBS), TLR2, TLR4, CD14 and MD-2 have been found up-regulated during active inflammation [76, 99]. Taken together, these observations point out that TLRs are clearly involved in inflammation, but their actual role during this process has been elucidated by combining transgenic mice for the MyD88 protein and inflammation models.

As expected, the lack of functional TLRs elicited by deletion of MyD88 impairs the immune response towards pathogenic bacteria, as well as the antimicrobial peptide production [83, 100]. Studies with inoculated bacteria showed that depending on the pathogenicity of the bacteria, the absence of MyD88 derives in increased susceptibility to infection and higher crypt destruction [83] or in a lack of inflammatory response and prompt resolution of the pathologic process [100]. However, as discussed in previous paragraphs, no pathogenic bacteria have been up-to-date identified as a causative of IBD. Hence, a new element was added to the system: the presence of an injurious stimulus. Work from the group of Medzhitov showed that recognition of commensal bacteria by TLRs is essential to overcome DSS-induced colitis [101]. MyD88$^{-/-}$, TLR4$^{-/-}$ and TLR2$^{-/-}$ mice showed high morbidity and mortality upon DSS treatment when compared to wild-type controls. Such observation was not due to bacterial overgrowth or increased leukocyte infiltration, but to an absence of production of cytoprotective factors, as well as a deregulation of IEC proliferation and differentiation [101]. Other groups have confirmed this increased susceptibility, and have additionally described increased epithelial barrier disruption due to loss of TJ proteins in MyD88$^{-/-}$ and TLR2$^{-/-}$ mice [102], and reduced acute inflammatory infiltrate in MyD88$^{-/-}$ and TLR4$^{-/-}$ mice, correlating with increased bacterial translocation to MLNs [103]. Thus, TLR signalling confers protection during acute injury by maintaining proper epithelial barrier function, inducing cytoprotective mediators and enhancing the defensive immune response. Therefore, exogenous administration of TLR ligands would not only be interesting as a therapeutic approach in an attempt to promote tolerogenic responses in physiologic

conditions, but also during inflammation, as these experiments demonstrate that TLR signalling is mandatory for maintenance of homeostasis during injury.

Following this line of evidence, several TLR ligands have been used as prophylactic and/or therapeutic tools [104]. In the same set of experiments previously referenced [101], the group of Medzhitov also demonstrated that commensal-depleted mice showed more morbidity and mortality upon DSS-treatment when compared to controls. In these animals, prophylactic oral administration of the TLR4 ligand LPS increased survival, whereas this effect was not found in TLR4$^{-/-}$ mice [101]. In contrast, in specific pathogen-free conditions, treatment of mice with a TLR4 blocking antibody ameliorates DSS-induced colitis by reducing the recruitment of APCs to the colon [105]. Oral TLR2 ligand administration has also been proven to be beneficial for colitic animals, as it improves barrier integrity, decreases apoptosis of IECs and accelerates recovery after inflammatory injury [80, 102]. In the same vein, TLR9 ligand administration ameliorates the severity of DSS-colitis, but only when the nucleotide is inoculated in a route that allows for a systemic delivery [106]. Other beneficial and detrimental effects have been described for other TLR ligands, and have been reviewed elsewhere [104].

Besides their early effects in regulating secretion of antimicrobial peptides, inducing expression and redistribution of TJ proteins or controlling microbial translocation by promoting an effective immune response, TLRs have been also involved in the later phases of inflammation. Indeed, TLRs are necessary for repair responses and ulcer healing after injury. In the recovery phase after DSS administration (usually from days 7 to 14 after the beginning of colitis induction), MyD88$^{-/-}$ and TLR4$^{-/-}$ mice have decreased numbers of proliferative epithelial cells [107, 108], while MyD88$^{-/-}$ and TLR2$^{-/-}$ mice display increased numbers of apoptotic cells when compared to wild-type DSS-treated mice [80, 102]. Furthermore, blockade of TLR4 signalling by means of intraperitoneal administration of a neutralisation antibody also reduces the number of proliferating cells in the recovery phase [105]. In all cases, the overall phenotypic result in these animals is a delayed recovery, with worse clinical and histopathologic signs of colitis and higher mortality rates.

The mechanisms involving TLRs in repair responses seem to be receptor-specific. On one hand, TLR2 activity seems to rely on its stabilising effect on TJ-associated barrier

integrity, as well as on its proliferative and maturation-promoting effects in goblet cells [80, 102]. On the other hand, TLR4 has been demonstrated to act through cyclooxygenase (COX)-2 [68, 107]. This isoform of the enzyme, which is induced under inflammatory conditions, synthesises prostaglandins from arachidonic acid. As prostaglandins are not only important in inflammation, but also in maintenance of epithelial integrity, this enzyme plays an important role in epithelial repair. TLR4 stimulation with LPS or hyaluronic acid increases COX-2 expression in colonic epithelial cell lines and macrophages, and MyD88$^{-/-}$ and TLR4$^{-/-}$ mice treated with DSS are not capable of up-regulating COX-2, in contrast with wild-type mice [68, 107]. Up-regulation of COX-2 is essential for the synthesis of prostaglandin (PG) E2, which has been shown to be crucial to rescue these transgenic animals from the detrimental signs they show due to their impaired healing mechanisms [68, 107].

Interestingly, recent investigation has reported that persistent signalling by pro-inflammatory cytokines induces the synthesis of inhibitors of the Wingless-Int (Wnt)/β-catenin proliferative pathway [109]. Since this signalling cascade is the most important controlling enterocyte proliferation and survival [110], its cytokine-induced blockade disrupts self-renewal of the intestinal epithelium, exacerbating mucosal inflammation [109]. TLR4 has been associated to proliferative responses, as MyD88$^{-/-}$ and TLR4$^{-/-}$ mice exhibit decreased epithelial proliferation [103, 107, 108], and mice over-expressing TLR4 in IECs show increased susceptibility to tumorigenesis [111, 112]. Indeed, activation of TLR4 in IEC lines elicits production of different ligands of the epidermal growth factor receptor and the β-catenin pathway [112, 113]. Since, promotion of the Wnt/β-catenin signalling cascade during experimental colitis has shown beneficial results in both prophylactic and therapeutic regimes of administration [114, 115], its activation through TLR4 could be also useful during experimental inflammation.

In summary, TLRs are clearly involved in IBD and colitic processes, as supported by several evidences. Signalling of these receptors plays important roles in combating microorganism colonization of the inner intestinal layers, inducing tolerance and promoting epithelial repair and wound healing after injury. Exogenous administration of TLR ligands in prophylactic and therapeutic regimens might be helpful to improve the colitis features, but the route of administration and the treatment schedule are critical to obtain beneficial effects.

3) Enteric nervous system

3.1) General aspects

The ENS is an integrated neuronal network embedded in the wall of the GI tract. One singular property of such network is that it contains all the components necessary to assemble reflex pathways, and is thus capable of controlling the functions of the GI tract independently of the central nervous system (CNS) [116]. For this reason, the ENS is considered the "brain-in-the-gut". However, the integrated neural control of the GI functions is based in coordination of the ENS intrinsic reflexes with reflexes that involve motor and sensory pathways of the sympathetic and parasympathetic nervous systems, as well as the CNS [117].

The human ENS contains more than 100 million neurons, and 4 to 10 times more glial cells that provide them support (aside of developing other different functions) [118]. Neurons and glial cells are the only cell types that constitute the ENS. They organise in small ganglia that are connected by nerve bundles forming two major plexuses, namely the myenteric or Auerbach's plexus, and the submucous or Meissner's plexus (Figure 4). The myenteric plexus lies between the longitudinal and circular muscle layers, and extends the whole length of the gut, while the submucous plexus is located in the submucosal layer of the small and large intestines. The myenteric plexus is involved in the control of the GI motility through innervation of the muscle layers. The submucous plexus innervates the epithelium, intestinal endocrine cells, submucosal blood vessels and cells from the immune system, thus controlling the epithelial barrier function, the secretion of water and electrolytes, the blood flux in the submucosa and, to some extent, the response of the local immune system [117]. All these functions can be further controlled by the CNS through the extrinsic innervation, which enters the gut via the vagus, mesenteric and pelvic nerves, and is largely composed by afferent fibres that connect the gut with the brain [117].

The ENS reflexes are driven by intrinsic afferent or sensory neurons, which contact interneurons that project orally or rectally. Interneurons synapse with different motor neurons that subsequently release neurotransmitters in their nerve endings. These neurotransmitters diffuse into surrounding tissues and bind to their corresponding receptors, which are expressed in muscle, epithelium, endothelium and immune cells,

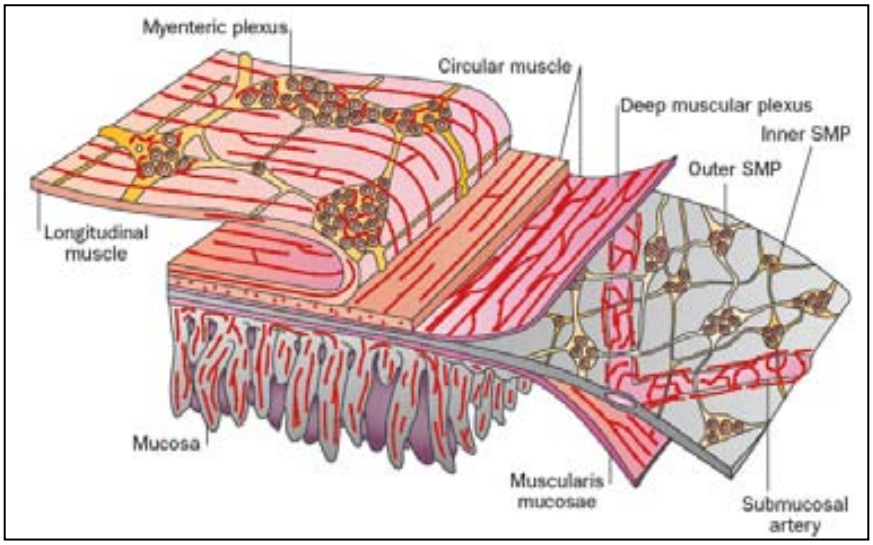

Figure 4 - Organisation of the ganglionated plexuses of the human ENS. The two major plexuses are represented. Submucous plexus has an outer and inner component. **SMP**, submucous plexus.

From Furness, J.B. 2012. The enteric nervous system and neurogastroenterology. Nature reviews. Gastroenterology and hepatology. 9: 286-294.

thus modulating their functions. Several different neurotransmitters have been described, determining the chemical coding of neurons [119]. Among them, acetylcholine (ACh), vasoactive intestinal peptide (VIP) or the sympathetic neurotransmitter norepinephrine (NE) have been widely studied because of their ability to induce anti-inflammatory phenotypes in immune cells [120]. Conversely, other molecules such as substance P (SP), also secreted by ENS neurons, have been shown to contribute to the pro-inflammatory events observed in IBD [121]. In either case, there is a general conception that neurons are directly involved in the immune responses of the GI tract and in the pathogenesis of IBD.

Classic conception of the glia has been that of its etymologic meaning, that is, *glue*. Nevertheless, enteric glial cells (EGC) ensheath the neuronal cell bodies within the ganglia as well as the interganglionic processes, providing support to the neuron somas and nerve bundles, as no connective tissue is found in the ENS [122]. The study of intestinal glial functions beyond those related to structural support was motivated by

the similarities observed between EGCs and CNS astrocytes in morphology, specific marker expression and interactions with neighbouring cell types [122]. Actually, EGCs are considered the counterparts of the CNS astrocytes in the ENS. From subsequent investigations, it is currently known that EGCs contain neurotransmitter precursors, uptake and degrade neuroligands and have trophic effects on neurons by secreting neurotrophins [118, 121, 122]. However, and most importantly, EGCs participate in the GI homeostasis through maintenance of the integrity of the epithelial barrier and the endothelium [123, 124], as CNS astrocytes do by controlling the blood-brain barrier [125]. Indeed, EGCs are found in close contact with IECs and endothelial cells [118, 126], and are thus able to modulate their functions through paracrine release of substances [127]. Involvement of the EGCs in IBD pathogenesis was first proposed by Geboes and colleagues, who described MHC class II expression in EGCs from the submucous and myenteric plexuses of CD patients [128]. This possibility has been also supported by emerging evidence that demonstrates IL-6 expression in EGCs [129], thus positioning this cell type as non-professional APCs in the innate immune response.

Besides neuronal and EGC inputs, the ENS activity is also conditioned by neighbouring cells such as myofibroblasts, mast cells and resident macrophages. Among these cells, muscularis macrophages have been thoroughly studied because of their involvement in motility disorders such as postoperative ileus [130]. These cells are embedded within the longitudinal muscular layer [131, 132], express TLRs and can respond to MAMPs as well as DAMPs [130]. Upon recognition of such substances, muscularis macrophages trigger pro-inflammatory responses characterised by secretion of TNF-α, IL-6, IL-1β, monocyte chemoattractant protein (MCP)-1, inducible nitric oxide synthase (iNOS) and COX-2 [130, 133], which might alter neuronal and muscular sensitivity to electric stimuli, leading to impaired contractility [134, 135]. Thence, the role of these cells during homeostasis and inflammation must also be taken into consideration when studying the ENS.

3.2) ENS, innate and adaptive immunity

As already stated, the innate immune system relies on constitutive and inducible mechanisms to control microbial penetration into host tissues. In the GI tract, the epithelial barrier represents the main constitutive mechanism to avoid microorganism invasion, whereas dendritic cells, resident macrophages and the gut-associated lymphoid tissue lead the innate response towards invasion. In this context, several studies demonstrate that the ENS modulates the correct function of both types of mechanisms, and might therefore be considered as a part of the innate immune system.

The role of the ENS, and specifically EGCs, in maintaining the integrity of the epithelial barrier was shown in an experimental animal model of glial ablation [123]. Selective glial destruction resulted in patchy lesions of severe inflammation and haemorrhage involving different segments of the intestine. Interestingly, initiation of these pathological changes was characterised by epithelial lesions and increased intestinal permeability, as well as dilation of submucosal capillaries [123, 127]. This model, which shows similarities with some animal models of IBD, was subsequently used to demonstrate that EGCs produce substances like S-nitrosoglutathione (GSNO), which modulate TJs, increasing the transepithelial resistance and reducing the permeability of the epithelial barrier [127]. Similarly, other studies have shown that TJs can also be regulated through release of neurotransmitters like VIP, pointing out that neurons are also implicated in the control of intestinal permeability [136]. In addition to keeping the epithelial barrier function, the ENS might also participate in controlling bacterial overgrowth through the secretion of ions and modulation of motility, which contribute to clearing high bacterial burdens [137].

EGCs have been also implicated in preservation of the vascular integrity. In a different animal model of targeted glial ablation using an autoimmune approach, the destruction of the glial fibrillary acidic protein (GFAP)-expressing cells (that is, EGCs) led to extensive submucosal oedema and vascular inflammation that spread disrupting the intestinal mucosa, eventually developing fulminant enterocolitis and haemorrhagic necrosis [124]. Taken together, these findings strongly suggest the participation of the ENS in the proper performance of the GI constitutive innate immunity through two basic

mechanisms: controlling bacterial overload and preserving the integrity of the barriers that separate the high antigenic burdens dwelling the gut lumen from the recruited professional immune cells.

Modulation of the immune response is usually driven by cell-to-cell interactions and the cytokines released to the microenvironment. In the context of the GI tract, resident cells of the immune system might also be influenced by the cytokines secreted by non-immune cells. In this regard, some studies have shown EGCs produce IL-1β, IL-6 and TNF-α in response to challenges with pro-inflammatory cytokines and bacteria [129, 138]. Thus, EGCs would be capable of shaping the innate and adaptive immune responses providing an immunostimulatory milieu through secretion of cytokines.

In parallel, it has been also described that neurons condition immune cells through neurotransmitter release. Neuronal control of immune cells can be achieved via mechanisms involving the participation of the autonomic nervous system or via local interaction with the ENS. The cholinergic anti-inflammatory pathway, described by the group of Tracey [139], represents perhaps the clearest example of how neurons are capable of controlling immune cell function. Upon TLRs stimulation, production of cytokines like IL-1 activates afferent signals in sensory neurons of the vagus nerve. The "Inflammatory Reflex", which involves interneurons in the CNS, is driven through the motor fibres of vagus nerve, which project axons in the splenic nerve. Stimulation of the splenic nerve leads to the release of epinephrine, which subsequently induces ACh liberation by a subset of T lymphocytes expressing β2-adrenoceptors [140]. Released ACh interacts with the α7 nicotinic ACh receptor (nAChR) expressed in macrophages, down-regulating the production of TNF-α, IL-1, IL-6 and IL-8 without altering the anti-inflammatory cytokines IL-10 and TGF-β (for review, [141]). This effect on α7nAChR-expressing cells could also occur in the GI mucosa, where they lie in close proximity to cholinergic neurons and fibres [142].

The secretion of the sympathetic nervous system neurotransmitter NE has been proposed as another pathway to modulate immune cell functions. Actually, NE has been shown to act via β2-adrenoceptors to reduce LPS-mediated activation of NF-κB *in vitro* [143], and seems to mediate the vagal "Inflammatory reflex" on splenocytes and T cell subsets *in vivo* [140]. In addition, the sympathetic innervation of lymphoid structures

such as Peyer's patches results interesting as it could mediate regulatory responses like those observed by Vida and colleagues in the spleen [140].

Conditioning effects that would not need extrinsic participation are those related with VIP, SP and nitric oxide (NO). VIP is a 28-amino-acid peptide primarily expressed in nerve fibres and ganglia along the gut. VIP mediates vascular and smooth muscle relaxation, absorption of water and ions and secretion by enterocytes [144]. Its receptors, VPAC1 and VPAC2, are expressed in several different cell types, including DCs. Immature DCs treated with VIP for 24 hours have been shown to induce Th2-type cytokine production in T CD4+ cells, increasing IL-4, IL-5 and IL-10 production and decreased IFN-γ [145]. In the same study, LPS-matured DCs concomitantly incubated with VIP showed reduced expression of the costimulatory molecules CD80/86, which induced anergic T cells that did not proliferate [145]. Subsequently, VIP-generated DCs were used to induce Treg cells [146], pointing out that such neurotransmitter has important roles in tolerance and modulation of inflammation. Conversely, the 11-amino-acid peptide SP, which is expressed in the ENS plexuses as well as in intrinsic and extrinsic sensory neurons, has been related to pro-inflammatory responses. Besides its effects in muscle contraction, vasodilatation and enteric secretion, interaction of SP with neurokinin (NK) receptors 1 and 2 stimulates the secretion of TNF-α, IL-1β, IL-2 and IL-6 in macrophages. In addition, SP also induces the release of chemokines by leukocytes and mast cell degranulation (for reviews, [147, 148]). NO is a gaseous mediator that has a wide variety of functions ranging from chemical stress - through generation of reactive nitrogen oxide species - to immunomodulation (for review, [149]). NO donors have well known anti-inflammatory properties in macrophages and DCs through inhibition of the NF-κB pathway [150]. Activation of the soluble guanylyl cyclase, which is a downstream signalling target enzyme, elicits TGF-β production [151], which might additionally inhibit pro-inflammatory responses. Furthermore, in the nervous tissue and in physiologic conditions, NO is synthesised in neurons by the constitutive neuronal isoform of the NO synthases (nNOS). Such isoform has been shown to regulate NF-κB activation, as well as induction of the iNOS, thereby playing modulatory roles [152, 153]. In contrast, studies in iNOS chimeras demonstrate that this isoform enhances the inflammatory response, especially when expressed in non-hematopoietic cells [154]. Therefore, over-expression of iNOS in EGCs during inflammation [155] may lead to

production of NO amounts that might result in opposite effects, enhancing the immune responses.

Finally, and independently of neurotransmitter mediation, neurons themselves can secrete chemokines like IL-8 when incubated with toxin B of *Clostridium difficile* or under IL-1β stimulation, which might result in chemoattraction of immune cells to challenged areas [156].

3.3) ENS in inflammation and IBD

Taking into consideration the relationships described between the ENS and the immune system, the involvement of the former in inflammatory disorders such as IBD is expected. Indeed, there is abundant literature supporting the idea that the ENS is not only a bystander but plays an active role during inflammation. However, as a result of the damage induced by inflammatory processes, the ENS undergoes plastic changes affecting the structural, functional or chemical phenotype of neurons. These plastic changes ultimately impair important GI functions like motility and secretion, as observed in IBD patients [157].

An active participation of the ENS in inflammation was initially suggested since both neurons and EGCs acquire activated phenotypes after exposure to cytokines and other inflammatory mediators. In this respect, the group of Sharkey demonstrated that incubation of guinea-pig ileum samples with IL-1β or PGE2 increases expression of c-Fos, a transcription factor necessary for the formation of AP-1, in neurons of the submucosal ganglia. Of note, double-labelling immunohistochemistry revealed that such activated neurons were NOS and/or VIP containing neurons [158, 159]. Both types of neurotransmitter have been found altered in IBD [160]. In the same vein, IL-8 expression in neurons and EGCs from human submucosal ganglia has been reported after IL-1β challenge [156], and EGCs challenged with LPS and/or cytokines do proliferate and increase expression of IL-6, NO and MHC class II [129, 161]. Indeed, MHC class II expression is observed in EGCs of inflamed and uninflamed areas of IBD specimens [128]. MHC class II immunoreactivity of EGCs is positively correlated with the presence of inflammatory infiltrate around the plexuses and nerve fibres, indicating that these cells have an important role in inflammation and perhaps in spreading it throughout the gut [160].

Other evidence concerning the involvement of the ENS in the immune response emerges from animal model studies. Perhaps the clearest demonstration of this fact is that reported by Margolis and colleagues, who have shown that enteric neuronal density determines the severity of the inflammatory response [162]. In their study, they used neuron specific enolase (NSE)-noggin mice, which have 150% of enteric neurons, and *Hand2*$^{+/-}$ mice, which have about 59% of enteric neurons compared to their littermates. Chemical colitis was induced with TNBS and DSS and, in both cases, intensity of colitis was found to be significantly greater in NSE-noggin mice, and significantly milder in *Hand2*+/- animals, than the colitis developed in their wild type littermates [162]. Such observations could be related to enhanced production of SP or its receptor NK-1, as both situations have been reported in IBD patient specimens [160]. Indeed, administration of NK-1 receptor antagonists has proven to be useful in ameliorating colitis [163]. Thus, the cited works support the idea that the effect of enteric neurons is probably pro-inflammatory. However, an immunomodulatory role for EGCs cannot be ruled out, since they secrete neurotrophins when incubated with pro-inflammatory stimuli [164], and neurotrophin blockade has been proven to worsen experimental colitis [165]. In addition, the ENS might be involved in the cholinergic anti-inflammatory pathway, by mediating its effects locally [118]. Therefore, since the vagal-mediated effects have been demonstrated in experimental models of colitis and post-operative ileus [166, 167], a possible anti-inflammatory tone should not be discarded.

Besides the active participation of the ENS in the inflammatory response, there is also an intrinsic adaptive response to the harmful microenvironment generated in these processes. Enteric neurons are very plastic, what is thought to contribute to the preservation of the ENS structures even when the architecture of the intestine is severely altered, like happens in IBD [168]. GI samples of IBD patients often display structural abnormalities such as hypertrophy and/or hyperplasia of neurons, whereas hyperplastic to hypoplastic changes have been described in EGC populations [124, 160]. Hypertrophy and thickened nerve fibres are more frequent in CD, and are associated to axonal damage and necrosis [160]. Hyperplasia of neuronal cell bodies is a common finding in CD and UC patients [160, 162]. Along with these morphologic alterations, phenotypic changes in the neurochemical coding of neurons have been also reported. The number of SP-positive myenteric neurons has been found increased up to three-

fold in tissue specimens of UC patients, in both inflamed and non-inflamed segments. Of note, many of these neurons colocalised with cholinergic neurons [169]. Neuronal NOS-positive myenteric ganglia and nerve fibres have been also described to display higher immunoreactivity in whole-mount preparations of small intestine of CD patients when compared to controls [170]. Concerning VIP, an increase or decrease of VIP immunoreactivity has been reported depending on the methodology used, but in all cases it seems that the VIP innervation pattern is altered [160, 170, 171]. Taken together, all these abnormalities in the neurotransmission are probably involved in the impaired motility and increased nociception observed in IBD patients.

Some of the observations summarised have led some researchers to hypothesise that the neural tissue may play a role not only in active inflammation, but also in predisposition to develop IBD [160]. First, although neuronal hyperplasia is a common feature of CD and UC, neurons are post-mitotic cells and no evidence supports that cytokines induce enteric neurogenesis. Thus, it has been proposed (and shown in animal models) that an excess of neuronal tissue could predispose to IBD [162]. Other evidence supporting this observation is that some IBD-associated susceptibility genes are neuronal genes [162]. Second, perineural inflammation is often very dense, and expression of MHC class II by EGCs is positively correlated with the leukocyte infiltrate, especially with CD8+ T lymphocytes [128]. Precisely CD8+ T lymphocytes are the effector cells mediating the inflammation observed in the model of Cornet and colleagues, where they target a viral protein expressed in EGCs [124]. Third, inflammatory lesions and neurochemical phenotypic changes are usually found in affected and unaffected areas of inflammation. This damage pattern might indicate a role for ENS cells in the propagation of, at least, CD [121, 160]. Fourth and finally, CD biopsies show reduced EGC network in involved and non-involved specimens when compared to control biopsies [124]. Defective EGC function has been associated with increased permeability of the epithelial barrier and subsequent inflammation in experimental animal models [123, 124], and alterations in permeability are frequently observed in IBD patients as well as in their first-degree relatives [24]. Therefore, the ENS should be taken into account when designing future studies addressing not only disturbances in motility and nociception, but also the pathogenesis of IBD and the treatment of the inflammatory response.

3.4) ENS and TLRs

The study of the expression and function of TLRs in the ENS has been motivated by the description of these receptors in different cells of the CNS. Microglia, astrocytes, oligodendroglia and even neurons have been shown to express such receptors [172, 173]. In the CNS, these receptors have been shown to mediate the innate immune response to infection, and are also related to autoimmunity, neurodegeneration, apoptosis and tissue damage responses [174].

Since EGCs are of neuroectodermal origin, they share many morphologic and functional similarities to CNS astrocytes, but not microglia [122]. Given that several different studies have described expression of TLRs in astrocytes [175-177], it is reasonable to hypothesise that EGCs might express these receptors too, as these cells are probably exposed to bacterial ligands when localised in the subepithelial compartment [126]. Subsequently, some groups have demonstrated expression of TLR3, TLR4 and TLR7 in the myenteric plexus of the ENS colocalising not only with EGCs, but also with neurons [178, 179]. However, data about the putative functions of TLRs in the ENS are scarce. Recent work by Anitha and colleagues has shown that TLR4 signalling is necessary to promote survival of intestinal nitrergic neurons in ENS primary culture and mice [180], supporting the anti-apoptotic role observed in previous studies with CNS neurons [181]. Nevertheless, the putative role of these receptors in mediating the neuro-immune interactions observed in IBD has not been addressed up-to-date.

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Hypotheses and objectives

In the immunologically unique environment existing in the GI tract, preservation of immune tolerance to resident microbiota is fundamental to maintain homeostasis. Since cross-talk between microbes and host's cells is mediated by TLRs, dysregulation of such receptors might be involved in the pathogenesis mechanisms triggering IBDs. TLR2 and TLR4, two of the most studied TLRs in the gut, are increased during inflammation in infiltrating immune cells. TLR2 activation in immunocytes has been associated to both pro-inflammatory and immunomodulatory responses, whereas stimulation of TLR4 is mainly associated to pro-inflammatory responses. Modulation of the activity of both receptors is possible through administration of their ligands, as their expression and functions show cross-regulation phenomena, hyporesponsiveness and tolerance in particular situations. These properties make these receptors an interesting target to modulate deregulated immune responses.

The ENS plays crucial functions in maintaining the gut homeostasis by innervating several different cell types. During inflammation, it has been classically considered a plastic bystander, but emerging data suggest an active role for this structure in inflammation. Indeed, EGCs display activated phenotypes during inflammation and express MHC class II in IBD specimens. Initial immunolocalisation studies point out that of TLR2, TLR4 and TLR9 are expressed in the intrinsic plexuses of the ENS. In the CNS, these receptors are preferentially expressed in microglia and astrocytes, and participate in innate immune responses to infection, autoimmunity, apoptosis and tissue repair. Their roles in the ENS are still to be elucidated.

Hypotheses

On the basis of this schematic background, this work was aimed to our studies were conceived to corroborate the following hypotheses:

1) Challenging TLR2 through local administration of its ligand zymosan might down-regulate expression of this receptor and trigger immunomodulatory responses in immunocytes, leading to attenuation of inflammation.

2) Stimulation of TLR4 through intracolonic administration of its ligand LPS might provoke subsequent down-regulation of this receptor, reducing severity of colitis.

3) TLRs expressed in the ENS, and especially in EGCs, confer this tissue the capability to respond to invading microorganisms, inducing the release of soluble factors that chemoattract immune cells and prime their subsequent responses to MAMPs.

Objectives

In order to accept or reject these hypotheses, we intended to accomplish the following objectives:

1) To characterise TLR2 expression during homeostasis and following induction of experimental inflammation by means of DSS administration.

2) To establish a pro-inflammatory dose of zymosan that causes inflammation in naïve mice after intracolonic administration. In parallel, to find a sub-inflammatory dose of zymosan that induces cross-responsive and/or tolerogenic responses in naïve mice.

3) To determine the effects of two additional higher doses of zymosan or LPS (200 and 500 µg of each) given intracolonically and in two alternate days to naïve or DSS-treated mice. To evaluate the expression of TLR2 and TLR4 after challenge, as well as other inflammatory mediators, for establishing the mechanisms linking TLR stimulation with colitis amelioration.

4) To study the expression and distribution of TLR2, TLR4 and TLR9 in the ENS during homeostasis, and to assess their functionality in terms of NF-κB activation and inflammatory mediator production in ENS and EGC culture models. To investigate possible interactive and cross-regulation responses.

5) To analyse the involvement of the TLR-induced ENS phenotypes in chemoattraction of immunocytes and priming of their subsequent responses in terms of cytokine production.

Results

Chapter 1

Zymosan intracolonic administration does not induce colitis but enhances the sub-inflammatory effects of ethanol

Abstract

Toll-like receptors (TLR) are involved the preservation of tolerance to commensal microflora, but some evidence indicate a role for these receptors in the onset of inflammation. TLR2 activation, through its ligand zymosan, has pro- and anti-inflammatory effects. Our aims were 1) to investigate whether single or repeated intracolonic administration of ethanol and zymosan can exert effects on the immune response *in vivo*, and 2) to characterise TLR2 expression during dextran-sodium sulphate (DSS)-induced inflammation. Single instillation of 30% ethanol (Et30) and zymosan (Et30+Zym) induced transient acute inflammatory effects, including interleukin (IL)-1β and IL-10 up-regulation in colon tissue at 3 hours post-administration, and increased plasma serum amyloid A levels at 24 hours. Such effects disappeared faster in Et30+Zym- than in Et30-treated mice, suggesting that zymosan may induce a tolerogenic response. No effects were observed 7 days after instillation. Repeated intracolonic administration at days 0, 3 and 6 caused multi-focal areas of inflammation, shortening of colon length and IL-1β up-regulation that were more consistent in Et30+Zym100- than in Et30-treated groups. On the other hand, DSS-induced colitis was characterised by significant up-regulation of TLR2 that was mainly located in remaining crypts and infiltrating cells in *lamina propria* and submucosa. Results of this work suggest that zymosan could trigger both pro- or anti-inflammatory effects provided that epithelial barrier function is altered by other events, and that TLR2 is a dynamic receptor which can be targeted to ameliorate outcomes of inflammation.

Keywords

Toll-like receptor, zymosan, experimental colitis, inflammation

Introduction

The gastrointestinal (GI) tract mucosa represents the largest surface of the organism exposed to the external milieu. The GI tract harbours a large number of microbes which has been estimated in as much as 100 trillion, most of them residing in the distal parts of the bowel, especially in the colon [1]. In such microenvironment, the existence of defence mechanisms that promote tolerance to the commensal microflora while triggering an adequate immune response to pathogenic microorganisms is essential [2]. Several mechanisms are involved in avoiding penetration of commensal microbiota into the host tissues. These include the secretion of mucus and antimicrobial substances, the strengthening of the epithelial barrier through apical junctional complex proteins or the immune surveillance by antigen presenting cells (APC) [3]. All these mechanisms are highly dynamic and must be able to sense and respond fast to changes in luminal microbial burdens. Therefore, they must be modulated by receptors allowing for an early recognition of microbial-associated molecular patterns (MAMP) while additionally orchestrating the subsequent suitable responses depending on the cell type [4]. Among these receptors, the Toll-like receptor (TLR) family has been thoroughly studied in the last decade since TLRs have been proposed to mediate the cross-talk between microorganisms and the host cells [5].

TLRs are transmembrane proteins that recognise highly conserved molecular structures in microbes and trigger different signalling pathways to finally induce the secretion of inflammatory mediators [4, 6, 7]. In the GI tract, TLRs are widely expressed and have been identified in the four intestinal epithelial cell lineages [8] and APCs [9, 10]. Recognition of commensal microflora through these receptors is crucial for the correct maturation of goblet cells [11], the secretion of antimicrobial peptides [12] and the increase of epithelial resistance to paracellular permeability [13]. In addition, TLRs can induce tolerogenic effects in dendritic cells [14, 15]. Thus, apparently TLRs contribute to the permissive phenotype exhibited towards resident microflora. However, different evidences also point out that TLRs might be directly involved in the onset of inflammation. First, it is well established that experimental inflammation depends on the presence of non-pathogenic bacteria, as transgenic animals reared in germ-free conditions do not develop spontaneous colitis [16]. Second, intact functionality of TLRs is necessary for the development of spontaneous colitis in interleukin (IL)-$10^{-/-}$ mice [17]. Third, MAMPs might

act as mediators of inflammation: lipopolysaccharide (LPS) intracolonic administration induces ileitis [18], flagellin exacerbates the signs of experimental colitis [19] and increased MAMP concentrations are found in colonic content after induction of colitis with dextran-sodium sulphate (DSS) [20]. Finally, increased expression of TLRs has been reported in different experimental models of colitis [21-23] as well as in inflammatory bowel diseases (IBD) [9, 24]. Hence, although TLR-mediated recognition is necessary for preserving homeostasis, their excessive or uncontrolled signalling may contribute to an inflammatory process.

TLR2, particularly, has been both related to the development of proinflammatory and immunomodulatory responses [14, 15, 23, 25]. It recognises a number of ligands, including lipoproteins, peptidoglycans and zymosan [25, 26]. Zymosan is a carbohydrate from the cell wall of yeasts that is recognised by TLR2 in cooperation with TLR6 and dectin-1 [27]. Stimulation with zymosan particles induces secretion of the chemokine IL-8 in epithelial cells [28], and production of TNF-α in macrophages [25]. Consequently, it has been used as an inflammagen for experimental models of peritonitis, since its local administration induces recruitment of leukocytes and production of cytokines and chemokines [29], and so could be useful with the same purposes in the bowel. However, it has been also involved in generation of tolerance, through priming of T cells by IL-10- and transforming growth factor (TGF)-β-secreting cells [15].

The aims of this study were: 1) to determine whether single or multiple alternate zymosan intracolonic administrations are capable of triggering proinflammatory or immunomodulatory responses in healthy mice, and 2) to characterise TLR2 expression in murine colon samples. As zymosan particles trapped in the mucus layers would not be able to contact epithelial cells and immune cells in the *lamina propria*, we administered them in a mixture containing ethanol in order to disrupt the mucus layers. Such procedure is routinely done for the induction of colitis by intracolonic instillation of trinitrobenzenesulfonic acid (TNBS) [30]. Our results demonstrate that, at the concentrations studied, repeated intracolonic administrations of zymosan may potentiate the sub-inflammatory effects of ethanol to settle a moderate colitis with higher reproducibility than that caused by ethanol administration.

Materials and Methods

Animals

For in vivo experiments, 13 to 15-week old C57Bl/6 female mice were purchased from Charles River (Les Oncins, France) and housed in specific pathogen-free conditions, under a controlled temperature (20±2ºC) and photoperiod (12h/12h light-dark cycle), with free access to food and water.

All procedures performed were approved by the Ethical Committee of the Universitat Autònoma de Barcelona (CEEA-UAB 561).

Zymosan dosage trials

Zymosan was purchased from Invivogen (San Diego, USA). This ligand was chosen for intracolonic administration experiments because of its affordability compared to other TLR2 ligands. Zymosan was initially sterilised in 100% ethanol, and subsequently mixed with endotoxin-free water in order to achieve final ethanol concentrations in the mixture of 10 or 30%, depending on the experiment. Since zymosan is not water soluble, zymosan suspensions were always vortexed immediately before its use in order to warrant the administration of consistent doses to anesthetised animals.

Depending on the schedule of each trial, summarised in their corresponding figures, animals were anesthetised by intraperitoneal injection of a mixture of ketamine 100 mg/kg (Imalgène 1000®; Merial Laboratorios, Tarragona, Spain) and xylazine 20 mg/kg (Rompun 2%®; Bayer HealthCare, Kiel, Germany) in NaCl 0.9% solution (10 mL/kg). Each animal was administered with 100 µL of a saline solution containing ethanol 10 or 30%, zymosan 0.1 mg/mL or zymosan 1 mg/mL by means of a customized catheter which had an ovoid bead attached 2 cm from the distal end. The bead was intended to prevent the solution from being expelled from the colon. This system ensured for the administration of 10 or 100 µg of zymosan per mouse. Mice were kept anesthetized in Trendelemburg position for 60 to 90 minutes.

Colitis induction

In order to compare the inflammatory effects of the mixture of ethanol and zymosan, middle-distal colitis was induced by administration of 3% DSS (36-50 kDa; MP

Biomedicals, Illkirch, France) in drinking water for 5 consecutive days. DSS solution was freshly prepared and replaced every 2 days.

Blood sampling and serum amyloid A determination

In some of the zymosan trial experiments, small blood samples were collected at different time points to follow up DSS colitis and the zymosan-associated response. Around 40 µL of blood were obtained from each animal by submandibular vein puncture, as described elsewhere [31]. After centrifugation, the resulting plasmatic fraction was subsequently used for the ELISA determination of serum amyloid A (SAA) following manufacturer's instructions (Life Technologies, el Prat de Llobregat, Spain). SAA is a lipoprotein involved in the acute phase response in vertebrates that is secreted by the liver under inflammatory conditions. It is considered an early and sensitive biomarker for injury and inflammation, as it increases 100 to 1000-folds in plasma after these stimuli, and declines rapidly to basal levels after recovery [32].

Euthanasia and tissue collection

On the scheduled times, depending on the zymosan trial, mice were euthanized by cardiac puncture exsanguination under isoflurane (Isobavet®; Schering-Plough, Sant Cugat del Vallès, Spain) anaesthesia. Colons were removed and, after measuring their length, were rinsed in ice-cold phosphate buffered saline (PBS) and cut into longitudinal pieces, which were further fixed in 4% paraformaldehyde or kept in RNAlater solution (Ambion, Applied Biosystems, Alcobendas, Spain). In the first zymosan trial experiment, an additional longitudinal piece was flash-frozen in liquid nitrogen to evaluate its myeloperoxidase (MPO) activity.

Myeloperoxidase activity determination

Leukocyte infiltration into the colon was determined as previously described for use in a 96-well plate [33]. Briefly, 50-70 mg of tissue were homogenised in a 50 mM hexadecyltrimethyl ammonium bromide (Sigma, Madrid, Spain) solution (1 mL/50 mg wet tissue) and centrifuged to obtain supernatants. MPO activity was assayed in supernatants by mixing them with a 1 mg/mL *o*-dianisidine hydrochloride solution (Sigma). Changes in optical density at 450 nm were measured at 3-minutes intervals for

15 minutes and compared to standards kinetics. One unit of MPO activity was defined as the amount that degraded 1 µmol of peroxide per minute at 25 °C.

Disease activity index

Animals were controlled daily for individual weight loss, stool consistency, faecal blood and general aspect in order to build up a disease activity index (DAI) to follow up colitis, as described previously [34]. Criteria used for the DAI scoring are further detailed in Table 1. Endpoint criteria are also shown in Table 1, and were applied as described elsewhere[35].

<table>
<tr><th>Body weight change</th><th>Score</th><th>Stool consistency</th><th>Score</th></tr>
<tr><td>< 0 % (gain)</td><td>0</td><td>Normal</td><td>0</td></tr>
<tr><td>0 – 2.5 % (loss)</td><td>1</td><td>Pasty</td><td>1</td></tr>
<tr><td>2.5 – 5 % (loss)</td><td>2</td><td>Very pasty but not liquid</td><td>2</td></tr>
<tr><td>5 – 7.5 % (loss)</td><td>3</td><td>Liquid, but still with a pellet shape</td><td>3</td></tr>
<tr><td>> 7.5 % (loss)</td><td>4</td><td>Very liquid</td><td>4</td></tr>
<tr><th>Animal aspect</th><th>Score</th><th>Bloody faeces</th><th>Score</th></tr>
<tr><td>Normal</td><td>0</td><td rowspan="4">Semi-quantitative evaluation depending on the quantity of blood observed</td><td rowspan="4">0 - 3</td></tr>
<tr><td>Coarse hair</td><td>1</td></tr>
<tr><td>Coarse hair, abnormal activity</td><td>2</td></tr>
<tr><td>Obvious dehydration</td><td>3</td></tr>
<tr><th>Endpoint criteria</th><th>Score</th><th colspan="2">Endpoint criteria</th></tr>
<tr><td>Body weight</td><td>0 - 3</td><td colspan="2" rowspan="4">A score of 3 in two out of the three parameters analysed is taken as an endpoint criterion</td></tr>
<tr><td>Aspect</td><td>0 - 3</td></tr>
<tr><td rowspan="2">Behaviour: aggressive or comatose?</td><td>Yes = 3</td></tr>
<tr><td>No = 0</td></tr>
</table>

Table 1 – Parameters and scores related to DAI calculation and endpoint criteria execution.

Histologic score

Histologic assessment of colitis was performed by an investigator blinded to the study design. Haematoxylin & eosin stained sections from mid-distal colons of each animal were evaluated as previously described [36]. Five different areas were evaluated as follows: for inflammation: 0, none; 1, slight; 2, moderate; and 3, severe; for extent of inflammation: 0, none; 1, mucosa; 2, mucosa and submucosa; and 3, transmural; for crypt damage: 0, none; 1, basal 1/3 damaged; 2, basal 2/3 damaged; 3, only surface

epithelium intact; and 4, entire crypt and epithelium loss. An additional point was added to areas showing blood cells out of capillaries. Each of these values was multiplied for the corresponding percentage of involvement of the studied area: 1, 1-25%; 2, 26-50%; 3, 51-75%; and 4, 76-100%.

Immunohistochemistry

For immunohistochemistry, paraffin-embedded tissue sections were deparaffinized and treated in boiling citric acid during 15 minutes for antigen retrieval. Endogenous peroxidase activity was blocked with a 5% solution of hydrogen peroxide in PBS, and avidin and biotin with the Avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA, USA). Slides were then incubated for 1 hour in phosphate buffered saline containing 5% bovine serum albumin and 1% Triton X-100. Overnight incubation at 4°C with TLR2 antibody (Imgenex, San Diego, USA) was followed by addition of biotinylated secondary antibodies, which were further detected by Vectastain ABC kit combined with 3-3'-diaminobenzidine peroxidase substrate kit (both from Vector Laboratories). Slides were examined through a Nikon Eclipse 90i microscope interfaced to a DXM 1200F camera (Nikon Corporation, Barcelona. Spain). Pictures were taken using the ACT-1 software (Nikon Corporation).

Real-time RT-PCR analysis

Total RNA from colon samples embedded in RNAlater solution was extracted using the RNeasy Mini Kit (Qiagen, Las Matas, Spain), quantified by optical densitometry and assessed for integrity by on-chip gel electrophoresis with the Experion™ System (Bio-Rad Laboratories, el Prat de Llobregat, Spain). 1 μg of RNA was retro-transcribed by using the Transcriptor First-strand cDNA Synthesis Kit (Roche Applied Science) for reverse-transcriptase polymerase chain reaction (RT-PCR). Primer sequences listed in Table 2 were designed to span introns using the Universal ProbeLibrary Assay design Center. PCR amplifications were performed using the LC480 SYBRGreen I Mastermix (Roche Applied Science) according to manufacturer's protocol, and run on a LightCycler 480 II instrument (Roche Applied Science). Absence of coamplification products was assured by generating a final melting curve for each reaction. mRNA level of expression of the genes of interest was corrected to that of the housekeeping gene β-actin and calculated by the ΔΔCt method [37].

Gene	Sense primer	Antisense primer	Reference
B-actin	CTAAGGCCAACCGTGAAAAG	ACCAGAGGCATACAGGGACA	ENSMUST00000031564.4
TLR2	ACCGAAACCTCAGACAAAGC	CAGCGTTTGCTGAAGAGGA	NM_011905.3
TLR4	GGACTCTGATCATGGCACTG	CTGATCCATGCATTGGTAGGT	NM_021297.2
IL-1β	TGTAATGAAAGACGGCACACC	TCTTCTTTGGGTATTGCTTGG	ENSMUST00000028881.6
IL-10	CAGAGCCACATGCTCCTAGA	TGTCCAGCTGGTCCTTTGTT	ENSMUST00000016673.4

Table 2 – List of primers used for real-time RT-PCR analysis.

Statistical analysis

Results are presented as mean values ± S.E.M. All data were compared by one-way or two-way ANOVA for comparison of more than two groups, followed by Tukey's post hoc test (unless otherwise stated). Data analysis and plot were performed with GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, USA). A P value < 0.05 was considered to be significant.

Results

Single intracolonic administration of zymosan does not induce colitis

In the first set of zymosan schedule and dose trial experiments, treatments were performed once at day 0 and experimental groups were followed up for 7 days (Fig. 1A). Zymosan doses chosen represented 10- to 100-fold the necessary amount of the product to achieve stimulation of macrophages in culture, as indicated by manufacturer. To compare the possible inflammatory responses and study TLR2 distribution during inflammation, an additional group of mice was given oral DSS to induce well-characterised experimental colitis. The number of animals used in this first trial was n= 7-8.

Neither ethanol 30% (Et30) solution nor the different doses of zymosan used (Zym 10 or Zym 100, representing the micrograms of product instilled) induced clinical signs of colitis during the follow-up period, as shown in body weight evolution and DAI (Figs. 1B and C). Contrastingly, 3% administration in drinking water for 5 days caused significant body weight loss (Saline+DSS= 76.4±2% vs. Saline= 103.6±1.9% of initial body weight on day 7, n= 7-8, $P<0.001$; Fig. 1B), and pasty and bloody faeces, which increased the DAI (Saline+DSS= 7.77±0.9 arbitrary units (AU) vs. Saline= -0.71±0.4 AU on day 7, n= 8, $P<0.001$; Fig. 1C). Determination of plasma SAA levels confirmed that, although there seemed to be an initial small SAA increase by day 1 in the groups treated with Et30, this effect had nearly disappeared by day 2 (Fig. 1D). Conversely, animals treated with DSS showed strong increases in this acute phase protein as colitis developed (Saline+DSS= 1369±265.3 mg/mL vs. Saline= 5.19±1 mg/mL on day 7, n= 4, $P<0.001$; Fig. 1D). Post-mortem findings corroborated the lack of colitis hallmarks in zymosan-treated mice, as these animals neither showed shortened colon length (Fig. 1E), nor leukocyte infiltration (Figs. 1F and G). DSS-treated mice did show shortened colon length (Saline+DSS= 5.46±0.17 cm vs. Saline= 7.26±0.23 cm, n= 7-8, $P<0.001$; Fig. 1E) and increased leukocyte infiltration, as demonstrated by MPO activity = (Saline+DSS= 104.6±31.6 U/mg tissue vs. Saline= 0.0016±0 U/mg tissue, n= 3-5, $P<0.001$; Fig. 1F) and histologic examination (Saline+DSS= 14.25±3.03 AU vs. Saline= 0.375±0.24 AU, n= 4, $P<0.001$; Fig. 1G).

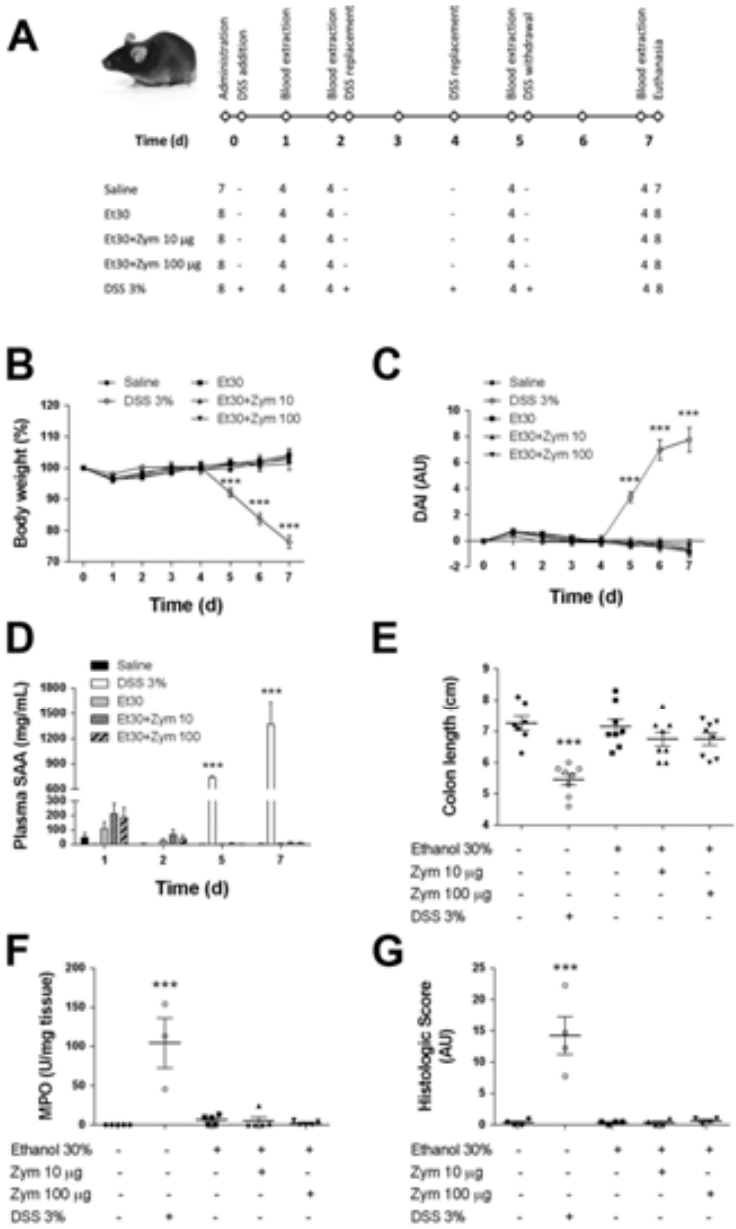

Figure 1 – Zymosan single intracolonic instillation compared to DSS-induced colitis. A) Trial schedule, experimental groups and number of animals used in each procedure. **B)** Changes in body weight percentage during the whole experiment (n= 7-8; ***P<0.001). **C)** DAI in arbitrary units (AU) (n= 7-8; ***P<0.001). **D)** Plasma SAA levels at the selected time points (n= 4; ***P<0.001). **E)** Colon shortening elicited by selected treatments (n= 7-8; ***P<0.001). **F)** MPO activity of colonic samples (n= 3-5; ***P<0.001). **G)** Histologic score of the haematoxylin & eosin preparations (n= 4; ***P<0.001).

TLR2 expression and distribution is altered by DSS colitis, but not by zymosan intracolonic instillation

Colonic expression of mRNA for TLR2, TLR4, IL-1β and IL-10 was studied to confirm the absence of inflammatory changes. Quantitative PCR assays revealed significant up-regulation of TLR2 in the DSS-treated group (Saline+DSS= 3.55±0.77 vs. Saline= 1.11±0.25 folds, n= 3-5, *P*<0.01; Fig. 2A), IL-1β (Saline+DSS= 30.47±7.7 vs. Saline= 1.12±0.27 folds, n= 3-5, *P*<0.001; Fig. 2C) and IL-10 (Saline+DSS= 2.61±0.19 vs. Saline= 1.14±0.31 folds, n= 3-5, *P*<0.05; Fig. 2D). However, no differences were observed for Et30 or zymosan-treated groups (Figs. 2A-D), further indicating that zymosan is not able to induce neither inflammatory nor immunomodulatory responses in normal healthy individuals.

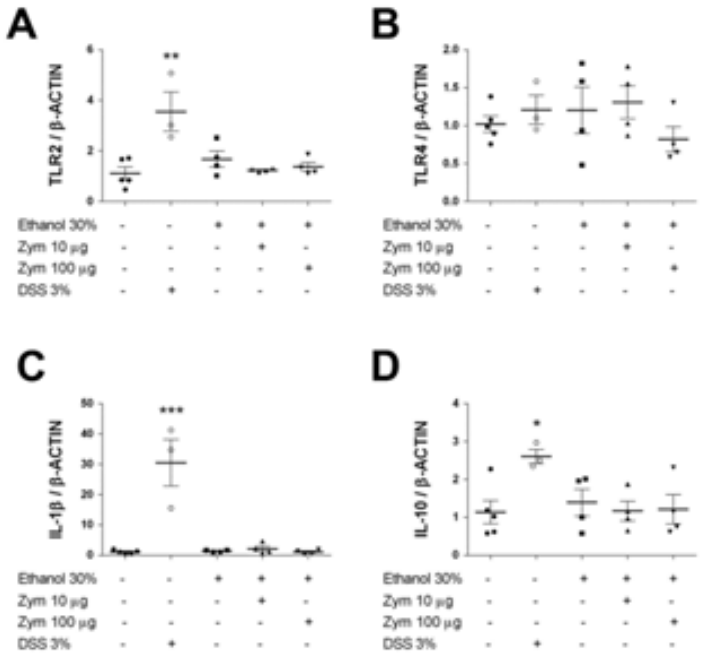

Figure 2 – Gene expression profile induced by zymosan single intracolonic instillation vs. DSS colitis. A) TLR2 mRNA expression (n= 3-5; ***P*<0.01). **B)** TLR4 mRNA expression (n= 3-5). **C)** IL-1β mRNA expression (n= 3-5; ****P*<0.001). **D)** IL-10 mRNA expression (n= 3-5; **P*<0.05).

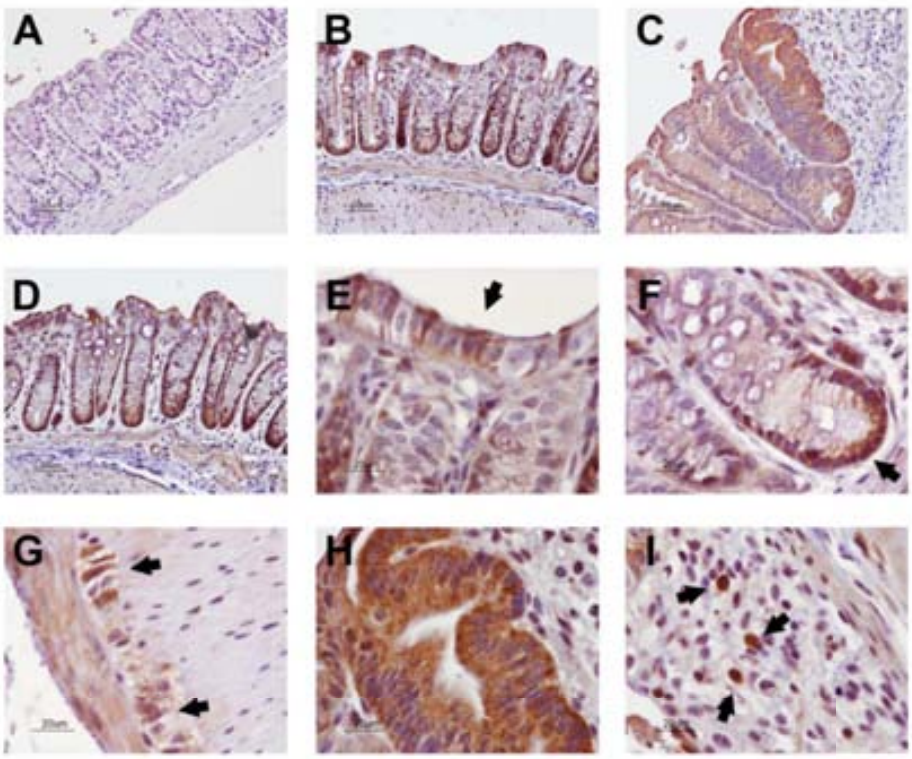

Figure 3 – TLR2 protein expression in control, DSS-treated and zymosan-treated mice. Micrographs demonstrating TLR2 immunoreactivity in **(A)** blocking peptide-incubated control sample (200x), **(B)** control sample (200x), **(C)** DSS-treated mouse slide (200x) and **(D)** Et30+Zym100-treated mouse preparation (200x). Increased magnification photographs showing in detail how TLR2 is expressed in normal conditions in **(E)** the crypt apical surface cells, as indicated by the arrow (600x), **(F)** the apical pole of colonocytes in the bottom of the crypt (600x), **(G)** the muscle layers and the cells in the enteric nervous system, as shown by arrows (600x). Increased magnification micrographs displaying **(H)** increased reactivity of this receptor in the basolateral and apical sides of all the epithelial cells along the crypt (600x) and **(I)** increased presence of positive immune cells infiltrating the *lamina propria* (600x).

In order to deepen into possible changes in location of TLR2, immunohistochemistry was performed. The specificity of this technique was validated by parallel incubation of the antibody with its blocking peptide (Fig. 3A). In saline-treated mice, TLR2 immunoreactivity was found mainly in epithelial cells from the lower portion of the crypt, and this staining disappeared as cells progressed to the crypt luminal surface (Fig. 3B and F), although some cells in this location also showed intense reactivity (Fig. 3E, arrow). The subcellular distribution was cytoplasmic, especially concentrating in the

apical side of the cells (Fig. 3E and F). Eventually, discrete cells from the *lamina propria* were also stained in the cytoplasm. Muscular layers and the myenteric plexuses showed also strong expression of this receptor in cellular somas (Fig. 3G, arrows). Et30 and Et30+Zym-treated animals did not show differences in the distribution pattern when compared to control saline-treated animals (representative picture in Fig. 3D). However, in DSS-treated colon sections, colonocyte expression of TLR2 was clearly increased in the whole crypt, from the lower to the upper portions, especially in hyperplastic crypts (Fig. 3C and H). In the same regard, the number of TLR2-positive cells in the *lamina propria* and submucosa was increased (Fig. 3I, arrows).

Ethanol administration induces short-term inflammatory changes that may be enhanced by zymosan

As already stated, plasmatic SAA levels were increased 24 hours after administration of the groups bearing ethanol 30% as vehicle when compared to saline controls (Fig. 1D). Therefore, in a second set of experiments, we sought to characterise this slight acute-phase response and to determine whether it could be due to short-term changes in TLR2 expression.

Animals received a single intracolonic administration and 3 or 24 hours later were sacrificed to assess plasmatic SAA concentration and colonic mRNA expression (Fig. 4A). SAA was increased only in some animals 3 hours post-administration, but this increase was statistically significant in all animals 24 hours post-treatment (Et30= 305±21.2, Et30+Zym10= 288.8±9 and Et30+Zym100= 283.6±10.3 mg/mL vs. Saline= 27.28±11.2 mg/mL, n= 3, $P<0.001$; Fig. 4B). This 10-fold raise was indicative of a sub-inflammatory process, since the values observed in DSS colitis are comparatively higher (Fig. 1D). However, the similar values obtained for these groups point out that such response was due to the effect of ethanol, rather than to that zymosan (Fig. 4B). Measurement of colon length showed no changes (Fig. 4C), as colon shortening takes place once inflammation and oedema are established and may thus take longer to appear. Analysis of TLR2 by quantitative PCR revealed time-dependent effects for the expression of this receptor in zymosan treated animals: transcript levels, which at 3 hours were slightly increased in comparison to controls, were significantly decreased after 24 hours (Et30+Zym10 3h= 1.5±0.27 folds vs. Et30+Zym10 24h= 0.72±0.11 folds, and Et30+Zym100 3h= 1.43±0.03 folds vs. Et30+Zym100 24h= 0.54±0.11 folds, n= 2-3,

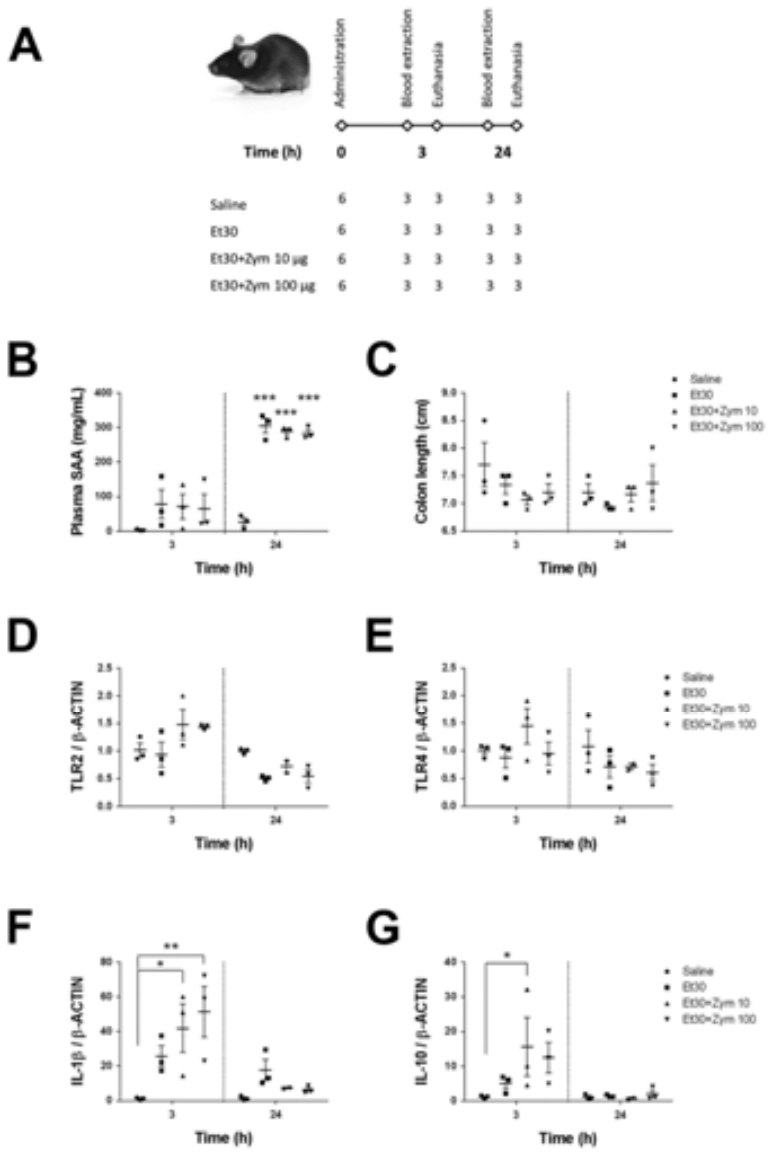

Figure 4 – Ethanol and zymosan-induced transient changes after single intracolonic instillation. A) Trial schedule, experimental groups and number of animals used in each procedure. **B)** Plasma SAA levels at the selected time points (n= 3; ****P*<0.001). **C)** Colon length at the end of experimental manipulations (n= 3). **D)** TLR2 transcripts at 3 and 24 hours (time-dependent effects were seen for Et30+Zym10 3h vs. 24h, and Et30+Zym100 3h vs. 24h, n= 2-3, *P*<0.05 and *P*<0.01, respectively). **E)** TLR4 mRNA expression (n= 2-3). **F)** IL-1β expression (n= 2-3; **P*<0.05 and ***P*<0.01; time-dependent effects were observed for Et30+Zym10 3h vs. 24h, and Et30+Zym100 3h vs. 24h, *P*= 0.064 and *P*<0.01, respectively). **G)** IL-10 transcripts (n= 2-3; **P*<0.05; time-dependent effects were observed with *P*<0.05). **D-G)** Values from a mouse from the group Et30+Zym10 24h were not included in studies due to poor RNA quality.

P<0.05 and *P*<0.01 respectively; Fig. 4D). Interesting results were also observed for cytokine expression. IL-1β transcripts at 3 hours were significantly increased when compared to saline-treated controls only when zymosan was added to ethanol (Et30+Zym10= 41.9±14 and Et30+Zym100= 51.4±14.7 folds vs. Saline= 1.23±0.44 folds, n= 3, *P*<0.05 and *P*<0.01 respectively; Fig. 4F). In addition, there was also a time-dependent decrease, since zymosan-treated mice showed faster decrease in mRNA levels from 3 to 24 hours when compared to ethanol-treated groups (Et30+Zym10 3h= 41.9±14 folds vs. Et30+Zym10 24h= 7.5±0.4 folds, and Et30+Zym100 3h= 51.4±14.7 folds vs. Et30+Zym100 24h= 6.3±1.2 folds, n= 2-3, *P*= 0.064 and *P*<0.01 respectively, Fig. 4F). Finally, IL-10 was only significantly increased at 3 hours in mice treated with the lowest dose of zymosan (Et30+Zym10= 15.6±8.4 folds vs. Saline= 1.13±0.3 folds, n= 3, *P*<0.05; Fig. 4G), although a 12.5-fold increase was also observed in the animals receiving the highest dose. A time-dependent significant decrease was also found (n= 2-3, *P*<0.05; Fig. 4G).

Taken together, these results indicate that after intracolonic instillations, animals treated with ethanol show short-term transcriptional changes in colonic tissue, demonstrating a transient inflammatory response that seems to be enhanced by the presence of zymosan. This early response is still not observed systemically in all animals at 3 hours, as SAA is not significantly increased. However, such sub-inflammatory state is locally resolved around 24 hours after administration, whereas systemic concentrations of SAA take a little more time to decrease to basal levels (Fig. 1D). Nevertheless, significant time-dependent effects concerning down-regulation of TLR2, IL-1β and IL-10 (Fig. 4D, F and G) observed in zymosan-treated groups might prompt an underlying tolerogenic effect induced by this TLR2 ligand.

Repeated administration of ethanol induces subclinical inflammation that might be potentiated by zymosan

Taking into account that the sub-inflammatory effects observed after a single administration of ethanol and zymosan were transient, we finally designed an experimental trial consisting of 3 different administrations separated in time. Animals were intracolonically treated on days 0, 3 and 6, and sacrificed on day 7 (Fig. 5A), and inflammation was assessed.

Analysis of the body weight percentage curves demonstrated that ethanol-treated mice evolved in a different way than the rest of groups, as they showed moderate decreases in body weight at different time points (Fig. 5B). Differences observed with zymosan-treated groups at days 2, 4 and 5 may suggest that simultaneous intracolonic administration of zymosan is beneficial for these animals (Fig. 5B). However, after the last administration performed at day 6, these animals stabilised to control levels, while those from the group treated with 100 µg of zymosan showed a significant weight decrease (Et30+Zym100 6d= 101±0.95 % vs. Et30+Zym100 7d= 90.1±1.1 %, n= 3, *P*<0.01; Fig. 5B) that was also different from that observed in the rest of groups (Et30+Zym100 7d= 90.1±1.1 % vs. Saline 7d= 98.8±0.2, Et30 7d= 96.6±0.6 and Et30Zym10 7d= 96.9±1.6 %, n= 3, *P*<0.01, *P*= 0.07 and *P*= 0.053 respectively). This marked weight loss was accompanied by a tendency towards colon shortening (Et30+Zym100= 6.4±0.09 cm vs. Saline= 7.2±0.3 cm, n= 3, *P*= 0.09). However, no clinical signs of colitis were found, and therefore the DAI was only dependent on weight loss (data not shown).

Concerning gene expression profiles, TLR2 mRNA levels remained unchanged (Fig. 5D), while TLR4 was significantly decreased in all groups treated with ethanol (Et30= 0.38±0.1, Et30+Zym10= 0.35±0.07 and Et30+Zym100= 0.46±0.07 vs. Saline= 1.01±0.09, n= 3, P<0.01; Fig. 5E). Transcripts of the inflammatory cytokine IL-1β were inconstantly increased in ethanol and zymosan 10-treated groups, as one of each three animals had similar values to controls (Fig. 5F). Colon tissue from zymosan 100-treated mice did show steady increases in this interleukin, but statistic significances could not be appreciated as big differences were observed between sample values in ethanol-treated mice (Fig. 5F). IL-10 did not show remarkable differences in expression compared to controls (Fig. 5G).

Taken together the results obtained for weight loss, colon shortening, TLR4 and IL-1β mRNA, it is reasonable to infer that ethanol might have sub-inflammatory effects that are potentiated by zymosan to promote moderate colitis. Actually, histopathologic evaluation of colons also supported this hypothesis. Mice treated with the highest dose of zymosan showed a significant increase in inflammatory lesions when compared to saline-treated controls (Et30+Zym100= 7.8±1.2 AU vs. Saline= 2.07±0.07 AU, n= 3, *P*<0.05; Fig. 6B), while other ethanol-treated groups did not. Indeed, mice treated with

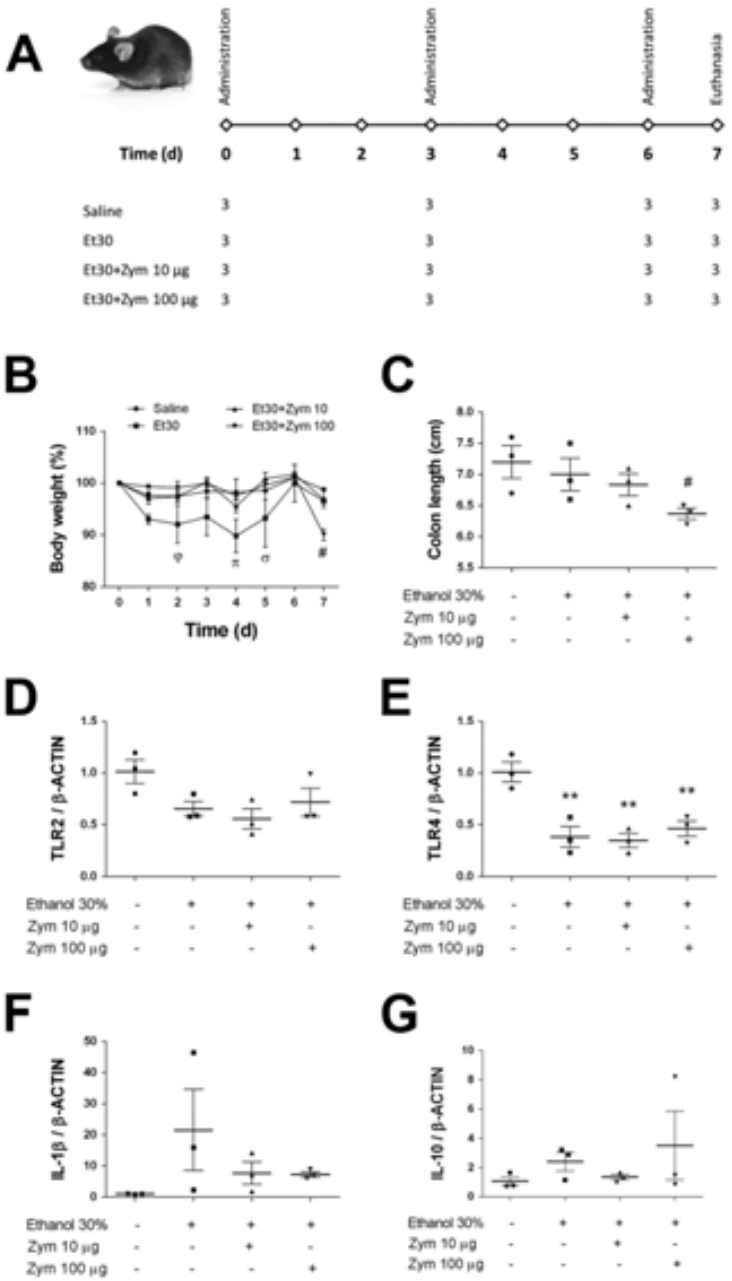

Figure 5 – Inflammatory parameters studied after repeated intracolonic administration of ethanol and zymosan. A) Trial schedule, experimental groups and number of animals used in each procedure. **B)** Body weight evolution during the whole experiment (n= 3; φ $P<0.05$ vs. Et30+Zym 100; π $P<0.05$ vs. Saline and Et30+Zym 100; σ $P<0.05$ vs. Et30Zym 10; # $P<0.01$ vs. Saline, $P= 0.07$ vs. Et30, and $P= 0.053$ vs. Et30+Zym 10). **C)** Colon length at the end of experimental manipulations (n= 3; #$P= 0.09$ vs. Saline). **D)** TLR2 mRNA expression (n= 3). **E)** TLR4 transcript levels (n= 3; **$P<0.01$). **F)** IL-1β expression (n= 3). **G)** IL-10 mRNA levels (n= 3).

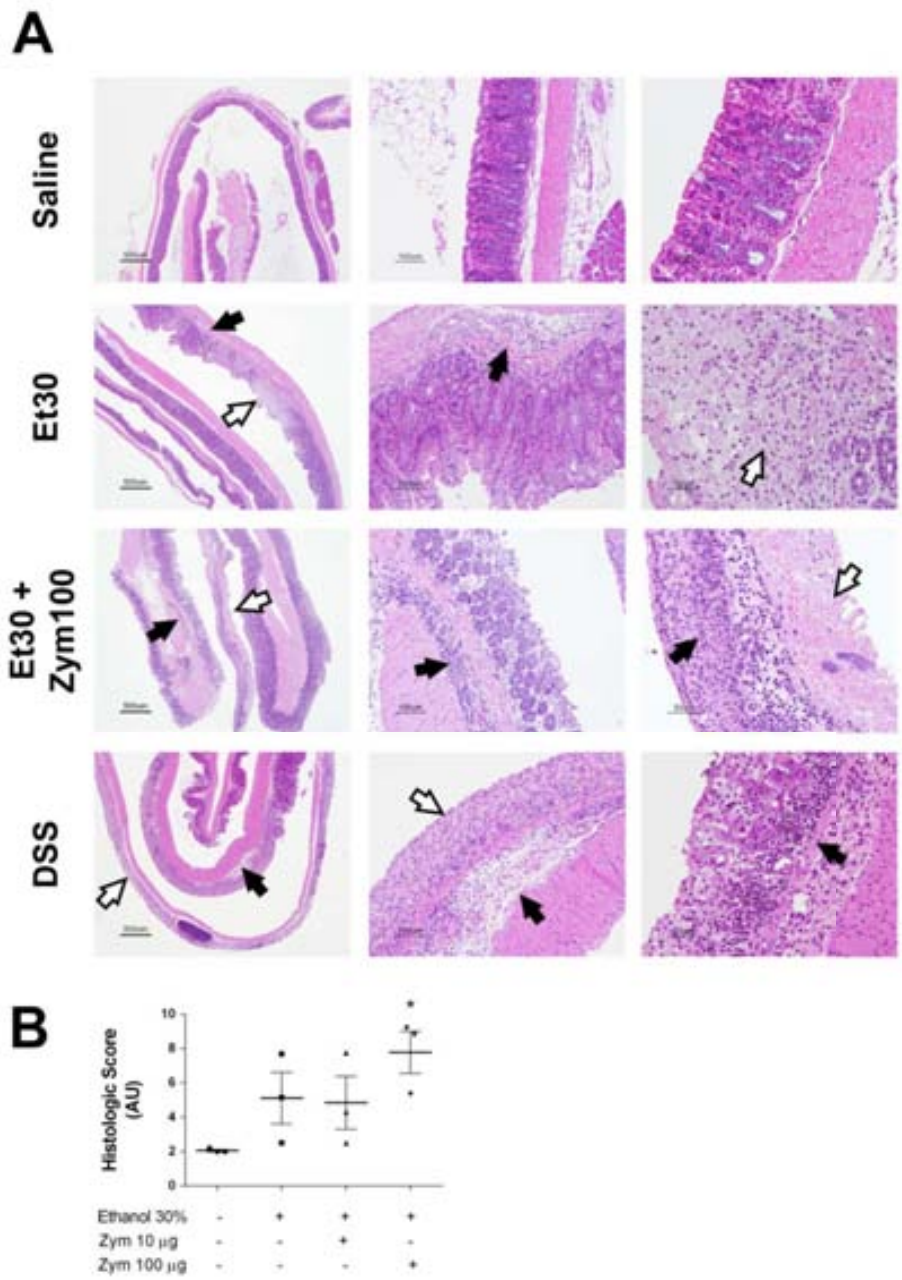

Figure 6 – Histologic findings in mice from the "repeated administration" trial study and DSS-treated mice from the first trial. A) Haematoxylin & eosin sections from the animals of study. From left to right, pictures were taken at a magnification of 20x, 100x and 200x. Black arrows point to areas with infiltration of leukocytes, while white arrows show areas of crypt architecture destruction and ulceration. **B)** Histologic score of the preparations (n= 3; **P*<0.05).

ethanol or 10 µg of zymosan showed inconstant inflammation, as like for IL-1β expression, one of each three mice did not develop any histopathologic sign of colitis (Fig. 6B). Typical lesions in inflamed animals were found in the mid-distal colon, showing a multi-focal pattern of distribution, and were characterised by leukocyte infiltration of submucosa and *lamina propria* and oedema (Fig. 6A, black arrows). In addition, different areas of ulceration with important loss of crypt architecture, as well as various foci of haemorrhage, could also be appreciated (Fig. 6A, white arrows). The extension of the lesions was generally not as important as that observed in DSS-treated animals (Fig. 6A, lower panels).

Discussion

TLRs play key roles in discriminating self from non-self motifs, and might therefore be involved in most innate immune responses to microorganisms through the recognition of the microbial conserved structures known as MAMPs [4]. Since experimental colitis is dependent on TLR functionality [17] and their expression is increased in animal models of colitis and in IBD patients [9, 21, 22, 24], it is reasonable to hypothesize that stimulation of these receptors might be an important factor in triggering such noxious inflammatory responses. Our present work suggests that mechanisms maintaining the intestinal epithelial barrier function in the lower GI tract are capable of isolating the host from a highly antigenic environment. However, alteration of these mechanisms by disruption of the mucus layers can induce inflammation, which might be further enhanced by zymosan co-administration. In addition, we report increased expression for TLR2 in DSS-treated mice when compared to control mice. This finding indicates that TLR2 is dynamic and might be considered as a possible target to enhance tolerogenic responses and attenuate inflammation of the bowel.

Zymosan is an insoluble component of the yeasts that induces TNF-α production in cultured macrophages upon recognition by TLR2/TLR6 heterodimers and dectin-1 [25, 27]. Accordingly, doses of 10 to 1000 µg per animal in a single injection have been used to induce peritonitis, [29], while doses of 25 to 100 µg have been used to obtain tolerogenic effects [15]. Peritoneum is a sterile cavity and is highly reactive to microorganisms, but the distal colon harbours a great amount of microbes and is thus tolerant to them. In this regard, an important drawback in selection of zymosan doses was taking as a reference macrophage responses in culture, as chosen doses for intracolonic instillation were the comparable to mid and lower doses used in induction of peritonitis [29]. In fact, multi-focal areas of inflammation in colon have been described 3 hours after intracolonic administration of 25 mg of zymosan in rats [38, 39]. Such amount is 250-fold higher than the highest dose used in the present study, i.e. 100 µg, which might in part explain why a single co-administration with ethanol did not trigger any immune response in our first trial. Another important point that may have accounted for this lack of effect is the chosen schedule. SAA measurements at 24 and 48 hours suggested that there was an acute-phase process that disappeared soon (before 48 hours; Fig. 1D and 4B). This transient inflammatory process and its fast-resolving course were

confirmed by evaluation of IL-1β and IL-10 transcripts in colons of zymosan-treated animals at 3 and 24 hours after intracolonic instillation (Fig. 4F and G). These results indicated that although zymosan co-administration with ethanol induced short-term immune responses, subsequent re-administrations would be necessary to achieve the desired extended effect. Such idea is supported not only by the results of our last trial experiment, but also by other studies in which 3 mg of zymosan per mouse, given transanally for 3 consecutive days, induce only a brief accumulation of macrophages in the *lamina propria* that resolves by day 6 [40]. Thence, a persistent challenge with higher amounts of zymosan seems necessary to cause a moderate immune response.

DSS is administered continuously for 5 days in drinking water and causes alterations in the mucus layer, facilitating the access of bacteria to the epithelial lining and inducing colitis [41, 42]. Ethanol intracolonic instillation has been used in induction of TNBS- or zymosan-derived colitis because of its mucus disrupting effects [30, 39]. Thus, it would be reasonable to speculate that similar mechanisms of action may result in similar inflammatory responses. However, administration schedules for both chemicals were different in this study, and consequently important differences were observed in clinical signs, extent of the lesions, TLR expression and reproducibility of inflammation. Nevertheless, histologic examination of DSS-treated and ethanol-re-administered mice showed similarities in the infiltrate quality, consisting of mononuclear cells and neutrophils, as well as in crypt destruction and ulceration.

Repeated ethanol instillation was characterised by 1) a slight but significant loss in body weight after the first and second administration (Fig. 5B); 2) inconsistent inflammation, as demonstrated by IL-1β expression and histological findings (Fig. 5F and 6B); and 3) significant TLR4 down-regulation (Fig. 5E), which might correspond to a desensitisation of colonocytes caused by increased exposition to luminal TLR ligands [43]. Whether a real effect exists for zymosan in this study when co-administered with ethanol is controversial. Statistically, differences between Et30- and Et30+Zym100-treated mice were not seen during the whole study; only a trend could be appreciated after the third administration in the repeated challenge trial, at day 7 (Fig. 5B, $P = 0.07$). In this respect, another drawback in this final trial was the low number of animals used, what makes it difficult to obtain statistically significant results. Yet, Zym100-re-treated mice showed a trend towards shortening of colon (Fig. 5C), as well as reproducible IL-

1β overexpression and significant increase in histopathologic lesions (Fig. 5F and 6B) when compared to saline controls. Conceptually, such differences and tendencies, which were not observed in the Et30-treated group, might point out the existence of a sub-inflammatory process elicited by zymosan. However, such effect would be dependent on an initial injury to the epithelial lining, as described in other studies for zymosan [39] or flagellin [19]. Indeed, very high doses of zymosan in saline must be administered in mice to obtain a moderate and transient inflammatory infiltrate [40], and more potent ligands like LPS do not induce colitis, but ileitis, when administered intracolonically [18]. Taken together, these data indicate that the colonic epithelial barrier protects the host from the external milieu and preserves tolerance even when it is highly loaded with MAMPs. Hence, a therapeutic approach searching the induction of tolerogenic responses with such kind of molecules would be only suitable once the epithelial barrier function is compromised.

Another interesting point in this study concerning zymosan is the induction of a possible tolerogenic effect by this product. In the short-term trial, significant time-dependent changes were observed in IL-1β and IL-10 transcripts from 3 to 24 hours only in zymosan-treated animals (Fig. 4F and G). While the proinflammatory cytokine IL-1β was higher 3 hours after administration in zymosan-treated groups, at 24 hours it was lower (Fig. 4F). This faster decrease could be due to a more important induction of IL-10 (Fig. 4G), which is known to have immunomodulatory properties. In fact, zymosan stimulation has been reported to increase IL-10 production in dendritic cells, as well as TGF-β in cultured macrophages [15]. *In vivo*, immunological tolerance was induced by these cells through priming and differentiation of naïve T cells into T regulatory cells in a model of antigen-specific driven immune response [15]. In our experiments, this hypothetic tolerogenic effect was not persistent, but transient, and thus it would have been difficult to manage in our experimental design. Perhaps daily intracolonic instillations devoid of ethanol could sustain a tolerogenic response in individuals already suffering from GI inflammation; therefore, the use of zymosan as an inductor of tolerogenic responses must still be largely studied in order to find a possible application in IBD patients.

TLR2 plays a role in inflammation of the bowel that has not been elucidated yet. DSS-treated $TLR2^{-/-}$ animals are more susceptible to colitis, and show increased mortality

and morbidity [5], what is related to a decreased epithelial barrier function [44]. Thus, up-regulation of TLR2 observed in our DSS-treated animals (Fig. 2A and Fig. 3C) seems crucial for the resolution of colitis. Indeed, TLR2 expression has been reported to be low in physiologic conditions [45], concentrating in the crypt bottoms [21], although they have also been described in epithelium surface [46], as we have observed. During inflammation, this receptor is increased not only in immune cells infiltrating the *lamina propria* [9], but also in epithelial cells [21, 22]. Whether this up-regulation is promoted by the increasing concentration of TLR2 ligands in the lumen [20] or is secondary to cytokine secretion is not known. However, an interesting study demonstrates that in a spontaneous model of colitis, colonocytes from IL-$2^{-/-}$ mice express more TLR2 and respond in a stronger fashion to MAMPs than colonocytes from littermates, even before the onset of inflammation [23]. Thence, TLR2 overexpression can be seen as an initiating factor of inflammation, but given the tolerogenic effects described for zymosan in APCs [15] and the involvement of TLR2 in preservation of the epithelial barrier [13], this receptor emerges as an interesting target to address amelioration of colitis and improvement of the clinical course in IBD patients.

In conclusion, our experiments support that the epithelial barrier plays a very effective role in separating external milieu from the host, but once it is disrupted, zymosan could have short-term tolerogenic effects. However, in combination with ethanol, repeated instillations of zymosan may potentiate the sub-inflammatory effects of this solvent to establish a moderate patchy colitis. Nevertheless, important drawbacks in this work concerning experimental design should be addressed to obtain more robust conclusions. Finally, overexpression of TLR2 in DSS-treated animals could be considered as an opportunity to manage inflammation outcomes through administration of its ligands during active colitis.

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Chapter 2

Therapeutic intracolonic administration of lypopolysaccharide ameliorates acute colitis through induction of epithelial proliferation

Abstract

Targeting Toll-like receptors (TLR) during commensal-induced inflammation has emerged as an attractive management strategy since TLRs play important roles in microbial recognition and wound repair. However, their use in therapeutic regimes has seldom resulted in beneficial effects. Our aim was to evaluate the effects of two different doses of zymosan and lipopolysaccharide (LPS; TLR2 and TLR4 ligands, respectively) administered intracolonically during dextran-sodium sulphate (DSS)-induced colitis. Instillation of zymosan resulted in no beneficial or detrimental effects, whereas both doses of LPS ameliorated animal disease score, although significant reduction in colon shortening was only achieved with the lowest dose used. TLR2 and TLR4 mRNA expression decreased close to basal levels following LPS administration, in parallel with disease severity. Transcriptional profiles, however, were not improved when compared to DSS-treated mice, since LPS has important pro-inflammatory properties. Major histological findings in DSS+LPS-treated mice included preservation of the epithelial layer and larger crypts, suggesting that TLR4 activation in epithelial cells might play protective and/or proliferative roles during inflammation. An increased epithelial proliferative response was confirmed by overexpression of proliferating cell nuclear antigen, whilst changes in cyclooxygenase-2 expression did not correlate with the observed phenotypes. These results indicate that modulation of TLR4 activity in colonocytes might result in activation of the Wnt/β-catenin pathway, compensating crypt loss and thus improving the course of acute inflammation.

Keywords

Toll-like receptor, lipopolysaccharide, zymosan, inflammation, DSS-induced colitis, epithelial proliferation, cyclooxygenase-2

Introduction

Inflammatory bowel disease (IBD) and its two main forms, Crohn's disease (CD) and ulcerative colitis (UC), are characterized by recurrent and chronic inflammation of unknown etiology. Although its pathogenesis is not fully understood, it seems well established that tolerance to commensal bacteria that are normally present in the gastrointestinal (GI) tract is lost in genetically predisposed individuals, triggering a pro-inflammatory response that is further sustained by the persistence of the antigen in the milieu [1, 2]. Subsequent studies in different animal models of IBD have concluded that spontaneous colitis does not occur in the absence of non-pathogenic microbiota [3], suggesting that the altered recognition of microflora may result in a loss of tolerance and inflammation. Therefore, in recent years, the study of pattern recognition receptors, which have been shown to mediate the recognition of bacterial highly conserved motifs, has gained momentum. Among them, the Toll-like receptor (TLR) family has been widely investigated, since abrogation of the TLR signalling pathway in transgenic mice that develop microbial-dependent spontaneous colitis results in a lack of inflammatory phenotype, even in the presence of microflora [4]. In addition, several reports indicate that these receptors are up-regulated in inflamed areas during active IBD [5, 6], as well as in animal models of colitis [7], suggesting that deregulation of TLRs might be directly implicated in the pathogenesis of inflammatory processes.

TLRs are expressed in different cell types throughout the GI tract, including the four epithelial lineages [8] and antigen presenting cells (APC), such as dendritic cells (DC) and macrophages [5, 9]. The functions of these receptors depend largely on the cell type they are expressed in and on the TLR subtype. TLR2 and TLR4, two of the most important TLRs, are expressed in goblet cells, Paneth cells and absorptive enterocytes, where they are involved in proper cell maturation [10] and secretion of antimicrobial peptides [11]. In addition, they favour strengthening of the epithelial barrier and elicit antiapoptotic and proliferative effects [12, 13]. In APCs and immunocytes, these receptors are responsible of triggering protective inflammatory responses in the presence of penetrating microorganisms by stimulating the production of cytokines and chemokines and shaping the adaptive immune response [14].

Different work from various authors demonstrates that induction of tolerance in different cell types is feasible through repeated administration of TLR ligands. Intestinal

epithelial cells (IEC), for instance, display cross-regulation of TLRs: stimulation with TLR2 and TLR4 causes down-regulation of TLR4 and TLR5, reducing their subsequent activation [15]. Furthermore, prolonged incubation of IECs with the TLR2 ligand lipoteichoic acid or the TLR4 ligand lipopolysaccharide (LPS) induces hyporesponsiveness to subsequent TLR challenges through up-regulation of inhibitory molecules [16]. Similarly, LPS-induced tolerance is associated to down-regulation of TLR4 in macrophages [17], whereas different priming experiments in DCs with TLR2 and TLR4 ligands have shown that these cells increase production of interleukin (IL)-10 after TLR re-stimulation [18-20], thus promoting tolerogenic responses through regulatory priming of T cells [21]. In parallel, TLRs also play crucial roles during inflammation *in vivo*, as myeloid differentiation primary response protein (MyD)88$^{-/-}$, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice are highly susceptible to DSS-induced colitis [22]. Actually, these TLRs contribute to wound healing after acute inflammatory insults: TLR2 has been shown to increase maturation and lifespan of goblet cells [10], whereas TLR4 has been clearly implicated in epithelial repair through regulating cyclooxygenase (COX)-2 activity after DSS injury [23-25]. Indeed, TLR4$^{-/-}$ mice display impaired mucosal healing during DSS-colitis since they cannot up-regulate COX-2 expression, and thus lack the epithelial cytoprotective prostaglandin (PG) E2 [23].

Given the importance of TLRs in maintaining homeostasis and regulating inflammation, and the possibility of inducing tolerance or wound healing through administration of their ligands, they have been considered a potential target for novel therapies [26]. In this regard, prophylactic administration of TLR2 and TLR4 ligands has proven to be efficient in ameliorating the clinical signs of colitis [22, 27]. Therapeutic approaches, however, have obtained inconsistent results, from amelioration to aggravation of the signs of colitis [26].

Previous work from our group showed that TLR2 is up-regulated during DSS-induced colitis, and suggested possible roles for zymosan in both worsening inflammation and promoting tolerogenic responses. However, only low doses of zymosan were tested and were given in the absence of an inflammatory background. In order to evaluate whether intracolonic instillation of this TLR2 ligand can prompt beneficial or detrimental responses through modulation of altered TLR2 expression during colitis, DSS-treated mice were administered a higher dose of zymosan on two alternate days. Treatments with LPS were also assayed according to the same study design, as this

ligand is able not only to induce tolerance in different cell types [15-17, 20], but also to promote inflammation *in vivo* [28]. Our results indicate that, despite minor decreases in the pro-inflammatory profiles at the lowest doses assayed, zymosan had no ameliorating effects in DSS-colitis. Conversely, although inducing transient signs of inflammation, LPS instillation improved clinical signs of colitis by promoting IEC proliferation and increasing crypt preservation, possibly through activation of the Wnt/β-catenin pathway.

Materials and Methods

Reagents and antibodies

LPS administration was performed with a mixture of phenol-extracted LPS from *E. coli* O55:B5 and *S.typhosa* (1:1). Both LPS and zymosan were purchased from Sigma (Madrid, Spain). Carboxymethyl cellulose (CMC) was purchased from Roig-Farma (Barcelona, Spain), ketamine from Merial Laboratorios (Imalgène 1000®; Tarragona, Spain) and xylazine from Bayer HealthCare (Rompun 2%®; Kiel, Germany). Rabbit polyclonal anti-COX-2 antibody (1:5000 for western blot (WB)) and rabbit polyclonal anti-proliferating cell nuclear antigen (PCNA) (1:500 for WB and 1:600 for immunohistochemistry (IHC)) were from Abcam (Cambridge, UK), rabbit polyclonal anti-cyclin D1 (1:400 for IHC) was from Antibodies-online.com (Aachen, Germany), mouse monoclonal anti-β-actin (1:5000 for WB) was from Sigma, biotinylated goat anti-rabbit IgG (1:200 for IHC), from Life Technologies (El Prat de Llobregat, Spain), horseradish peroxidase (HRP)-linked goat anti-rabbit antibody (1:10,000 for WB), from Cell Signaling (Danvers, USA), and HRP-linked sheep anti-mouse IgG (1:100,000 for WB) was from GE Healthcare (Barcelona, Spain).

Animals

For in vivo experiments, 12-week old C57Bl/6 female mice were purchased from Charles River (Les Oncins, France) and housed in specific pathogen-free conditions, under a controlled temperature (20±2ºC) and photoperiod (12h/12h light-dark cycle), with free access to food and water.

All procedures performed were approved by the Ethical Committee of the Universitat Autònoma de Barcelona (CEEA-UAB 561).

Colitis induction and TLR ligand administration

Middle-distal colitis was induced by administration of 3% DSS (36-50 kDa; MP Biomedicals, Illkirch, France) in drinking water for 5 consecutive days. DSS solution was freshly prepared and replaced every other day. On days 2 and 4 since the beginning of the DSS treatment, animals were anesthetised by intraperitoneal injection of a mixture of ketamine 100 mg/kg and xylazine 20 mg/kg in NaCl 0.9% solution (10 mL/kg). In a first set of “low-dose” experiments (200 µg of ligand per mouse), 200 µL of a 1% CMC

solution containing no ligands, zymosan 1 mg/mL or LPS 1 mg/mL were intracolonically administered to anesthetised animals by means of a customised catheter that prevented the solution from being expelled from the colon. In a second set of "high-dose" experiments (500 µg of ligand per mouse), 250 µL of a 1% CMC solution containing no ligands, zymosan 2 mg/mL or LPS 2 mg/mL were intracolonically administered to anesthetised animals. The anesthetised animals were kept flat on a warm surface for the total duration of anaesthesia (60 to 90 minutes). This time ensured complete absorption of the 1% CMC solution carrying the TLR ligands. Solutions of zymosan and LPS were prepared fresh every day of administration. As experiments were performed on different lots of animals and instillations contained different vehicle volumes, results are presented in separate graphs.

Euthanasia and tissue collection

Seven days after induction of colitis, mice were euthanized by cardiac puncture exsanguination under isoflurane (Isobavet®; Schering-Plough, Sant Cugat del Vallès, Spain) anaesthesia. Colons were removed and, after measuring their length, were rinsed in ice-cold phosphate buffered saline (PBS) and cut into longitudinal pieces, which were further fixed in 4% paraformaldehyde or kept in RNAlater solution (Ambion, Applied Biosystems, Alcobendas, Spain).

Disease activity index

Animals were controlled daily for individual weight loss, stool consistency, faecal blood and general aspect in order to build up a disease activity index (DAI) to follow up colitis, as described previously [29]. Criteria used for the DAI scoring are further detailed in Table 1. Endpoint criteria are also shown in Table 1, and were applied as described elsewhere[30].

As symptoms of colitis usually appear during the fourth day, a global alternative DAI was calculated from day 4 to the end of experiments to evaluate the symptomatic phase of the disease. In this case, DAI time-course was plotted for each animal and the area under the curve (AUC) was calculated to compare overall clinical symptoms between groups.

Body weight change	Score	Stool consistency	Score
< 0 % (gain)	0	Normal	0
0 – 2.5 % (loss)	1	Pasty	1
2.5 – 5 % (loss)	2	Very pasty but not liquid	2
5 – 7.5 % (loss)	3	Liquid, but still with a pellet shape	3
> 7.5 % (loss)	4	Very liquid	4
Animal aspect	**Score**	**Bloody faeces**	**Score**
Normal	0	Semi-quantitative evaluation depending on the quantity of blood observed	0 - 3
Coarse hair	1		
Coarse hair, abnormal activity	2		
Obvious dehydration	3		
Endpoint criteria	**Score**	**Endpoint criteria**	
Body weight	0 - 3	A score of 3 in two out of the three parameters analysed is taken as an endpoint criterion	
Aspect	0 - 3		
Behaviour: aggressive or comatose?	Yes = 3		
	No = 0		

Table 1 – Parameters and scores related to DAI calculation and endpoint criteria execution.

Histologic score and epithelial surface and crypt measurements

Histologic assessment of colitis was performed by an investigator blinded to the study design. Haematoxylin & eosin sections from mid-distal colons of each animal were evaluated as previously described [31]. Five different areas were evaluated as follows: for inflammation: 0, none; 1, slight; 2, moderate; and 3, severe; for extent of inflammation: 0, none; 1, mucosa; 2, mucosa and submucosa; and 3, transmural; for crypt damage: 0, none; 1, basal 1/3 damaged; 2, basal 2/3 damaged; 3, only surface epithelium intact; and 4, entire crypt and epithelium loss. Each of these values was multiplied for the corresponding percentage of involvement of the studied area: 1, 1-25%; 2, 26-50%; 3, 51-75%; and 4, 76-100%.

For epithelial preservation studies, micrographs from each colon roll were taken at a low magnification (20x) through a Nikon Eclipse 90i microscope interfaced to a DXM 1200F camera (Nikon Corporation, Barcelona. Spain). Depending on their crypt damage, the surface of the epithelium along the total length of each sample was measured and classified into; intact areas, high preservation areas (minor inflammatory infiltrates with 3/4 to total crypt persistence), major loss areas (important inflammatory infiltrates with 1/2 or more of the crypt damaged) or total loss areas by an observer blinded to

the study design. Measurements were performed by means of the ImageJ software (National Institutes of Health, Bethesda, USA). Results are expressed as the percentage of each degree of epithelium covering over the total length of the colon sample.
For crypt morphometry, the height of at least 20 crypts was determined by an investigator blinded to the treatments by means of the ImageJ software (National Institutes of Health).

Immunohistochemistry

For IHC, paraffin-embedded tissues were deparaffinized and treated in boiling citric acid during 15 minutes for antigen retrieval. Endogenous peroxidase activity was blocked with a 5% solution of hydrogen peroxide in PBS, and avidin and biotin with the Avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA, USA). Slides were then incubated for 1 hour in PBS containing 5% bovine serum albumin and 1% Triton X-100. Overnight incubation at 4°C with PCNA or cyclin D1 antibodies was followed by addition of a biotinylated secondary antibody, which was further detected by Vectastain ABC kit combined with 3-3'-diaminobenzidine peroxidase substrate kit (both from Vector Laboratories).

Real-time RT-PCR analysis

Total RNA from colon samples embedded in RNAlater solution was extracted using the Nucleospin RNA II Kit (Macherey-Nagel, Düren, Germany) and quantified by optical densitometry. 750 ng of RNA were retro-transcribed by using the PrimeScript RT reagent Kit (Takara Bio Inc, Shiga, Japan) for reverse-transcriptase polymerase chain reaction (RT-PCR). Primer sequences listed in Table 2 were designed to span introns using the primer-BLAST design tool, unless otherwise stated. PCR amplifications were performed using the SYBR Premix Ex Taq (Takara) according to manufacturer's protocol, and run on a LightCycler 480 II instrument (Roche Applied Science). Absence of coamplification products was assured by generating a final melting curve for each reaction. mRNA level of expression of the genes of interest was corrected to that of the housekeeping genes β-actin or succinate dehydrogenase complex subunit A (SDHA), and calculated by the ΔΔCt method [32].

Gene	Sense primer	Antisense primer	Reference
B-actin	CTAAGGCCAACCGTGAAAAG	ACCAGAGGCATACAGGGACA	ENSMUST00000031564.4
SDHA	GCTTGCGAGCTGCATTTG	CATCTCCAGTTGTCCTCTTCCA	NM_023281.1
TLR2	ACCGAAACCTCAGACAAAGC	CAGCGTTTGCTGAAGAGGA	NM_011905.3
TLR4	GGACTCTGATCATGGCACTG	CTGATCCATGCATTGGTAGGT	NM_021297.2
TNF-α	GATCGGTCCCCAAAGGGATG	TGAGGGTCTGGGCCATAGAA	NM_013693.2
TGF-β1	TGGAGCAACATGTGGAACTC	CAGCAGCCGGTTACCAAG	NM_011577.1
COX-2	TGTGCAAGATCCACAGCCTA	TCTGGAGTGGGAGGCACTT	NM_011198.3
iNOS	CACCTTGGAGTTCACCCAGT	ACCACTCGTACTTGGGATGC	NM_010927.3

Table 2 – List of primers used for real-time RT-PCR analysis.

Western Blot

The flow-through of the samples used in RNA extraction was incubated overnight at -20°C with ice-cold acetone to precipitate protein. Such protein was further resuspended in a 10 M urea buffer and measured by means of the Bradford assay (Bio-Rad, California, USA). Two pools were prepared by mixture of different samples in each, and 10 µg of protein were separated on a 10% acrylamide gel containing 0.1% sodium dodecyl sulfate. After transferring to a nitrocellulose membrane with the iBlot™ Dry Blotting System (Life Technologies), membranes were blocked for 1 h at room temperature with 5% non-fat dry milk in Tris buffered saline (100 mM NaCl, 10 mM Tris, pH 7.5) with 0.1% Tween 20 (TBST). Overnight incubation at 4°C with primary antibodies diluted in a 5% non-fat milk solution in TBST was followed by detection with HRP-conjugated anti-rabbit or anti-mouse antibodies. Reactivity was visualized by enhanced chemiluminescent detection (ECL advance, GE Healthcare). Membranes were stripped for 15 min in Reblot buffer (Millipore), followed by extensive washing in TBST before reblocking with 5% non-fat dry milk in TBST and reprobing for β-actin determination. Bands were imaged in a LAS-3000 Imager (Fujifilm, Tokyo, Japan) and quantified with Multigauge 3.0 software (Fujifilm). Sample pool intensities were compared to those of CMC-treated mice and normalised to their amount of β-actin to assess proper loading of lanes.

Statistical analysis

Results are presented as mean values ± S.E.M. All data were compared using one-way or two-way ANOVA, followed by Tukey's post hoc test (unless otherwise stated). Data

analysis and plot were performed with GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, USA). A *P* value < 0.05 was considered to be significant.

Results

"Low-dose" intracolonic administration of LPS ameliorates DSS-induced colitis

Ligand doses were selected on the basis of literature and aiming to avoid excessive inflammatory responses. As a first approach, doses of 200 µg of zymosan or LPS were intracolonically administered to control or DSS-treated mice as shown in Fig. 1A.

Administration of 3% DSS in drinking water induced an acute colitis, as previously described [33]. Such inflammatory process was characterised by important weight loss (CMC+DSS= 74.2±1.08% vs. CMC= 95.6±1.7% of initial body weight on day 7, n= 4-7, *P*<0.001; Fig. 1B), increased DAI (CMC+DSS= 7.9±0.49 arbitrary units (AU) vs. CMC= 0.87±0.34 AU on day 7, n= 4-7, *P*<0.001; Fig. 1C) and colon shortening (CMC+DSS= 6.6±0.08 cm vs. CMC= 8.4±0.37 cm, n= 4-7, *P*<0.001; Fig. 1E).

Intracolonic instillation of the selected dose of LPS on days 2 and 4 significantly ameliorated the progression of the DSS-induced colitis. Body weight loss was significantly reduced at days 6 and 7 (CMC+DSS= 74.2±1.08% vs. CMC+DSS+LPS200= 85.1±2.14% of initial body weight on day 7, n= 7, *P*<0.001; Fig. 1B), and DAI was significantly decreased from days 5 to 7 (CMC+DSS= 7.9±0.49 AU vs. CMC+DSS+LPS200= 4.36±0.54 AU on day 7, n= 7, *P*<0.001; Fig. 1C). The overall DAI corresponding to the symptomatic phase of the disease was also improved in mice receiving LPS treatment (CMC+DSS= 14.26±0.87 AUxday vs. CMC+DSS+LPS200= 8.91±0.68 AUxday; n= 7; *P*<0.001, Fig. 1D), as well as colon shortening, that was milder (CMC+DSS= 6.6±0.08 cm vs. CMC+DSS+LPS200= 7.37±0.18 cm, n= 7, *P*<0.01, Fig. 1E). Such improvement could not be mimicked by zymosan administration (Fig. 1B-E), pointing out a preferential role for TLR4 in maintaining mucosal homeostasis during inflammation. Of note, neither LPS nor zymosan had apparent effects in the same evaluated parameters in non-inflamed mice.

In the same vein, histologic assessment of haematoxylin & eosin preparations demonstrated an improvement of the histologic scores of colitic animals that received intracolonic instillation of LPS (CMC+DSS= 17.83±1.26 AU vs. CMC+DSS+LPS200= 11.24±1.26 AU, n= 6-7, *P*<0.01; Fig. 2B). These animals showed reduced crypt

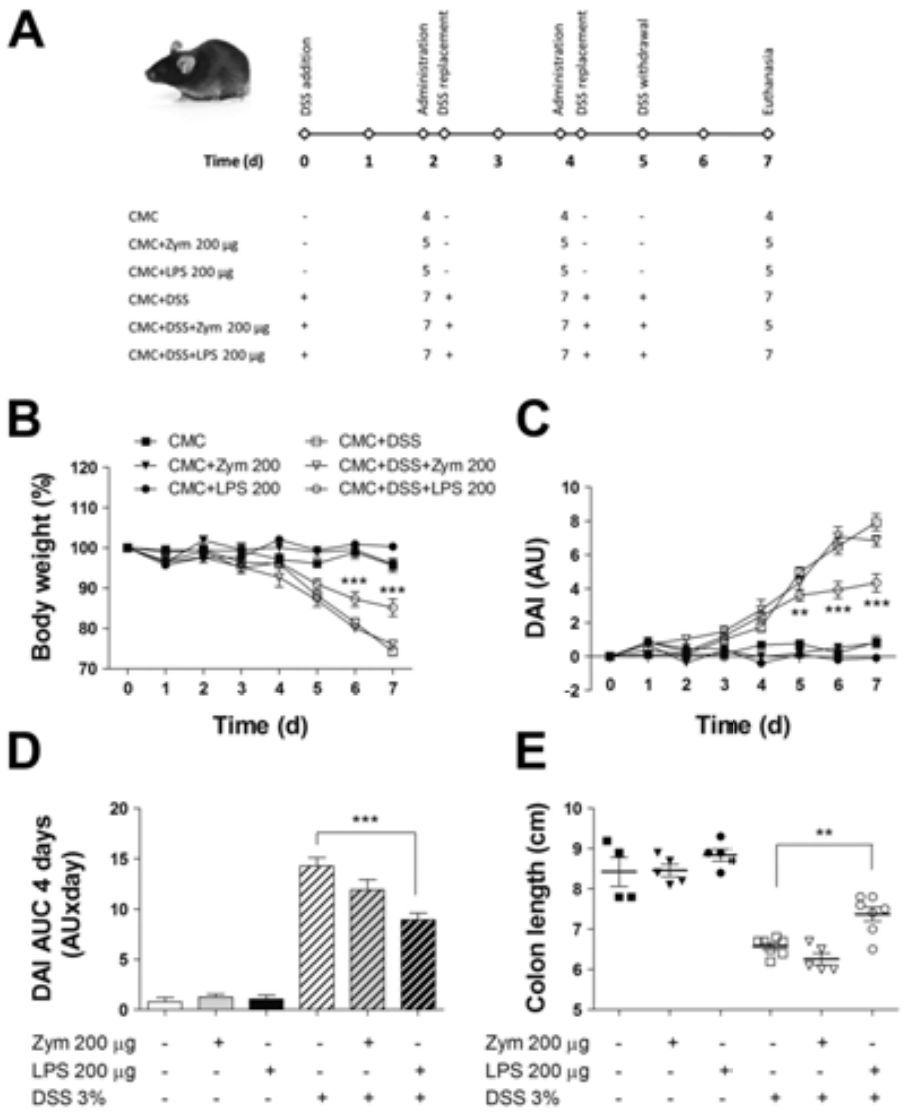

Figure 1 – "Low-dose" administration of TLR ligands in experimental colitis. A) Experimental design of the study, including treatment groups, number of animals and experimental manipulations. Differences in animal numbers on day 7 were due to mortality associated to colitis severity. **B)** Changes in body weight percentage during the whole experimental procedure (n= 4-7; ***P<0.001). **C)** DAI in arbitrary units (AU) (n= 4-7; **P<0.01 and ***P<0.001). **C)** DAI of the symptomatic phase of the disease (n= 4-7; ***P<0.001; one-way ANOVA followed by Bonferroni's post-hoc test for selected pairs of columns). **D)** Colon shortening elicited by inflammation (n= 4-7; **P<0.01).

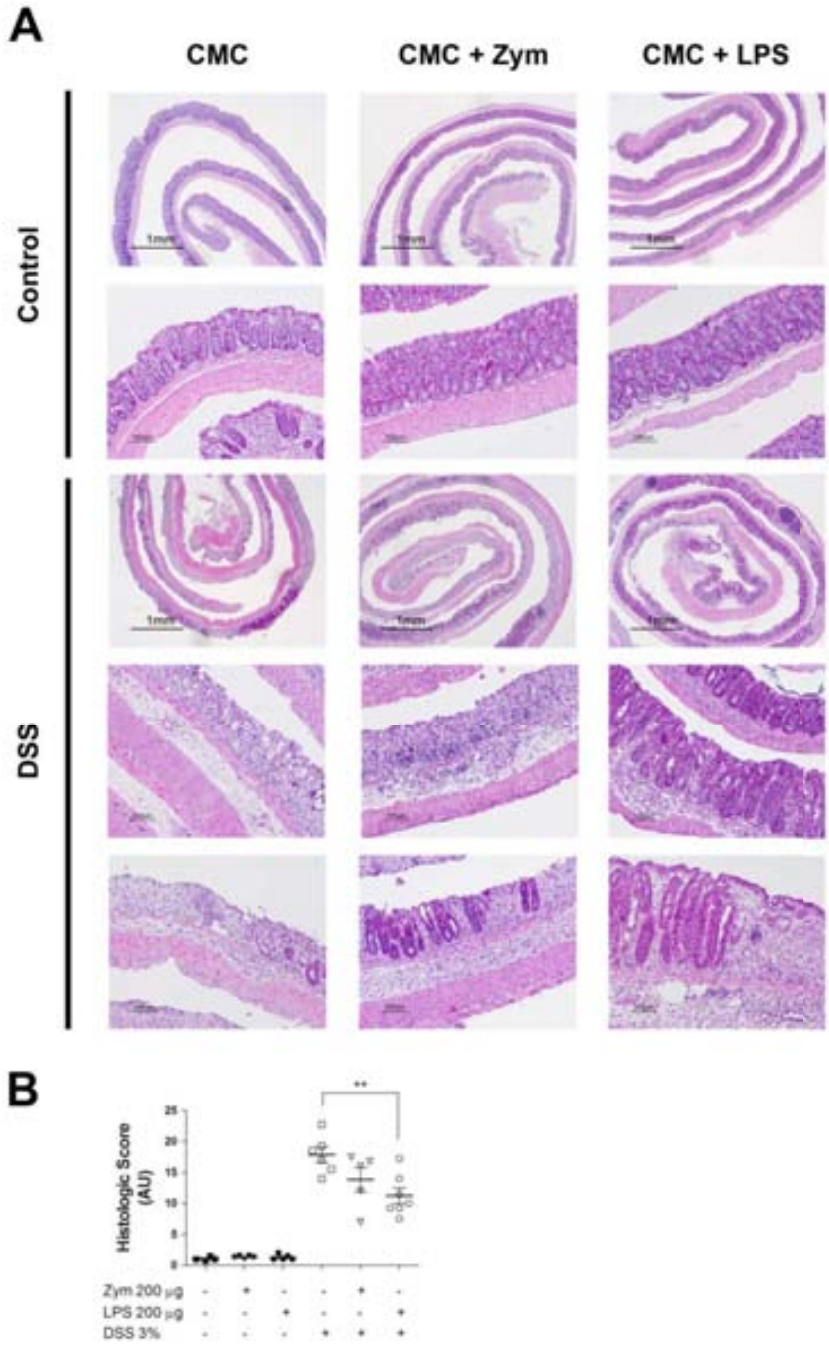

Figure 2 – Histologic findings in "low-dose" administration of TLR ligands experiments. A) Micrographs corresponding to the experimental groups. **B)** Histologic score in arbitrary units (AU) (n= 4-7; ***P*<0.01; one-way ANOVA followed by Bonferroni's post-hoc test for selected

destruction and less extensive areas of severe inflammation when compared to inflamed control mice (Fig. 2A). Zymosan intracolonic administration in DSS- treated mice did not show significant improvement of the microscopic lesions (Fig. 2A and B), confirming the results obtained in animal macroscopic evaluations. On the other hand, in the absence of DSS, neither intracolonic LPS nor zymosan induced microscopic changes in epithelium or in submucosal infiltration of immune cells (Fig. 2A).

Therefore, we can conclude that treating animals with repeated administrations of 200 μg LPS during development of colitis is effective in ameliorating clinical signs and histopathology. Conversely, despite increasing zymosan concentration above levels used in chapter 1 experiments, this TLR2 ligand showed no effects, neither in ameliorating nor worsening colitis.

Transcriptional analysis reveals TLR2 and TLR4 down-regulation after "low-dose" intracolonic instillation of MAMPs and similar pro-inflammatory profiles between DSS and DSS+LPS-treated mice

We next investigated the transcriptional profile of the colon samples of treated animals to characterise their inflammatory response and the expression of TLR2 and TLR4. According to the results obtained in time-course of clinical signs, macroscopic and microscopic parameters, non-colitic animals treated with CMC, zymosan or LPS did not show mRNA expression changes at the end of the study (Fig. 3A-E), pointing out that experimentally increased concentrations of these TLR ligands in the colonic lumen do not modify the immunologic homeostasis within the bowel. Conversely, after the epithelial barrier disruption exerted by DSS administration, treatment with zymosan and LPS caused a significant decrease in TLR2 (CMC+DSS= 5.75±1.12 folds vs. CMC+DSS+Zym200= 3.17±0.46 folds and CMC+DSS+LPS200= 2.18±0.05 folds, n= 4-5, $P<0.05$ and $P<0.01$, respectively; Fig. 3A) and TLR4 transcripts (CMC+DSS= 3.8±0.53 folds vs. CMC+DSS+Zym200= 2.13±0.17 folds and CMC+DSS+LPS200= 1.63±0.13, n= 4-5, $P<0.001$ for both; Fig. 3B). As TLRs have largely been related to inflammation, these results indicate that both zymosan and LPS would be able to reduce the immune response during DSS-induced colitis, perhaps exerting a significant tolerogenic effect. However, and in striking contradiction with the DAI, colon shortening and histologic assessment results, only zymosan treatment was able to significantly reduce the production of the pro-inflammatory mediators tumour necrosis factor (TNF)-α

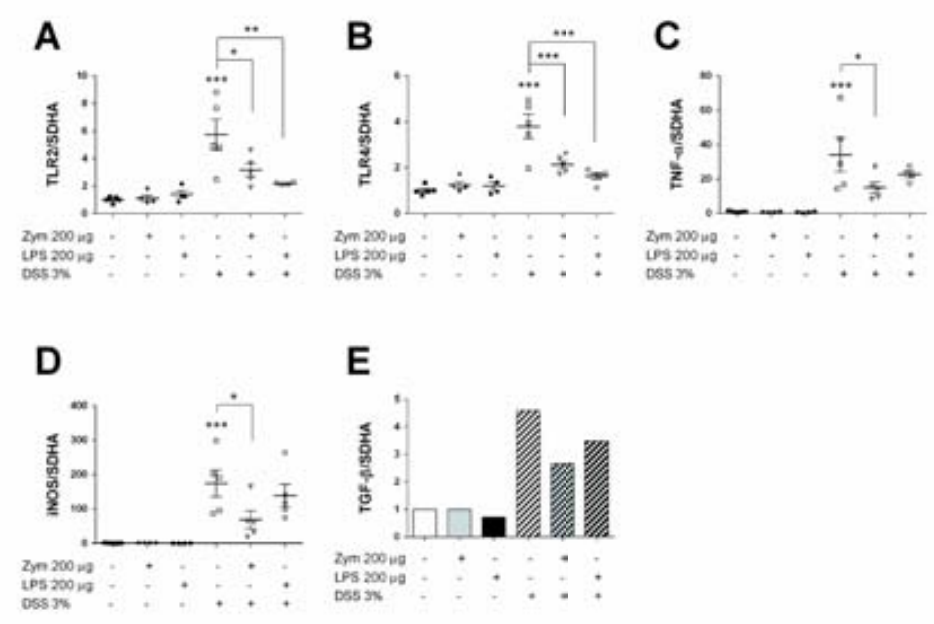

Figure 3 – Inflammatory transcriptional profile after "low-dose" administration of TLR ligands. A) TLR2 mRNA expression (n= 4-5; **P*<0.05, ***P*<0.01 and ****P*<0.001). **B)** TLR4 mRNA expression (n= 4-5; ****P*<0.001). **C)** TNF-α mRNA expression (n= 4-5; **P*<0.05 and ****P*<0.001). **D)** iNOS mRNA expression (n= 4-5; **P*<0.05 and ****P*<0.001). Statistics in **A-D** were performed by one-way ANOVA followed by Bonferroni's post-hoc test for selected pairs of columns to compare CMC+DSS vs. CMC-treated mice, and CMC+DSS vs. CMC+DSS+Zym or CMC+DSS+LPS. **E)** TGF-β mRNA expression from pools of samples corresponding to the animals used in transcriptional profiling studies.

(CMC+DSS= 34.16±9.77 folds vs. CMC+DSS+Zym200= 15.1±3.3 folds, n= 4-5, *P*<0.05; Fig. 3C) and inducible nitric oxide synthase (iNOS) (CMC+DSS= 174.7±38.9 folds vs. CMC+DSS+Zym200= 68.7±25.8 folds, n= 4-5, *P*<0.05; Fig. 3D). RNA expression of immunomodulatory molecules such as TGF-β analysed in sample pools showed a similar profile to that of pro-inflammatory cytokines, suggesting that their up-regulation was proportional to the severity of the inflammatory process, and not directly modified by TLR ligand instillation (Fig. 3E).

These observations concerning the pro-inflammatory transcriptional profile of the treatment groups are in conflict with the results obtained in disease activity time-course, macroscopic and microscopic parameters evaluated and TLR mRNA expression. However, as DSS administration induces a barrier leakage, the instilled TLR ligands are probably able to reach the immune cells in the *lamina propria* and submucosa, where

LPS possibly behaves as a stronger inducer of the inflammatory response in immunocytes when compared to zymosan. Indeed, upon stimulation with LPS and zymosan, macrophage cultures displayed different responses regarding pro-inflammatory mediator secretion. Whereas zymosan elicited mild increases in TNF-α and no nitric oxide (NO) production, the same doses of LPS provoked an important increase in liberation of both molecules ($P<0.01$ for TNF-α and $P<0.001$ for NO; Supplementary Fig. 1A and B, respectively). Therefore, this inflammatory profile observed in DSS+LPS-treated mice is probably a consequence of the pro-inflammatory properties of LPS on immune cells.

Intracolonically given "high doses" of LPS prompt transient signs of disease but ameliorate DSS colitis outcome

To ascertain whether higher doses of TLR ligands might aggravate or prompt beneficial effects on DSS colitis, and to corroborate the transcriptional findings observed with the lowest doses used, we subsequently administered mice with 500 µg of each ligand. Intracolonic instillation of 500 µg of LPS in DSS treated-mice caused a transient but significant loss of body weight on days 3 and 4, just after the first administration of this TLR4 ligand (CMC+DSS= 100.6±0.42% vs. CMC+DSS+LPS500= 91.9±1.8% of initial body weight on day 3, n= 6, $P<0.001$; Fig. 4A). This loss of body weight was translated into an increase in the DAI on days 3 and 4 (CMC+DSS= 0.22±0.21 AU vs. CMC+DSS+LPS500= 2.2±0.62 AU on day 4, n= 6, $P<0.001$; Fig. 4B). Interestingly, and even though this initial disease-like response, DSS+LPS500-treated mice showed a different trend in the symptomatic phase of the colitis, especially from day 5 to the end of the study. Indeed, while DSS- and DSS+Zym500-treated mice suffered an important increase in clinical signs of colitis during these days, DSS+LPS500-treated animals showed only a mild increase in their DAI. In addition, by day 7 this treatment group had a significantly lower DAI when compared to the inflammation control group (CMC+DSS= 5.4±0.78 AU vs. CMC+DSS+LPS500= 3.7±0.5 AU, n= 5-6, $P<0.01$; Fig. 4B). Such observation was further confirmed by the DAI of the symptomatic phase of the disease (CMC+DSS= 10.7±1.5 vs. CMC+DSS+LPS500= 6.4±0.91, n= 5-6, $P<0.01$; Fig. 4C). Although there was no improvement in the colon length (Fig. 4D), a significant amelioration of the histologic score was observed in colitic animals treated with intracolonic LPS (CMC+DSS= 13.8±2.12 AU vs. CMC+DSS+LPS500= 7.02±1.02 AU, n= 5-6, $P<0.01$; Fig. 5B),

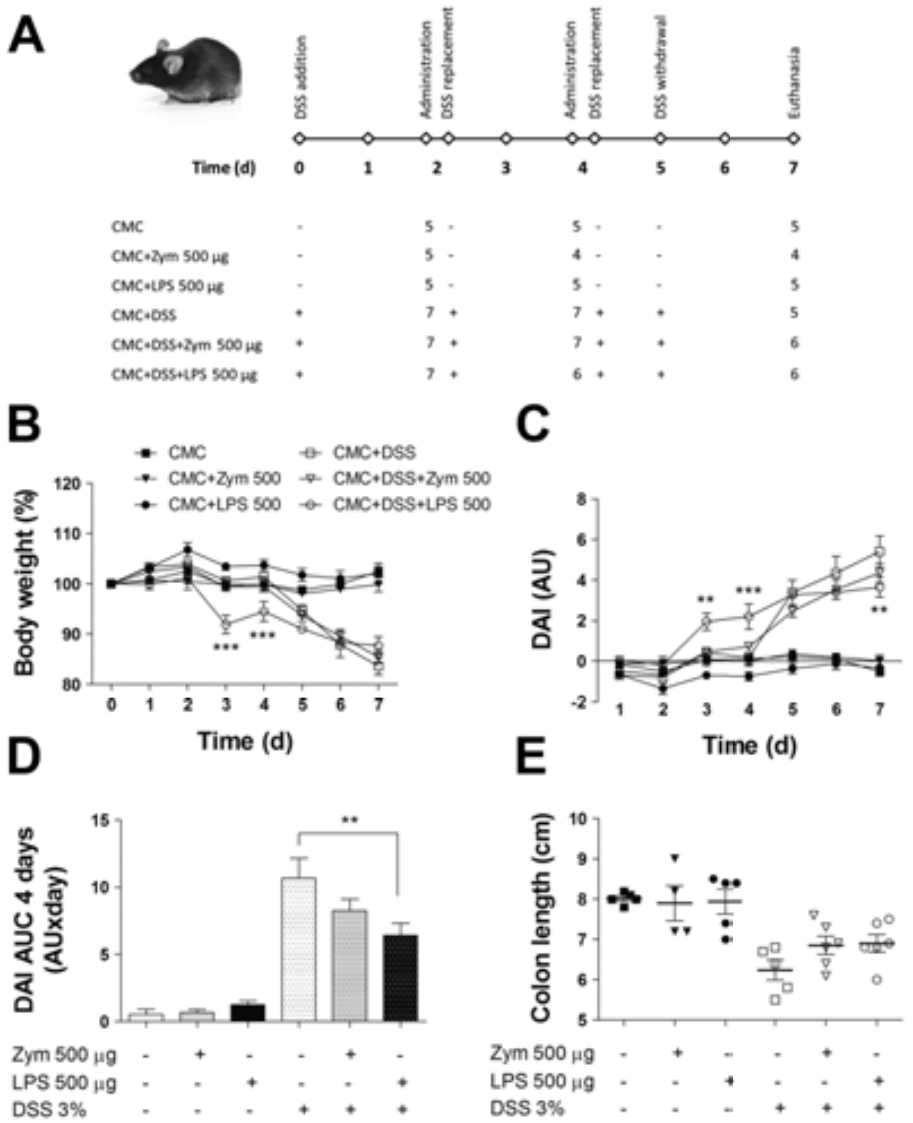

Figure 4 – "High-dose" administration of TLR ligands in experimental colitis. A) Experimental design of the study, including treatment groups, number of animals and experimental manipulations. Differences in animal numbers before day 4 were due to excessive anaesthesia on day 2, while differences on day 7 were due to mortality associated to colitis severity. **B)** Changes in body weight percentage during the whole experimental procedure (n= 4-7; ***P<0.001). **C)** DAI in arbitrary units (AU) (n= 4-7; **P<0.01 and ***P<0.001). **C)** DAI of the symptomatic phase of the disease (n= 4-7; **P<0.01; one-way ANOVA followed by Bonferroni's post-hoc test for selected pairs of columns). **D)** Colon shortening elicited by inflammation (n= 4-7).

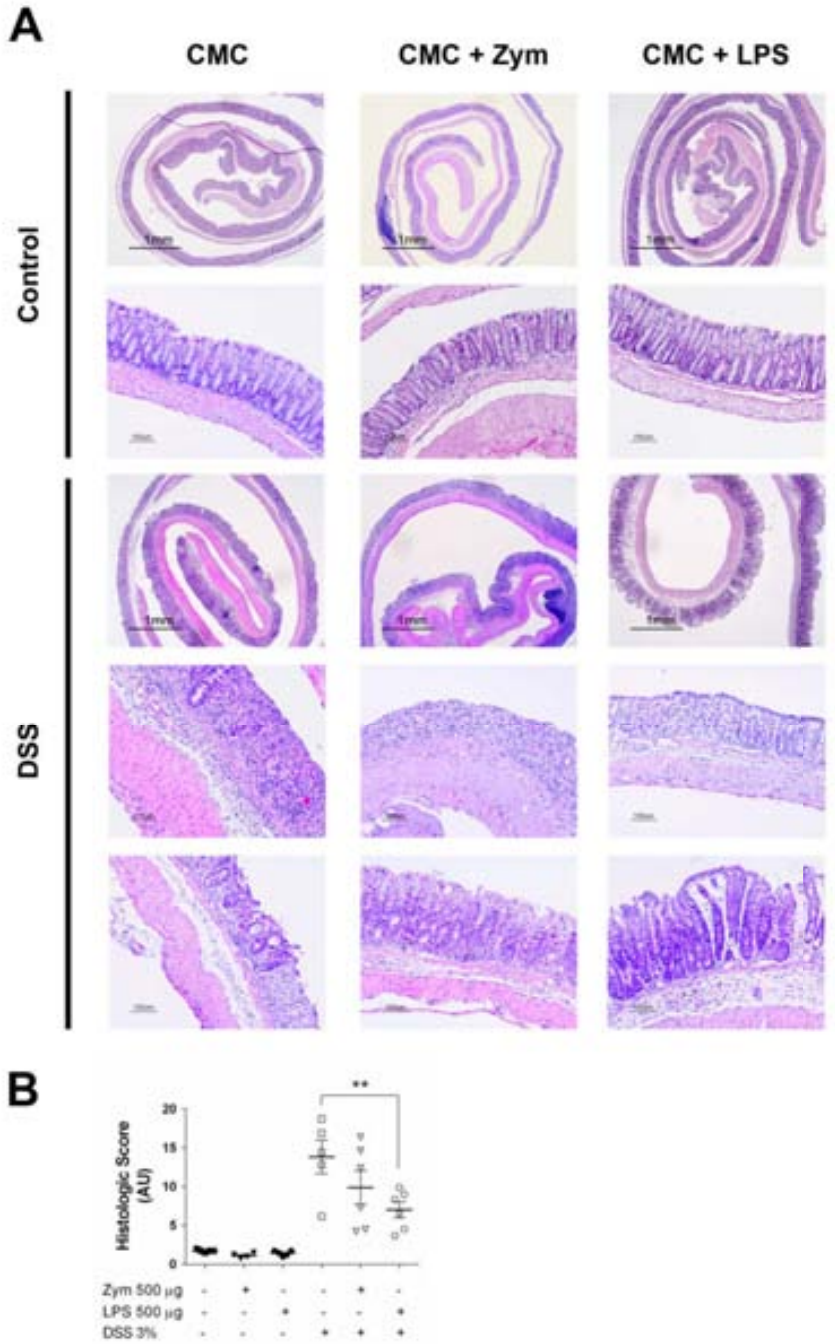

Figure 5 – Histologic findings in "high-dose" administration of TLR ligands experiments. A) Micrographs corresponding to the experimental groups. **B)** Histologic score in arbitrary units (AU) (n= 4-6; ***P*<0.01; one-way ANOVA followed by Bonferroni's post-hoc test for selected pairs of columns).

which was not mimicked by zymosan treatment (Fig. 5B). Microscopic evaluation of the DSS+LPS500 group revealed, as in the DSS+LPS200 group, an increased preservation of the architecture of the crypts, although apparent inflammation was present in submucosal areas. These observations indicate that LPS might have protective or proliferative effects on the epithelial barrier, as it was not seen in DSS- and DSS+Zym500-treated groups (Fig. 5A).

Intracolonic administration of "high doses" of MAMPs decreases TLR2 mRNA, but does not alter other inflammatory transcripts

We continued with the characterisation of the response by studying the inflammatory profile of the animals treated with DSS and the highest doses of zymosan or LPS. Similar to what we had observed in low-dose experiments, administration of zymosan or LPS even at high doses did not alter the transcriptional levels of the inflammatory mediators studied (Fig. 6A-E). Thus it seems that, provided that the epithelial barrier is kept intact, high bacterial burdens within the gut lumen do not elicit inflammation.

In DSS-treated animals, colonic instillation of 500 μg of zymosan did not cause a significant down-regulation in the production of TLR4, TNF-α and iNOS when compared to DSS+CMC-treated mice (Fig.6, B-D). However, such dose tended to decrease TLR2 mRNA expression (CMC+DSS= 5.11±0.82 folds vs. CMC+DSS+Zym500= 3.6±0.16 folds, n=3-6, *P*=0.054; Fig. 6A) and caused a slight up-regulation of TGF-β when compared to CMC-treated mice (CMC+DSS= 4.34 folds vs. CMC+DSS+Zym500= 3.02 folds, pool of RNA from all animals in each group; Fig. 6E).

These observations suggest that, whereas the 200 μg dose of zymosan might attenuate cytokine production, the 500 μg does not. In either case, however, this attenuating effect is not strong enough to result in an improvement of clinical signs of colitis, and therefore zymosan might not be useful to generate relevant beneficial responses.

In the same vein, administration of 500 μg of LPS elicited a significant decrease in TLR2 transcript levels (CMC+DSS= 5.11±0.82 folds vs. CMC+DSS+LPS500= 2.08±0.33 folds, n=3-6, *P*<0.001; Fig. 6A) and in TGF-β mRNA (CMC+DSS= 4.34 folds vs. CMC+DSS+LPS500= 3.06 folds, pool of RNA from all animals in each group; Fig. 6E), but no differences were observed for TLR4, TNF-α and iNOS expression (Fig. 6B-D).

To this point, although intracolonic instillation of either 200 μg or 500 μg of LPS had resulted in amelioration of the clinical signs in DSS-treated mice, the transcriptional

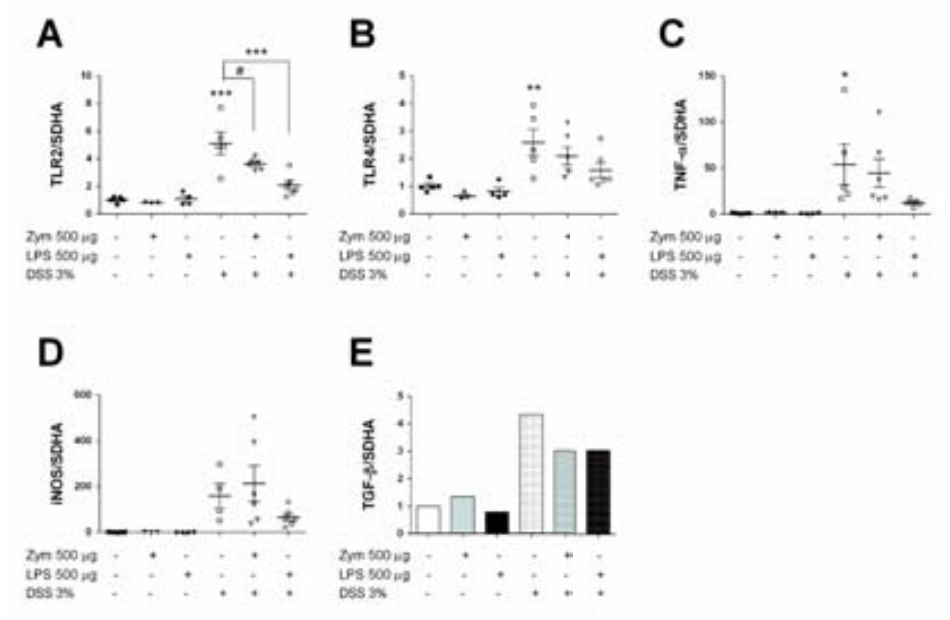

Figure 6 – Inflammatory transcriptional profile after "high-dose" administration of TLR ligands. A) TLR2 mRNA expression (n= 3-6; #*P*=0.054 and ***P<0.001). **B)** TLR4 mRNA expression (n= 3-6; **P<0.01). **C)** TNF-α mRNA expression (n= 3-6; *P<0.05). **D)** iNOS mRNA expression (n= 3-6). Statistics in **A-D** were performed by one-way ANOVA followed by Bonferroni's post-hoc test for selected pairs of columns to compare CMC+DSS vs. CMC-treated mice, and CMC+DSS vs. CMC+DSS+Zym or CMC+DSS+LPS. **E)** TGF-β mRNA expression from pools of samples corresponding to the animals used in transcriptional profiling studies.

profile of colonic tissue revealed that inflammation was similar in LPS-treated and untreated mice. Therefore, the improved phenotype observed in these groups was probably not dependent on tolerance induction, but on a different mechanism also implicated in maintenance of homeostasis.

COX-2-dependent cytoprotective effects do not seem to be involved in LPS-induced preservation of epithelial crypts

As preservation of crypt architecture was the main distinguishing feature between LPS-treated and untreated animals, we sought to quantify the percentage of the colon surface that was covered by preserved epithelial crypts. In both studies, LPS administration caused a significant increase in crypt preservation (CMC+DSS_{200}= 18.9±11.3 % vs. CMC+DSS+LPS200= 46.1±7.2 % and CMC+DSS_{500}= 29.3±11.1 % vs.

CMC+DSS+LPS500= 67.7±7.9 %, n= 4-6, *P*<0.05 in both cases; Fig. 7A and D, respectively) that was not observed in zymosan-treated groups.

Once we had ascertained that epithelial preservation was enhanced in LPS-treated animals, we focused on whether intracolonic instillation of this TLR4 ligand might induce cytoprotective or epithelial proliferative responses through up-regulation of COX-2, which was assessed by means of quantitative PCR and WB. COX-2 was increased during inflammation at transcript levels, although statistics did not show differences between CMC- and CMC+DSS-treated mice in the high dose study (Fig. 7B and E). COX-2 protein levels were more variable, but also displayed a trend to increase in DSS-treated groups (Fig. 7C and F). However, no differences could be observed in mRNA levels or in protein expression when comparing these DSS-treated groups (Fig. 7B, C, E and F).

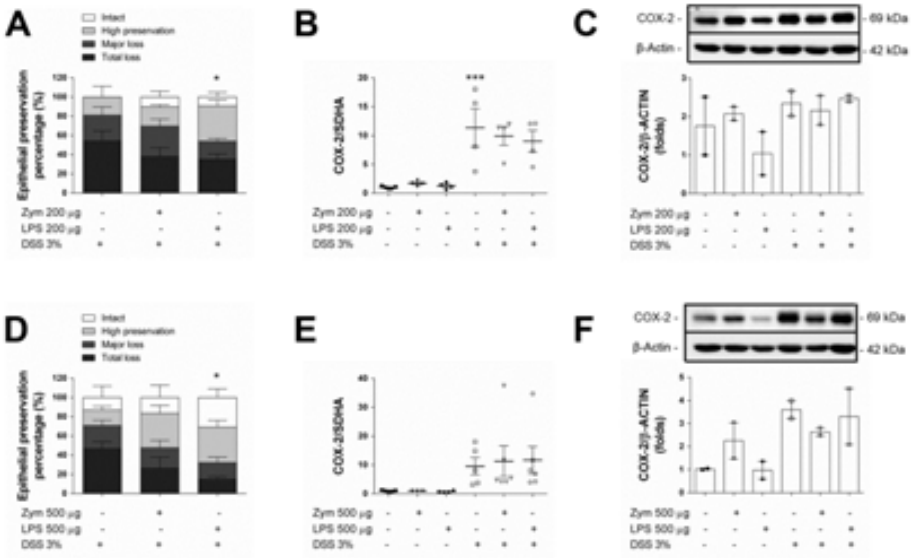

Figure 7 – Epithelial crypt preservation and COX-2 expression in low- and high-dose experiments. A) Quantification of the total mid-distal colon surface displaying preservation of epithelium in low-dose experiments (n= 4-6; **P*<0.05). **B)** COX-2 mRNA expression in low-dose experiments (n= 4; ****P*<0.001 vs. CMC-treated group). **C)** COX-2 protein expression in two different pools of animal samples from low-dose experiments. **D)** Quantification of the total mid-distal colon surface displaying preservation of epithelium in high-dose experiments (n= 4-6; **P*<0.05). **E)** COX-2 mRNA levels in high-dose experiments (n= 3-6). **F)** COX-2 protein expression in two different pools of animal samples from high-dose experiments. In **A-D**, the total length of colon surfaces showing intact and high-preserved epithelial lines was calculated in order to compare crypt persistence between groups. Comparison was performed against CMC+DSS group by means of a one-way ANOVA followed by Dunnett's test.

In the same vein, a pilot study based in quantification of COX-2-positive cells per area unit was performed in low-dose experiments to determine whether the observed differences might be explained by an increased number of COX-2 positive cells in areas of high crypt preservation. Such study corroborated that, upon inflammation, involved areas had increased numbers of COX-2-positive cells when compared to uninvolved areas (P<0.01 and P<0.001; Supplementary Fig. 2D). However, uninvolved areas in colitic mice samples had similar counts of COX-2-positive cells as control groups, and no differences could be seen between CMC, zymosan or LPS treatments in DSS-treated animals, indicating that LPS does not induce selective changes in COX-2 expression or distribution.

Taken together, all these data suggest that up-regulation of COX-2 does not seem to be the major pathway promoting epithelial preservation after DSS-induced injury in LPS-treated mice.

LPS intracolonic instillation is associated to increased epithelial proliferation

Previous data from other groups has demonstrated that MyD88$^{-/-}$ and TLR4$^{-/-}$ mice display reduced numbers of proliferative epithelial cells, suggesting that TLR4 plays an important role in promotion of epithelial cell division [23, 34]. Thence, we investigated whether there was an epithelial proliferative response secondary to LPS intracolonic instillation that might explain the epithelial preservation we observed.

Morphometric analysis of epithelial crypts revealed that LPS-treated mice had longer crypts when compared to CMC+DSS-treated animals in both low- and high-dose experiments (CMC+DSS$_{200}$= 159.7±13.1 µm vs. CMC+DSS+LPS200= 210.8±7.5 µm, and CMC+DSS$_{500}$= 177±5.7 µm vs. CMC+DSS+LPS500= 215.3±14.8 µm, n=4-6, P<0.01 and P<0.05, respectively; Fig. 8A and B). Conversely, zymosan instillation did not produce significant increases in the length of the crypts, although in areas where these structures were preserved, the crypt length values were similar to those from wild-type mice (Fig. 8A and B). In the same regard, the study of the general proliferation marker PCNA demonstrated that DSS+LPS-treated mice displayed increased expression levels of such protein, which were around 9.5 folds higher than in DSS-treated mice (CMC+DSS+LPS$_{200}$= 2.98 folds vs. CMC+DSS$_{200}$= 0.31 folds when compared to CMC controls, and CMC+DSS+LPS$_{500}$= 2.92 folds vs. CMC+DSS$_{500}$= 0.34 folds when compared to CMC controls; Fig. 8C and D), and 3 folds higher than in controls. IHC for PCNA in

DSS+LPS-treated mice showed positive reactivity along the whole axis of hyperplastic crypts (Fig. 8Eii), whereas control CMC-treated mice slides showed staining within the nuclei of IECs located in the base of the crypt that disappeared as these cells moved towards the lumen (Fig. 8Ei). These alterations in the expression patterns, in addition to the increased percentage of the epithelial surface covered by hyperplastic crypts, may account for the differences observed in these WB determinations. In contrast, although inflammatory cells also stained positive for PCNA (Fig. 8F), expression levels in these cells are possibly not so important, as DSS-treatment reduced the amount of PCNA to 0.3 folds when compared to the control group CMC (Fig. 8C and D).

Given that epithelial cell expansion is dependent on Wnt/β-catenin signalling [35] and that TLR4 has been shown to activate this cascade in IEC lines [13], we aimed to investigate the implication of this signalling cascade in the proliferative effects induced by LPS in our model. Immunostaining of cyclin D1, a downstream target of this proliferative pathway [36], revealed that this protein was dramatically increased in hyperplastic crypts of DSS-treated animals (Fig. 8H) when compared to naïve mice (Fig. 8G). Although these findings indicate that DSS-treatment elicits activation of the β-catenin proliferative pathway, cyclin D1 mRNA levels must still be quantified in order to address whether the activation of this proliferative pathway is increased after intracolonic administration of LPS.

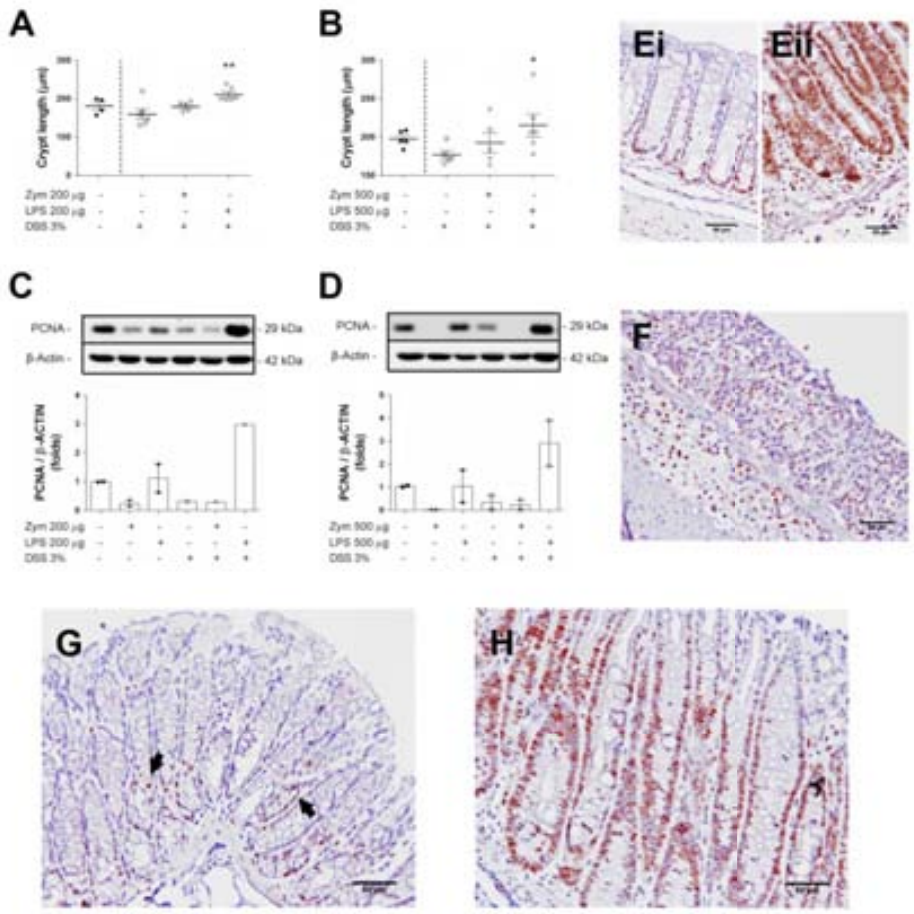

Figure 8 – Epithelial proliferation in low- and high-dose experiments. A) Morphometric analysis of crypt length in low-dose experiments (n= 4-6; ***P*<0.01 when compared to DSS control group). **B)** Morphometric analysis of crypt length in high-dose experiments (n= 4-6; **P*<0.05 when compared to DSS control group). **C)** PCNA protein expression in two different pools of animal samples from low-dose experiments. **D)** PCNA protein expression in two different pools of animal samples from high-dose experiments. **E)** Epithelial localisation of the proliferation marker PCNA in CMC- **(i)** and CMC+DSS+LPS-treated mice **(ii)**. **F)** Localisation of the PCNA protein in immune cells infiltrating mucosal and submucosal layers. **G)** Cyclin D1 expression in CMC-treated mice (black arrows point to normal positive staining). **H)** Cyclin D1 expression in colonocytes from hyperplastic crypts observed in CMC+DSS+LPS-treated mice. **(E-H)** all scale bars correspond to 50 µm.

Discussion

Given the unique milieu existing within the GI tract, different mechanisms have been set up in order to maintain hyporesponsiveness to the high bacterial burdens dwelling in the lumen [9, 15, 16, 37]. Such mechanisms are actually strongly influenced by microbial signals, and are therefore mainly controlled by pattern recognition receptors. Since TLRs are sensors of microbial components that are up-regulated during GI inflammation [5-7], show cross-regulation phenomena [15-17] and can modify DC transcription profiles to attenuate pro-inflammatory responses [19, 20], they have emerged as interesting targets aiming to promote tolerance. Moreover, the role they play in wound healing and epithelial preservation during inflammation [12, 25, 38] has also motivated investigation of their usefulness to treat inflammatory deregulated processes such as IBD. Different interventions involving TLR ligand administration have been carried out following prophylactic and therapeutic approaches in murine models of inflammation, resulting in amelioration or aggravation of the disease [26]. In this work, in order to avoid possible extraintestinal manifestations that might result from systemic administration of TLR ligands, we investigated the effects of the intracolonic instillation of zymosan and LPS in a therapeutic regime during DSS-induced colitis. Our observations indicate that zymosan does not trigger beneficial responses, whereas LPS has ameliorating effects in colitis by eliciting proliferative actions on IEC. Further work must be performed to identify the mechanisms involved in this LPS-mediated proliferation, but some of our findings suggest that these effects might be driven by the TLR4-mediated activation of the Wnt/β-catenin pathway.

The DSS model of GI inflammation has been thoroughly characterised and has good reproducibility [33, 39]. This polymer alters the inner mucus barrier, facilitating the entrance of bacteria that trigger the inflammatory response [40]. Thus, this model is very interesting to study the interactions between TLRs and the intestinal microflora, and how modulation of these receptors might result in improvement of the inflammatory process. Permeability is increased as soon as 24 hours after initiation of the DSS regime, and reaches its highest level by days 3 and 5, in parallel with the highest cytokine production levels [39]. Hence, we modified the administration schedules previously used (chapter 1) to ensure maximal contact between instilled ligands and IECs and immune cells within the *lamina propria*. Even though this therapeutic approach is quite different

from those aiming to induce tolerance, which are usually based on daily administrations [26], previous work from our group has demonstrated interesting results in suppression of cytokine production following similar administration timings [41]. The data presented in this report support the usefulness of this timing for LPS administration, but raises the question of whether a single instillation could result in improved clinical outcomes that might settle a basis towards the treatment of IBD patients. Nevertheless, prolonged TLR4 signalling is probably detrimental, since transgenic mice that overexpress this receptor in IECs are more susceptible to intestinal inflammation [42].

Assays using lower ligand doses resulted in increased beneficial effects when compared to those elicited by the higher doses. Indeed, 200 µg of zymosan caused significant down-regulation of TNF-α and iNOS transcripts (Fig. 3B-D), and treatment with 200 µg of LPS reduced the colon shortening (Fig. 1E), which is indicative of a reduction of inflammation [41]. Conversely, doses of 500 µg zymosan did not produce remarkable effects, while 500 µg LPS caused transient weight loss and increased DAI during days 3 and 4 (Fig. 4B and C). These transient signs of inflammation were probably due to the activation of TLR4 from hematopoietic origin, as DSS-treated animals show impaired barrier functions [39, 40], but could be also secondary to an inflammatory process within the small intestine [28]. This last possibility, however, is less likely, since CMC+LPS500-treated animals did not show any sign of transient inflammation during the whole experimental period. Interestingly, a second administration of 500 µg LPS on day 4 did not enhance these signs of inflammation, but improved them as shown by attenuated DAI (Fig. 4C and D). This finding might be suggestive of an induction of tolerance, but given the transcriptional profiles observed in DSS-treated groups and the effects in IEC proliferation, it is probably more related to the induction of a proliferative burst [43] through epithelial TLR4 stimulation.

Deregulation of TLR2 and TLR4 expression during inflammation is a major finding in IBD patients and mouse models of colitis [5-7, 44]. Therapeutic approaches addressing down-regulation of pro-inflammatory cytokines such as TNF-α, IL-1β or interferon-γ have been accompanied by reduction in TLR2 and TLR4 mRNA levels [41, 44]. This suggests that these cytokines amplify TLR expression and that reduction of disease severity decreases TLR expression to homeostatic levels. In this regard, our results show that modulation

of TLR2 and TLR4 was more important in LPS-treated colitic groups (Figs. 3A-B, and Fig. 6A-B), which additionally showed significant amelioration in DAI (Figs. 1D and 4D), colon length (Fig. 1E) and histological score (Figs. 2B and 5B). This suggests that expression of these receptors is associated to the severity of the disease, and not to a selective recognition of the TLR ligand. Unexpectedly, DSS+LPS200-treated animals did not show significant reduction of TNF-α and iNOS expression when compared to DSS+Zym200-treated mice, which in turn did not display clinical improvement. Thus, cytokine expression levels cannot be taken as a single reliable indicator of the progression of the disease, since the overall response elicited by TLR ligands relays on their interaction with different cell types. Hence, while activation of both TLR2 and TLR4 would contribute to mucosal healing by inducing antiapoptotic, protective and proliferative events in IECs [10, 12, 22-24], recognition of LPS by immunocytes would elicit more pronounced pro-inflammatory responses than zymosan (Supplementary Fig. 1A and B). Therefore, in zymosan-treated colitic mice low protective responses in IECs (Fig. 7A and D) combined with mild pro-inflammatory activation of immunocytes would terminate in a reduced expression of cytokines, which would not be important enough to improve the disease phenotype. Conversely, in LPS-treated colitic mice, high preservation of the crypt architecture would reduce exposure to luminal bacteria and diminish water and blood loss, thus ameliorating the signs of colitis. In parallel, LPS-mediated activation TLR4 in immunocytes would increase inflammatory transcripts to similar levels as those observed in DSS-treated animals. This explanation is additionally supported by the fact that, upon DSS-administration, treatment with a TLR4 blocking antibody results in reduced inflammatory infiltrate and decreased release of TNF-α and IL-6, but animals experience increased lethality during the recovery phase of inflammation due to impaired IEC proliferation and mucosal healing [24]. Therefore, special care must be taken concerning the target cell type when designing new therapeutic approaches, as the same receptor can act in very diverse ways in different cell populations.

Transcript levels of the immunomodulatory cytokine TGF-β, which is up-regulated during tolerogenic and regulatory responses [21, 37, 45], were not increased after zymosan or LPS treatment. Such finding, taken together with the reduction in TLR expression irrespective of the administered ligand, suggests that tolerance induction was not

achieved by these treatments. Conversely, the reported increases in crypt length and in proliferative marker expression within IECs after TLR4 stimulation show that these cells play a pivotal role in the disease improvement we report. Indeed, among strategic interventions addressing administration of TLR ligands, those achieving beneficial results relay on induction of protective effects on IECs [12, 26, 46]. Contrastingly, management of the immune system has been better accomplished through systemic injections of TLR antagonists or neutralising antibodies, further remarking the different outcomes obtained when targeting different cell types [24, 47].

So far, therapeutic improvement had been only achieved with administration of the TLR2/TLR1 synthetic ligand Pam3CSK4 [12]. This ligand activates antiapoptotic pathways in primary IECs and has been shown to increase epithelial resistance through strengthening of tight junction proteins [12, 48], thus maintaining barrier integrity. In our experiments, however, the TLR2 ligand zymosan did not exert similar effects, perhaps due to the different timing of experiments, route of administration or different selectivity of target receptors (TLR2/TLR6). Contrastingly, instillation of LPS in a therapeutic regime did result in ameliorating effects, which indicates that targeting TLR4 can also be considered a successful approach to the therapeutic management of acute colitis. To this point, TLR4 modulation had been only addressed in prophylactic studies. Repeated administration of LPS before DSS treatment down-regulated TLR4 [27] or restored cytoprotective factor secretion [22], attenuating the signs of colitis. The precise mechanism of action driving therapeutic effects of LPS needs to be further described, but on the basis of the available data it seems to be associated with epithelial preservation and promotion of IEC proliferative responses (Figs. 7 and 8).

COX-2 plays an important role in production of inflammatory mediators, as well as in secretion of cytoprotective substances such as PGE2. LPS induces PGE2 in IEC lines, and expression of COX-2 and PGE2 is decreased in TLR4$^{-/-}$ mice [23]. In addition, administration of hyaluronic acid, another TLR4 ligand, improves DSS-induced colitis in wild type, but not in TLR4$^{-/-}$ or COX-2$^{-/-}$ mice [25]. All these findings supported that COX-2 could be orchestrating the responses we observed. However, DSS+LPS-treated animals did not show increases in protein or in transcript levels of this molecule, suggesting that the preservation of crypt architecture we observed is not mediated by this protein

(Fig. 7), even though transient up-regulation on days following ligand instillation cannot be dismissed.

TLR4 has additionally been implicated in regulation of epithelial proliferation. MyD88$^{-/-}$ and TLR4$^{-/-}$ mice undergoing DSS treatment display shortened crypts and decreased proliferation of the IEC progenitor lineages [34, 38, 49], whereas mice overexpressing TLR4 in the epithelium have longer crypts and more proliferating IECs [13]. According to these data, our results show that DSS+LPS-treated mice displayed enlarged colonic crypts (Fig. 8A and B) and had dramatically increased PCNA protein levels (Fig. 8C and D). In the same vein, PCNA staining was usually concentrated in colonocytes (Fig. 8Ei, Eii and F), demonstrating that these were the main proliferating cells. Interestingly, PCNA overexpression in LPS-treated mice was only detected after DSS-treatment, which might indicate that epithelial disruption could favour contact between LPS and stem cells from the crypt bottom, perhaps promoting their expansion. Indeed, mice overexpressing TLR4 in IECs have increased numbers of progenitor cells [13]. Moreover, strong cyclin D1 positive staining was observed in the DSS-treated groups, evidencing that β-catenin signalling was activated at day 7 in proliferating crypts (Fig. 8G and H). Unfortunately, transcript levels must still be determined to compare expression of this β-catenin downstream target between DSS- and DSS+LPS-treated mice. It is reasonable to hypothesise that DSS+LPS-treated mice will have increased cyclin D1 transcripts, as activation of the Wnt/β-catenin canonical pathway through different mechanisms has been associated with reduced signs of inflammation associated to IEC proliferation [50, 51], as we observed in this experimental group. Furthermore, it has been shown that prolonged exposure to pro-inflammatory cytokines leads to inhibition of the Wnt/β-catenin signalling, reducing IEC proliferation and increasing apoptosis from days 4 to 6 [43]. Since administration of 500 µg LPS at day 4 modified the severity of the resulting inflammation, we might speculate that a proliferative burst was induced at this time point through TLR4-mediated activation of this proliferative pathway [13], thereby ameliorating the effects of colitis.

In conclusion, our study demonstrates that instillation of LPS during colitis results in improved clinical signs due to induction of epithelial proliferative responses. Our findings are consistent with the model proposed by the group of Abreu, in which TLR4 would directly activate the Wnt/β-catenin pathway [13], driving a proliferative stimulus

that would counteract the severe inhibition of epithelial proliferation caused by prolonged exposure to pro-inflammatory cytokines after DSS treatment [43, 50, 51]. Nevertheless, thorough research must still be performed to confirm that this is the main mechanism involved in such response and to evaluate possible crypt architecture irregularities derived from Wnt uncontrolled activation [51]. Additionally, for future purposes in development of therapeutic modulation of TLR4, important refinement should be done in administration protocols and epithelial cell targeting, in order to decrease the inflammatory responses elicited by LPS in immunocytes and to reduce the risk of initiating pro-tumorigenic responses [13, 42].

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Supplementary materials and methods

Reagents

Culture media, foetal bovine serum and antibiotics were from Life Technologies (El Prat de Llobregat, Spain). Two different types of zymosan were used in macrophage stimulation for comparison of their effects; purified zymosan used in the experiments of the first chapter (Zym_1) was purchased from Invivogen (San Diego, USA), whereas a different batch of this TLR2 ligand, used in the experiments of chapter 2 (Zym_2), was obtained from Sigma (Madrid, Spain). For use in cell culture, zymosan powder was sterilised in 100% ethanol, which was subsequently eliminated by centrifugation, aspiration and evaporation. Remaining zymosan was resuspended in phosphate-buffered saline. Lipopolysaccharide (LPS) was prepared by 1:1 mixture of LPS from E. coli O55:B5 and S. typhosa, both purchased from Sigma. Antibodies used for immunostaining of cyclooxygenase (COX)-2 were rabbit polyclonal anti-COX-2 antibody (1:500), from Abcam (Cambridge, UK), and biotinylated goat anti-rabbit IgG (1:200), from Life Technologies. RAW 264.7 mouse peritoneal macrophages were purchased from the American Type Culture Collection.

Cell culture stimulation

RAW264.7 cells, grown to confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum, were scraped from flask and seeded in 24-well plates (Falcon, BD, San Agustín de Guadalix, Spain) at a density of $3x10^5$ cells cm^{-2}. Such cells were grown to confluence for 2 more days, and then stimulated in serum-free DMEM medium for 24 hours with 500 ng of LPS, Zym_1 or Zym_2. Additional doses of 100 µg of both zymosan batches were also used to compare nitric oxide (NO) production of each. At the end of stimulation supernatants were harvested, centrifuged and frozen until proinflammatory mediator determinations.

TNF-α ELISA and nitrite quantification assays

Culture supernatants were assayed for tumour necrosis factor (TNF)-α by means of the TNF-α OptEIATM ELISA Set (from BD) following manufacturer's instructions. Absorbance readings were performed at 450 nm in an Infinite 200 plate reader (Tecan, Schweiz, Switzerland). Final values were related to the total protein amount of each

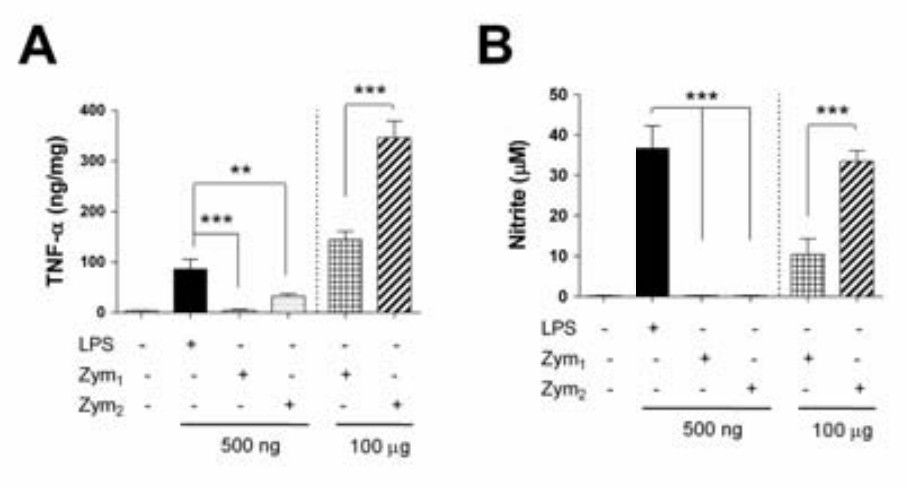
sample, which was determined by using the BCA protein assay kit (Pierce, Rockford, USA).

NO release to culture supernatants was quantified by the measurement of nitrite, one of its stable reaction products, by means of the Griess reagent. Equal volumes of supernatant and Griess reagent (Sigma) were mixed in a 96-well plate and incubated for 10 min in the dark. Optical density of the reaction was read at 550 nm in an iEMS MF plate reader (Labsystems, Helsinki, Finland). NO concentrations in experimental samples were calculated from a standard concentration-absorbance curve.

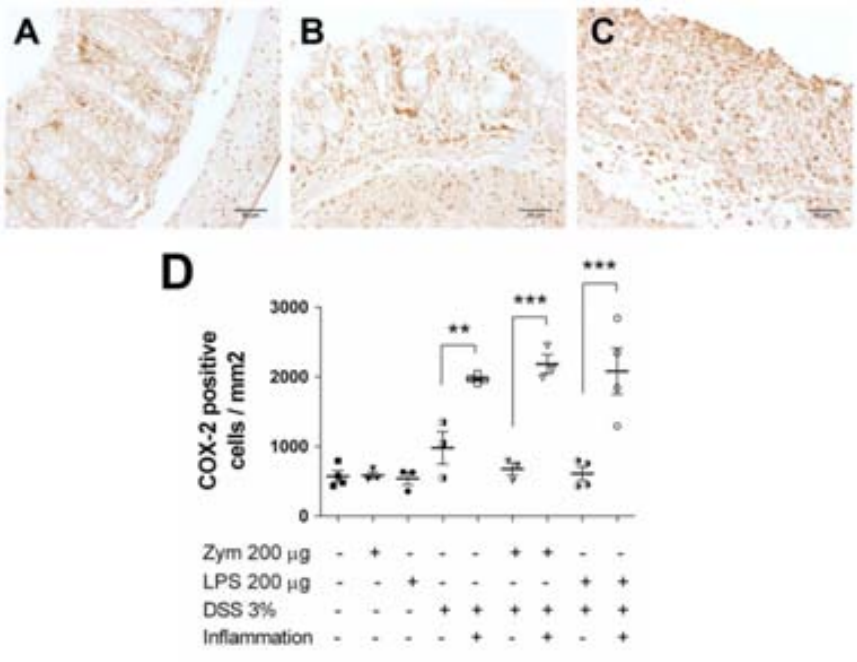
COX-2 immunoreactive cell counting

Immunohistochemistry for COX-2 was performed as described in chapter 2 for determination of proliferating cell nuclear antigen and cyclin D1. For quantitative determination of COX-2-expressing cells, a minimum of 10 pictures from different representative mucosal areas of each animal colon slide were taken by means of a Nikon Eclipse 90i microscope interfaced to a DXM 1200F camera (Nikon Corporation, Barcelona, Spain) at a magnification of 200x. COX-2-immunoreactive cells in the *lamina propria* and mucosa were counted by manually defining regions of interest and applying cell counting filters based on protein expression intensities by means of the ImageJ software (National Institutes of Health, Bethesda, USA).

Supplementary figures



Supplementary Figure 1 – Differential proinflammatory mediator release in RAW 264.7 macrophage cultures upon LPS and zymosan batch stimulation. A) TNF-α production (n=6; ***P*<0.01 and ****P*<0.001; one-way ANOVA followed by Bonferroni's post-hoc test for selected pairs of columns). **B)** NO production (n=6; ****P*<0.001; one-way ANOVA followed by Bonferroni's post-hoc test for selected pairs of columns).



Supplementary Figure 2 – COX-2 immunoreactivity and positive cell counts per area unit. A) Representative micrograph of an area corresponding to the mucosa of wild type mice. **B)** Representative micrograph of an inflammation-uninvolved area from DSS-treated mice. **C)** Micrograph from an inflammation-involved area from a DSS-treated mouse. Scale bars correspond to 50 µm. **D)** Total immunoreactive-cell counts per square mm of mucosa (n=3-4; ***P*<0.01 and ****P*<0.001; one-way ANOVA followed by Bonferroni's post-hoc test for selected pairs of columns).

Chapter 3

Role of macrophages and enteroglia in the enteric nervous system responses to bacterial motifs via Toll-like receptors

Abstract

Toll-like receptors (TLR) are expressed in neurons and glial cells (EGC) of the intrinsic plexuses of the enteric nervous system (ENS); their role, however, is still to be elucidated. The aim of this study was to characterise the expression and functionality of TLR2/4/9 in the ENS. We studied TLR distribution in murine ENS plexuses, as well as expression changes during experimental inflammation. Assessment of receptor functionality was performed in a rat ENS primary culture and in a rat EGC line upon stimulation with different TLR ligands. TLR2/4/9 were identified in the intrinsic plexuses of the murine ENS in basal conditions, whereas up-regulation of TLR4 was associated to gliosis in uninflamed areas during experimental colitis. Treatment of macrophage-containing ENS culture with TLR ligands led to activation of the nuclear factor (NF)-κB pathway, but only TLR4 challenge induced the release of interleukin (IL)-6, tumour necrosis factor alpha, monocyte chemoattractant protein (MCP)-1 and nitric oxide. Costimulation with TLR ligands resulted in a synergistic increase of cytokine secretion and promoted chemoattraction of RAW 264.7 macrophages. In contrast, in the EGC line, activation of NF-κB was only achieved after TLR4 stimulation, which subsequently enhanced IL-6 and MCP-1 secretion, as well as chemoattraction of macrophages. No interactions were identified in these cells during TLR costimulation. These results demonstrate that reactive EGCs contribute to inflammation through TLR4 recognition, but their involvement in inflammation must not be over-estimated, since activation of resident *muscularis* macrophages might account for most of the responses observed in ENS cultures.

Keywords

Enteric glial cell, *muscularis* macrophage, enteric nervous system, Toll-like receptor 4, DSS colitis, inflammation, chemoattraction.

Introduction

The enteric nervous system (ENS) is the largest component of the peripheral nervous system and the most complex, since it can function without central nervous system (CNS) inputs. It is constituted by two cell types, neurons and enteric glial cells (EGC), organized in two major ganglionated plexuses, namely the submucosal (Meissner's) and the myenteric (Auerbach's) plexuses [1]. Surrounding the ENS plexuses, myofibroblasts, mast cells and resident macrophages interact with neurons and EGCs, modulating their activity through secretion of different mediators [2, 3]. The ENS controls motility, secretion and blood flow in the gastrointestinal (GI) tract, participates in maintenance of the epithelial barrier and modulates various processes of the local immune system [1, 4]. Although a number of studies have demonstrated its capacity to undergo structural and phenotypic plastic changes during inflammatory responses [5], growing evidence indicates that the ENS is not only a bystander, but an active player in GI inflammation. Indeed, it has been shown that the number of enteric neurons determines the severity of the inflammatory process in two models of chemically-induced colitis [6], suggesting a pro-inflammatory role for neurons. Moreover, biopsies from patients with inflammatory bowel disease (IBD) often display dense perineural inflammation and expression of major histocompatibility complex (MHC) class II in EGCs, which is positively correlated with the extent of leukocyte infiltration [7].

Neuro-inflammation has been extensively studied in the CNS, where two resident cell types orchestrate the innate immune response against invading microorganisms: myeloid-derived microglial cells and neuroectodermal astrocytes [8]. In the ENS, the involvement of resident macrophages and *muscularis* macrophages - the counterparts of microglial cells in the GI tract - during innate immune responses has been thoroughly described [3, 9]. Nevertheless, there is increasing interest in the study of the immune functions of EGCs, since they share embryologic, phenotypic and functional properties with astrocytes [10], which are known to secrete cytokines and chemokines during CNS inflammation [11-13]. In addition, increases in the EGC markers glial fibrillary acidic protein (GFAP) and the calcium-binding protein S100β have been associated with pathologies such as ulcerative colitis (UC) [14]. EGCs express MHC class II in specimens from IBD patients [15] and release cytokines and nitric oxide (NO) upon stimulation with IL-1β,

tumour necrosis factor (TNF)-α or combinations of interferon (IFN)-γ and lipopolysaccharide (LPS) in culture conditions [16-18].

Toll-like receptors (TLR) are transmembrane receptors that contain an extracellular domain, which allows for selective detection of different microorganism-associated molecular patterns (MAMP) and damage-associated molecular patterns (DAMP), and a cytoplasmic signalling domain. Upon MAMPs binding, and after homo or hetero-dimerisation of the receptor, the cytoplasmic domain recruits different adapter proteins to trigger a variety of signalling pathways that ultimately activate transcription factors such as nuclear factor-κB (NF-κB), activating protein-1 (AP-1) and IFN regulatory factors, which in turn promote the production of pro-inflammatory cytokines [19, 20]. TLRs are expressed in most human tissues [21] including the GI tract [22], where they have been proposed to mediate the cross-talk between host cells and commensal microflora [23] due to the key role they play in the innate immune response and the shaping of the adaptive immune responses [20]. In addition, TLRs have been also associated to pathologic conditions such as IBD [24]. Expression of such receptors in CNS astrocytes and neurons [11, 12, 25] led some authors to hypothesize that their presence in the ENS could further link this tissue to GI inflammation [26, 27]. Although Barajon *et al* reported expression of TLR3/4/7 in enteric neurons and EGCs in the myenteric plexuses of the mouse intestine [27], data about the putative role of TLRs in the ENS are scarce. Recent work by Anitha and colleagues has shown that TLR4 signalling promotes survival of intestinal nitrergic neurons [28], whereas the presence of different TLRs in EGCs supports their involvement in microorganism and damage-induced inflammatory responses [18, 29]. Indeed, Esposito *et al* have demonstrated a direct participation of EGCs in experimental inflammation through activation of the S100β/TLR4 axis, settling the first evidence connecting EGC recognition of DAMPs with GI inflammation [29].

To characterise the TLR-mediated responses of the ENS and, specifically, of EGCs, we first studied TLR2/4/9 expression and distribution in mouse colon and in different cell cultures in basal and inflammatory conditions. Second, we addressed *in vitro* the functional responses of the ENS and EGCs upon single or combined stimulation with MAMPs. Finally, in order to evaluate a potential role in attraction of immunocytes, we evaluated the chemotactic activity of supernatants from MAMP-stimulated ENS or EGC cultures on RAW 264.7 macrophages. Our results demonstrate that 1) *In vivo*, TLR2/4/9

are expressed within the ENS in physiologic conditions, 2) activation of EGCs in the intrinsic plexuses during inflammation is accompanied with TLR4 up-regulation, 3) ENS and EGC cultures are differentially activated depending on the specific subtype of TLR stimulated, 4) TLR expression is also up-regulated *in vitro* under inflammatory conditions, 5) potential interactions between agonists and different cell types may result in enhanced responses, and 6) LPS-stimulated ENS and EGC supernatants have chemoattractant properties on macrophages. Overall, our findings give further support to the conception that TLRs might mediate the immune functions of the ENS, and suggest that recognition of MAMP by EGCs could be involved in promotion of immune cell homing after bacterial invasion of the GI mucosa.

Materials and Methods

Reagents and antibodies

All culture media, foetal bovine serum, antibiotics, N-2 supplement and 4',6-diamidino-2-phenylindole (DAPI) were from Life Technologies (El Prat de Llobregat, Spain). Trypsin, DNase I, gelatine and Bay 11-7082 were from Sigma (Madrid, Spain). The synthetic diacylated lipopeptide Pam2CSK4, a TLR2/6 specific agonist, was purchased from Invivogen (San Diego, USA). LPS stimulation of TLR4 was performed with a mixture 1:1 of LPS from E. coli O55:B5 and S.typhosa, both purchased from Sigma. Phosphorothioate-modified type B CpG oligonucleotides (ODN) 1826 5'-TCCATGACGTTCCTGACGTT-3' and 1826 control (cODN) 5'- TCCATGAGCTTCCTGAGCTT-3' were synthesised by Tib-Molbiol (Berlin, Germany) and used to stimulate TLR9. The primary antibodies used for immunostaining were rabbit polyclonal anti-TLR2 (1:100; Imgenex, San Diego, USA), rabbit polyclonal anti-TLR4 (1:50; Imgenex), mouse biotinylated monoclonal anti-TLR9 (1:50; Imgenex), chicken polyclonal anti-GFAP (1:500, Antibodies-online, Aachen, Germany) and mouse monoclonal anti-S100β (1:1000, Abcam, Cambridge, UK). Secondary antibodies used were biotin-XX goat anti-rabbit IgG (1:200, Life Technologies) for immunohistochemistry, and Alexa Fluor 568 goat anti-rabbit IgG (1:500, Life Technologies), Alexa Fluor 594- conjugated streptavidin (1:250, Life Technologies), CF488A donkey anti-chicken IgY (1:2000) and CF488A donkey anti-mouse IgG (1:500, both from Biotium, Hayward, USA) for immunofluorescence. For western blotting, rabbit monoclonal anti-phospho-IκBα (1:1000; Cell Signaling Technology, Danvers, USA), mouse monoclonal anti-GFAP (1:5000; Sigma), mouse monoclonal anti-β-actin (1:5000; Sigma), horseradish peroxidase (HRP)-linked goat anti-rabbit IgG (1:10,000; Cell Signaling Technology) and HRP-linked sheep anti-mouse IgG (1:5000 for detection of GFAP and 1:100,000 for β-actin; GE Healthcare, Barcelona, Spain) antibodies were used.

Animals

For in vivo experiments, 12-week old C57Bl/6 female mice were purchased from Charles River (Les Oncins, France) and housed in specific pathogen-free conditions, under a controlled temperature (20±2ºC) and photoperiod (12h/12h light-dark cycle), with free access to food and water. Middle-distal colitis was induced by administration

of 3% dextran sulphate sodium salt (DSS) (36-50 kDa; MP Biomedicals, Illkirch, France) in drinking water for 5 consecutive days. A disease activity index (DAI) was build up to follow up colitis as described in Supplementary data. Seven days after induction of colitis, mice were euthanized by cardiac puncture exsanguination under isoflurane (Isobavet®; Schering-Plough, Sant Cugat del Vallès, Spain) anaesthesia. Colons were removed, rinsed in ice-cold phosphate buffered saline (PBS) and cut into longitudinal pieces, which were further fixed in 4% paraformaldehyde or kept in RNAlater solution (Ambion, Applied Biosystems, Alcobendas, Spain). RNAlater-preserved samples of middle-distal colon where later dissected under a Leica Wild M3Z stereomicroscope (Leica, Heerbrugg, Switzerland) to separate mucosal and submucosal layers (SBL; including the submucosal ENS plexus) from muscular layers (ML; including the myenteric ENS plexus).

For in vitro experiments, pregnant Sprague-Dawley rats purchased from Charles River were killed by CO_2 inhalation followed by cardiac puncture exsanguination. Pregnant uteri were removed and kept in ice-cold PBS for further dissection.

All animal procedures performed were approved by the Ethical Committee of the Universitat Autònoma de Barcelona.

Cell cultures

Isolation and culture of rat ENS primary culture was performed as described elsewhere [30]. Briefly, intestines of rat embryos (E16) were removed and finely diced in PBS. Tissue fragments were digested with trypsin and DNase I, and cells obtained were counted and seeded at a density of $2.4x10^5$ cells cm^{-2} on 48-well plates, previously coated with a 0.5% gelatine solution in sterile PBS. Stimulation was performed for 24 hours after 15 day-culture in serum-free medium (DMEM-F12 (1:1)) containing 1% of N-2 supplement.

The JUG2 EGC line was kindly donated by Dr. Michel Neunlist. This cell line was obtained from rat ENS culture by trypsinization of cells morphologically resembling EGCs by means of a cloning cylinder. These cells were seeded in a flask and, after 1 month, they were immunoreactive for glial but not for neuronal or myofibroblast markers [31]. The macrophage cell line RAW 264.7 was purchased from the American Type Culture Collection.

JUG2 EGCs and RAW 264.7 cells were cultured in DMEM supplemented with 10% heat-inactivated foetal calf serum. JUG2 cells were seeded at a density of 50,000 cells cm^{-2} in

24 or 48-well plates (Falcon, BD, San Agustín de Guadalix, Spain), and grown to confluence for 2 days before stimulation experiments.

Stimulation experiments were performed in ENS primary culture or JUG2 EGCs for 24 hours with either 100 ng/mL Pam2CSK4, 100 ng/mL LPS, 1 µM ODN 1826 or 1 µM cODN 1826. For NF-κB inhibition experiments, cultures were pre-treated for 1 hour with 15 µM Bay 11-7082 before MAMP-stimulation.

For costimulation experiments, ligands were added to cultures for a total 24 hour-stimulation according to the following protocol: 1) Pre-LPS: Pam2CSK4 or ODN 1826 were added 4 hours before LPS; 2) Co-LPS: Pam2CSK4 or ODN 1826 were added simultaneously with LPS; and 3) Post-LPS: Pam2CSK4 or ODN 1826 were added 4 hours after LPS. Comparison between expected additive effects and measured effects of TLR ligand combinations was calculated according to the model of functional interaction, represented by the following equation as described in [32]:

$$E(ODN+LPS)_{expected} = E(ODN)_{measured} + E(LPS)_{measured} – E(ODN)_{measured} * E(LPS)_{measured}$$

Immunohistochemistry and immunofluorescence

For immunohistochemistry, paraffin-embedded tissues were deparaffinized and treated in boiling citric acid during 15 minutes for antigen retrieval. Endogenous peroxidase activity was blocked with a 5% solution of hydrogen peroxide in PBS, and avidin and biotin with the Avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA, USA). Slides were then incubated for 1 hour in PBS containing 5% bovine serum albumin and 1% Triton X-100. Overnight incubation at 4°C with TLR2, TLR4 or TLR9 antibodies was followed by addition of biotinylated secondary antibodies for TLR2 and TLR4, or addition of the Vectastain ABC kit for detection of biotinylated TLR9. Slides were revealed with the DAB peroxidase substrate kit (both from Vector Laboratories) and examined through a Nikon Eclipse 90i microscope interfaced to a DXM 1200F camera (Nikon Corporation, Barcelona. Spain). Pictures were taken using the ACT-1 software (Nikon Corporation).

For immunofluorescence, fixed whole-mount fragments of mouse submucosa or ENS cultures and JUG2 EGCs grown on cover-slips were blocked for 1 hour in PBS containing 4% horse serum, 1% Triton X-100 and 0,01% NaN3. Mouse submucosal whole-mounts were incubated overnight at 4°C with mixtures of either TLR2 or TLR4 with GFAP antibody, whereas ENS cultures were incubated with mixtures of TLR2 or TLR4 with

S100β antibody. Secondary antibodies to rabbit IgG, chicken IgY or mouse IgG were used to detect bound primary antibodies. For TLR9 immunodetection, samples were treated with the Avidin/biotin blocking kit (Vector Laboratories) followed by blocking in PBS with 4% horse serum. Primary antibody was added overnight alone or in a mixture with GFAP antibody, and fluorochrome-conjugated streptavidin was used to stain bound TLR9 antibody. Colocalisation of TLR9 and S100β was performed by sequential staining of S100β and its secondary anti-mouse IgG antibody followed by TLR9-streptavidin, to avoid cross-linking of the anti-mouse antibody with the TLR9 antibody.
All samples were mounted in Vectashield aqueous anti-fading mounting medium (Vector Laboratories) and analysed under a Zeiss LSM 700 confocal laser microscope (Carl Zeiss, Madrid, Spain).

Real-time RT-PCR analysis

Total RNA from JUG2 EGCs and ENS culture was extracted using the RNeasy Mini Kit (Qiagen, Las Matas, Spain), quantified by optical densitometry and assessed for integrity by on-chip gel electrophoresis with the Experion™ System (Bio-Rad Laboratories, el Prat de Llobregat, Spain). 100 ng of RNA were retro-transcribed by using the Transcriptor First-strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany) for reverse-transcriptase polymerase chain reaction (RT-PCR). Primer sequences listed in Table 1 were designed to span introns using the Universal ProbeLibrary Assay design Center (https://roche-applied-science.com/sis/rtpcr/url), and checked for specificity through BLAST search. PCR amplifications were performed using the LC480 SYBRGreen I Mastermix (Roche Applied Science) according to manufacturer's protocol, and run on a LightCycler 480 II instrument (Roche Applied Science). Absence of coamplification products was assured by generating a final melting curve for each reaction and by loading PCR products on a denaturing 2% agarose gel, stained with SYBR safe (Life Technologies) and visualized under UV transillumination. Specificity of the primers was also determined by sequencing these amplification products. mRNA level of expression of the genes of interest was corrected to that of the housekeeping genes S6 or β-actin and calculated by the ΔΔCt method.
In order to compare mRNA expression levels of the receptors in basal conditions, absolute mRNA levels were estimated by determining the difference between the cycle threshold (Ct) of the target receptor and the Ct of the housekeeping gene, as described

elsewhere [11, 18]. According to their ΔCt to the S6 gene, genes were classified as high-expressed (ΔCt less than 5 cycles), intermediate-expressed (ΔCt from 5 to 15 cycles), low or rare-expressed (ΔCt superior to 15 cycles) and undetectable (ΔCt superior to 40 cycles).

Gene	Sense primer	Antisense primer	Reference
mβ-actin	CTAAGGCCAACCGTGAAAAG	ACCAGAGGCATACAGGGACA	ENSMUST00000031564.4
mSDHA	GCTTGCGAGCTGCATTTG	CATCTCCAGTTGTCCTCTTCCA	NM_023281.1
mTLR2	ACCGAAACCTCAGACAAAGC	CAGCGTTTGCTGAAGAGGA	NM_011905.3
mTLR4	GGACTCTGATCATGGCACTG	CTGATCCATGCATTGGTAGGT	NM_021297.2
mTLR9	TCCCAACATGGTTCTCCGTC	CCAGAGTCTCAGCCAGCACT	NM_031178.
rS6	CCAAGCTTATTCAGCGTCTTGTTACTCC	CCCTCGAGTCCTTCATTCTCTTGGC	NM_017160
rTLR2	CAGATGGCCAGAGGACTCA	AATGGCCTTCCCTTGAGAG	ENSRNOT00000013025.3
rTLR4	GGATGATGCCTCTCTTGCAT	TGATCCATGCATTGGTAGGTAA	NM_019178.1
rTLR9	TCCGTGACAATCACCTCTCTT	GGTCCAGGTCTCGCAGATT	NM_198131.1

Table 1 – List of primers used for real-time RT-PCR analysis. The codes "m" and "r" are used in the gene names to design the target species, mouse or rat, respectively.

Western Blot

Cell cultures were harvested in RIPA lysis buffer (Millipore, Madrid, Spain) containing 2 mM sodium orthovanadate, phosphatase inhibitor cocktail 3 (Sigma) and a tablet of Complete™ protease inhibitors cocktail (Roche Applied Science). Submucosal and muscular layer protein was precipitated with acetone from the RNeasy Mini Kit used for RNA extraction following manufacturer's instructions. Protein samples (30 µg) were separated on a 10% acrylamide gel containing 0.1% sodium dodecyl sulfate and transferred to a nitrocellulose membrane with the iBlot™ Dry Blotting System (Life Technologies). Membranes were blocked for 1 h at room temperature with 5% non-fat dry milk in Tris-buffered saline (100 mM NaCl, 10 mM Tris, pH 7.5) with 0.1% Tween 20 (TBST), and incubated overnight at 4°C with primary antibodies in a 5% BSA solution in TBST. Bound antibodies were detected with HRP-conjugated anti-rabbit or anti-mouse antibodies, and visualized by enhanced chemiluminescent detection (ECL advance, GE Healthcare). Membranes were stripped for 15 min in Reblot buffer (Millipore), followed by extensive washing in TBST before reblocking with 5% non-fat dry milk in TBST and

reprobing for β-actin determination. Bands were imaged in a LAS-3000 Imager (Fujifilm, Tokyo, Japan) and quantified with Multigauge 3.0 software (Fujifilm). To allow comparison between different membranes, the density of the bands was referred to that of untreated controls and normalized to the amount of β-actin in the same sample.

IL-6, TNF-α and MCP-1 ELISA

Culture supernatants were centrifuged, aliquoted and frozen, and further assayed with the corresponding BD OptEIATM ELISA Sets (BD), following manufacturer's instructions. Final cytokine or chemokine values were related to the total protein amount of the sample, which was determined by using the BCA protein assay kit (Pierce, Rockford, USA).

Nitrite quantification assay

NO release to culture supernatants was quantified by the measurement of nitrite, one of its stable reaction products, by means of the Griess reagent. Equal volumes of supernatant and Griess reagent (Sigma) were mixed in a 96-well plate and incubated for 10 min in the dark. Optical density of the reaction was read at 550 nm in an iEMS MF plate reader (Labsystems, Helsinki, Finland). NO concentrations in experimental samples were calculated from a standard concentration-absorbance curve.

Migration studies

24 hours after stimulation of ENS primary culture with MAMPs, conditioned supernatants were centrifuged, placed into 24-well plates and left to equilibrate for an hour with the transwell insert. Then, 100,000 RAW 264.7 macrophages were seeded in the upper chamber of the 8 µm-pore transwell inserts, and allowed to migrate for 4 hours at 37°C and 5% CO_2. After fixation in 4% paraformaldehyde, cells on the upper surface of the transwell membrane were removed by rubbing with a sterile cotton swab, and cells on the lower surface were stained with DAPI. The average number of migrating cells was determined by counting 8 fields per membrane at 100x under a Carl Zeiss Axioskop 40 FL epifluorescence microscope equipped with a Zeiss AxioCam MRm camera (Carl Zeiss, Germany). Each experiment was performed in duplicate.

Statistical analysis

Results are presented as mean values ± S.E.M. of at least three independent experiments. All data were compared using Student's t-test for comparisons of two means, and one-way or two-way ANOVA when comparing more than two groups, followed by Bonferroni's post hoc test (unless otherwise stated). Where stated, randomised block design analysis was performed to minimize the variability due to differences between individual culture responses. Data analysis and plot were performed with GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, USA). Randomised block design analyses were performed with Minitab 15 Statistical Software (Minitab Inc., Pennsylvania, USA). A P value < 0.05 was considered to be significant.

Results

TLR2/4/9 are expressed in the intrinsic plexuses of the ENS

Expression of TLR2/4/9 was assessed in mouse colon by means of real-time RT-PCR, immunohistochemistry and immunofluorescence. All three receptors were expressed in mucosal and submucosal layers, as well as in muscular layers (Fig.1J). TLR2/4 displayed similar expression: they were immunodetected in epithelial cells, resident leukocytes and eventually in endothelial cells of the submucosal layer (Fig. 1A and B). In the smooth muscle cells within the *muscularis mucosae*, the circular and longitudinal muscular layers and the myenteric plexus, a strong immunoreactivity to these receptors was also observed (Fig. 1D and E, arrows). TLR9, however, was selectively localised in enterocytes from the upper part of the crypts (Fig. 1C), in discrete cells within the submucosal layer and in the ENS plexuses (Fig. 1F).

Whole-mount preparations from the submucosal plexus were additionally prepared in order to assess the cellular localisation of these receptors. TLR2 staining was mainly observed in neuronal fibres and interganglionic bundles (Fig. 1Gi), although a small part of its staining colocalised with the glial marker GFAP (Fig. 1Gii and iii, open arrows). TLR4 was expressed preferentially in neuronal somata as well as in discrete fibres (Fig. 1Hi) but, as described for TLR2, colocalisation with EGCs was also present in discrete areas (Fig. 1Hii and iii, open arrows), indicating that enteric glia can also express these receptors in physiologic conditions. TLR9 immunolabeling was found expressed in both neurons and glia (Fig. 1Ii, ii and iii).

Chemically-induced colitis promotes reactive gliosis and TLR4 over-expression in muscular layers

Administration of 3% DSS in drinking water induced a colitis characterised by body weight decrease, loss of stool consistency and occasional rectal bleeding (summarized in Suppl. Fig. 1B). Macroscopic post-mortem findings included colon shortening (DSS= 6.66±0.14 vs. control= 8.5±0.21 cm; n=8, P<0.001; Suppl. Fig. 1A), whereas microscopic studies revealed extensive leukocyte infiltration and oedema combined with moderate to severe crypt damage (Suppl. Fig. 1E). Inflammation was circumscribed to the mucosal and submucosal layers, as determined by myeloperoxidase (MPO) activity (MPO_{DSS}= 0.45±0.09 vs. $MPO_{control}$= 0.02±0.01 units/mg of wet tissue; n=4, P<0.01;

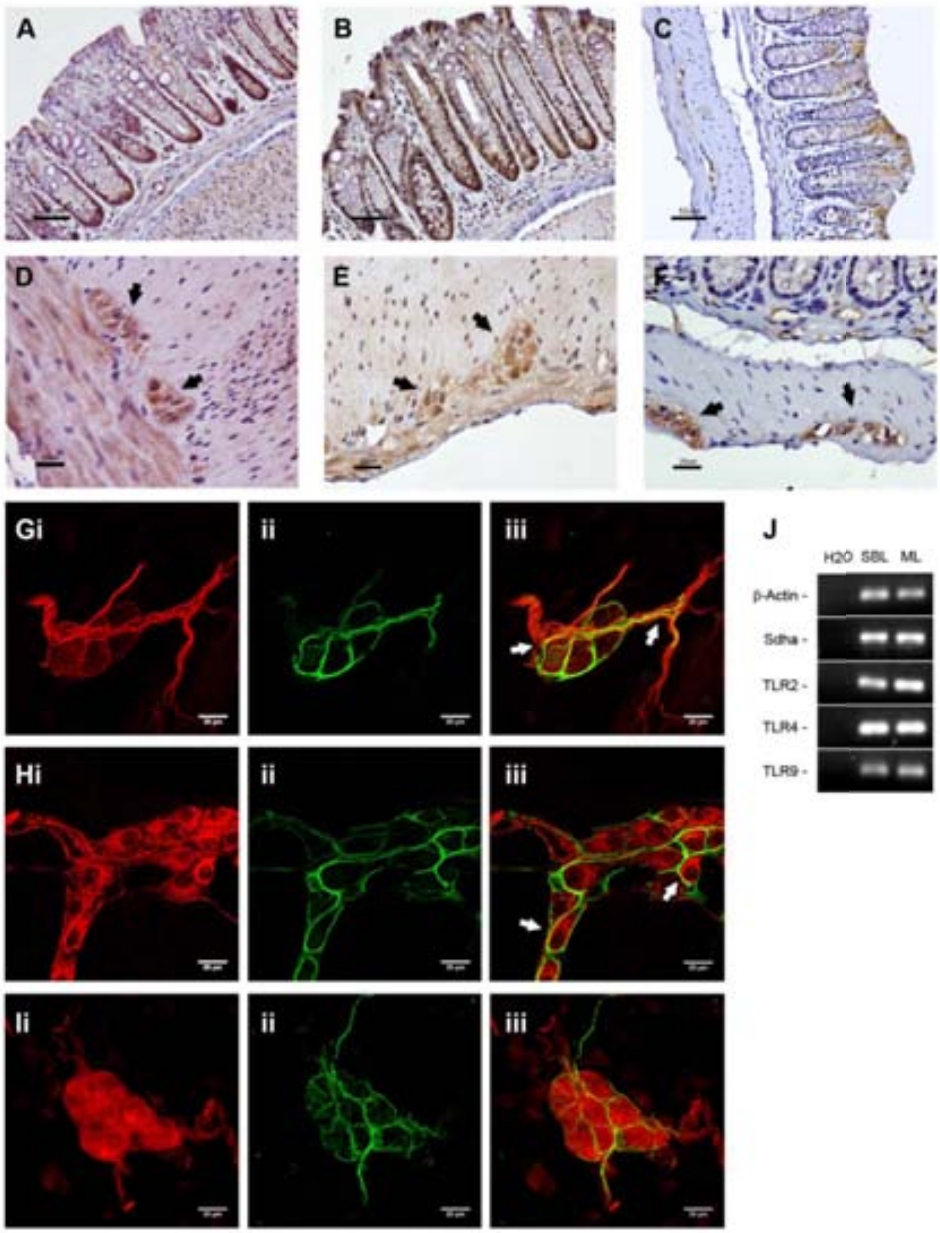


Figure 1 – TLR2/4/9 expression and distribution in submucosal and muscular layers of murine colon. Immunoreactivity for TLR2 **(A)**, TLR4 **(B)** and TLR9 **(C)** in colonic mucosa and submucosa of wild type mice. Scale bars correspond to 50 µm. Detailed view of the positive staining for TLR2 **(D)**, TLR4 **(E)** and TLR9 **(F)** in the myenteric plexus cells (black arrows). Scale bars correspond to 20 µm. Staining within submucosal plexus ganglions **(i)** for TLR2 **(G)**, TLR4 **(H)** and TLR9 **(I)**, EGCs distribution as determined by the GFAP marker **(ii)** and colocalisation images **(iii)**. White arrows point to areas where the EGC marker GFAP colocalises with TLRs. Scale bars correspond to 20 µm. **(J)** Agarose gel showing specific products of real-time RT-PCR for the assayed genes in submucosal layers (SBL) and muscular layers (ML); water was used as no-template control (H2O).

Suppl. Fig. 1G), inducible NO synthase (iNOS; $iNOS_{DSS}$= 2.56±0.58 vs. $iNOS_{control}$= 1.15±0.09 folds; n=4, *P*<0.05; Suppl. Fig. 1H) and cyclooxygenase (COX)-2 expression ($COX\text{-}2_{DSS}$= 8.19±1.37 vs. $COX\text{-}2_{control}$= 2.06±0.64 folds; n=4, *P*<0.01; Suppl. Fig. 1I). TLR2 and TLR9 were also up-regulated in these layers ($TLR2_{DSS\text{-}SBL}$= 2.07±0.19 vs. $TLR2_{control\text{-}SBL}$= 1±0.04 folds, n=5-6, *P*<0.001, Fig. 2D; $TLR9_{DSS\text{-}SBL}$= 2.97±0.34 vs. $TLR9_{control\text{-}SBL}$= 1.43±0.45 folds, n=5-6, *P*<0.05, Fig. 2D), in coincidence with increased TLR2- and TLR9-expresing leukocytes (Fig. 2A and C) and stronger staining of TLR2 in dysplastic crypts (Fig. 2A).

Although TLR4 was also expressed in homing immunocytes within the submucosa (Fig. 2B), transcripts for this receptor were only up-regulated within the muscular layers ($TLR4_{DSS\text{-}ML}$= 3.44±0.66 vs. $TLR4_{control\text{-}ML}$= 1.11±0.23 folds, n=5-6, *P*<0.01; Fig. 2E). Of note, such up-regulation was neither accompanied by apparent leukocyte infiltration

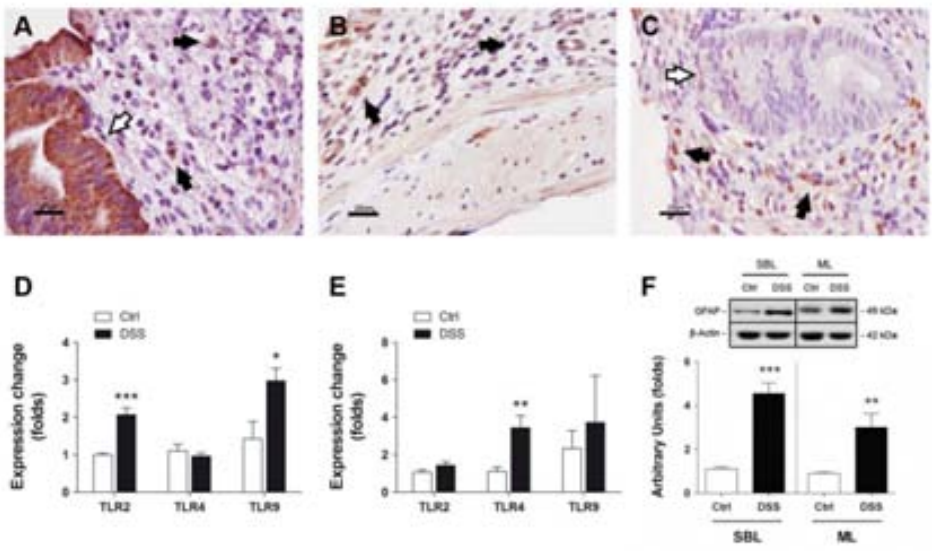


Figure 2 –TLR expression changes and reactive gliosis during experimental colitis. Immunoreactivity for TLR2 **(A)**, TLR4 **(B)** and TLR9 **(C)** in colonic mucosa and submucosa of DSS colitic mice. White arrows point to dysplastic crypts, and black arrows show positive infiltrating leukocytes. Scale bars correspond to 20 µm. **D)** TLR expression changes in mucosal and submucosal layers (n=5-6; DSS-treated (DSS) vs. control (Ctrl) mice, **P*<0.05 and ****P*<0.001, two-tailed t-test for each). **E)** TLR expression changes in muscular layers (n=5-6; ***P*<0.01, two tailed t-test). β-actin was used as housekeeping gene because its variations between Ctrl and DSS-treated animals were minimal compared to those observed for succinate dehydrogenase complex subunit A (SDHA). **F)** GFAP protein expression in submucosal layer and muscular layer of control and DSS mice (n=4; SBL-DSS vs. SBL-Ctrl, ****P*<0.001, ML-DSS vs. ML-Ctrl, ***P*<0.01; one-tailed t-test for each).

(Suppl. Fig. 1E-G) nor alterations in *muscularis* macrophage activation markers (such as iNOS and COX-2; Suppl. Fig. 1H and I) [33]. Contrastingly, increased expression of GFAP ($GFAP_{DSS\text{-}ML}$= 3±0.63 vs. $GFAP_{control\text{-}ML}$= 0.91±0.06 folds; n=4, P<0.01; Fig. 2F), which is considered a hallmark of reactive glial cells [17, 34], was associated to TLR4 over-expression. Taken together, these observations indicate that even when no activation of resident or infiltrating macrophages is occurring, there is an active immune response in the muscular layers involving TLR4 and EGCs, thence suggesting that such cells might participate in the inflammatory process through this receptor.

TLR2/4/9 are expressed in ENS primary culture and the JUG2 EGC line

In order to study the function of the TLRs expressed in the ENS, we used a previously characterised *in vitro* primary culture model [30]. However, in addition to neurons, EGCs and myofibroblasts, we detected ionised calcium binding adapter molecule (IBA)-1-expressing resident macrophages within the culture (Suppl. Fig. 3i). Therefore, an alternative culture of pure EGCs was used to specifically evaluate their contribution to the inflammatory response.

TLR2/4/9 were expressed in both ENS and JUG2 EGC cultures (Fig. 3A). In ENS primary cultures, these three receptors had similar expression levels and, according to previous work [11, 18], the three genes could be categorised as intermediate-expressed genes (Fig. 3B). Distribution of TLRs in ENS culture revealed similarities with ganglia from the mouse submucosal plexus. TLR2 labelling was intensively accumulated in neuronal somas and nerve bundles and, to a lesser extent, in EGC cytoplasm and perinuclear areas (Fig. 3Di-iii). Resident macrophages were also labelled with TLR2 (data not shown). The highest TLR4-immunoreactivity was observed in neuronal somas (Fig. 3Ei) and discrete nerve fibres, while TLR9 was mainly located in glial processes, and less intensively in neurons (Fig. 3Fi-iii).

Although colocalisation with EGCs was not observed for TLR4 in ENS cultures, in JUG2 EGCs TLR4 was the most expressed gene, followed by TLR9 and TLR2 (Fig. 3C). The expression of the three genes was intermediate, although TLR2 expression levels were the lowest observed (ΔCt=14.4 cycles; Fig. 3C). Concerning their distribution, TLR2 was found in the cytoplasm and perinuclear areas (Fig. 3G), whereas TLR4 was equally expressed in the cytoplasm and the nucleus of EGCs (Fig. 3H). Both receptors showed a

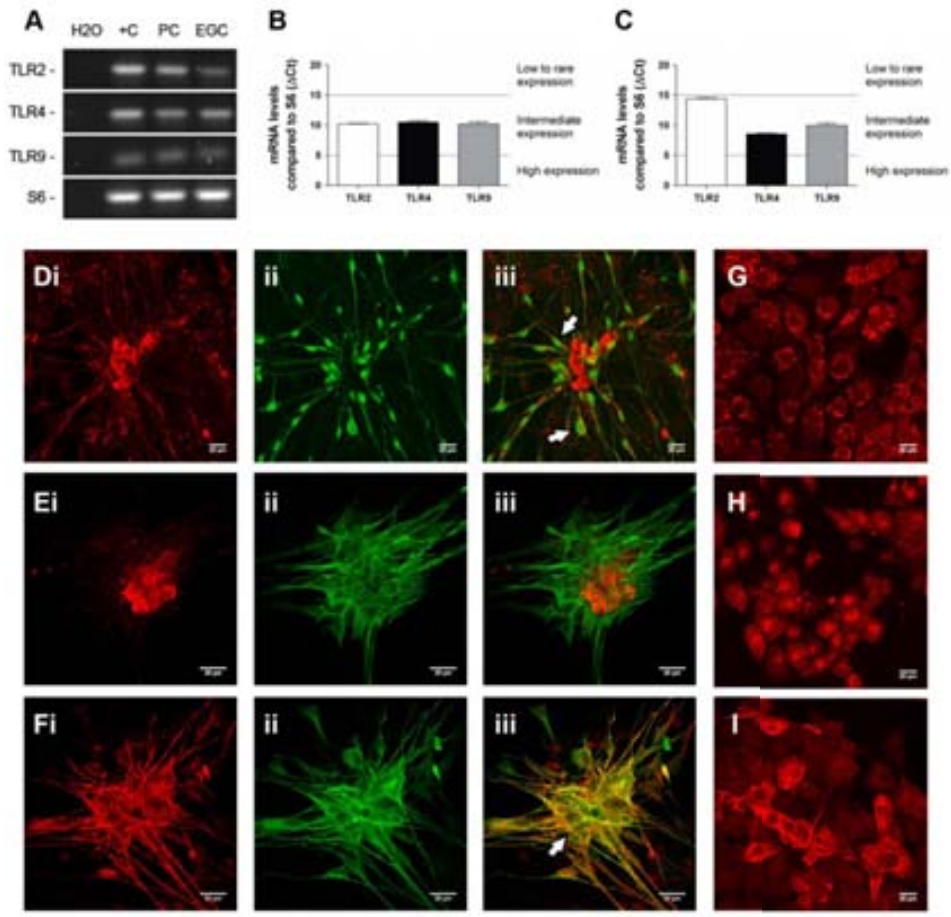


Figure 3 – TLR2/4/9 expression in ENS primary cultures and JUG2 EGCs. A) Agarose gel showing specific products of real-time RT-PCR for the assayed genes in ENS culture and JUG2 EGCs; water was used as no-template control (H2O), and rat colon cDNA as positive control (+C). **B)** TLR relative expression in ENS primary culture in basal conditions (n=7). **C)** TLR mRNA expression in JUG2 EGCs in basal conditions (n=8). Immunostaining of TLR2 **(D)**, TLR4 **(E)** and TLR9 **(F)** in ENS primary cultures **(i)**, localisation of EGCs as determined by the S100β glial marker **(ii)** and merged images showing colocalisation of such markers **(iii)**. White arrows point to areas of colocalisation. Immunolabeling of TLR2 **(G)**, TLR4 **(H)** and TLR9 **(I)** in JUG2 EGCs. All scale bars correspond to 20 µm. Differences in picture sizes are due to the use of an optical zoom of 1.5x in different experiments.

dotted immunostaining, while TLR9 stained the whole cytoplasm of the cells in a fibrous pattern (Fig. 3I).

MAMP-stimulated TLRs signal through the NF-κB pathway to induce a pro-inflammatory microenvironment in ENS culture

Phosphorylation of the inhibitor of κB (IκB) was used as an indicator of the NF-κB activation induced by exposure of cultures to TLR ligands. All TLRs described were functional in ENS culture, as each of their selective ligands and also their combinations activated the NF-κB pathway (Fig. 4A). Of note, LPS-treated cultures pre-incubated with 15 μM Bay 11-7082, as well as cODN 1826-treated cultures, did not exhibit phosphorylation of IκB. This confirms the specificity of the pathway in the former case and the selective stimulation of TLR9 by the CpG motifs in ODN 1826 in the latter. Activation kinetics observed for all MAMPs were similar, lasting from 1 to 48 hours and peaking at 4-8 hours (Fig. 4B). However, activation intensity was ligand-specific, with LPS being the most potent inducer ($LPS_{0.1}$= 386.6±61.8, $Pam2CSK4_{100}$= 328.3±29.14 and $ODN\ 1826_1$= 110±15.98 AUxhour; Suppl. Fig. 2A-E).

To further evaluate the functionality of TLRs in the ENS, secretion of cytokines and chemokines to culture supernatant was determined. Pro-inflammatory cytokines IL-6 and TNF-α, the inflammatory mediator NO and the chemokine monocyte chemoattractant protein (MCP)-1 were selected, as they have been demonstrated to be induced in the ENS intrinsic plexuses [35] or EGCs [16, 17, 36] after an inflammatory insult.

LPS addition to ENS culture elicited a marked increase in all inflammatory mediators analysed 24 hours after stimulation ($IL\text{-}6_{LPS}$= 3.85±0.68 vs. $IL\text{-}6_{control}$= 0.03±0.03 ng/mg of protein, n=7, *P*<0.001, Fig. 4C; $TNF\text{-}\alpha_{LPS}$= 3.64±0.37 vs. $TNF\text{-}\alpha_{control}$= 0 ng/mg of protein, n=8, *P*<0.001, Fig. 4D; $MCP\text{-}1_{LPS}$= 1.06±0.21 vs. $MCP\text{-}1_{control}$= 0.1±0.06 μg/mg of protein, n=8, *P*<0.001, Fig. 4E; NO_{LPS}= 13.64±1.42 vs. $NO_{control}$= 2.05±0.18 μM, n=8, *P*<0.001, Fig. 4F). In contrast, neither Pam2CSK4 nor ODN 1826 were able to induce secretion of such molecules, as their values remained close to the basal ones (Fig. 4C-F). Such results suggest that Gram-negative signalling induces pro-inflammatory responses in the ENS, while Gram-positive structures and bacterial DNA do not, despite they do activate the NF- κB pathway.

Inhibition of the NF-κB pathway by Bay 11-7082 significantly decreased production of all studied mediators, but did not completely abrogate it ($IL\text{-}6_{LPS+Bay}$= 0.92±0.26 ng/mg of protein, n=4, *P*<0.001 vs. $IL\text{-}6_{LPS}$, Fig. 4C; $TNF\text{-}\alpha_{LPS+Bay}$= 1.63±0.46 ng/mg of protein, n=4, *P*<0.001 vs. $TNF\text{-}\alpha_{LPS}$, Fig. 4D; $MCP\text{-}1_{LPS+Bay}$= 0.41±0.07 μg/mg of protein, n=4,

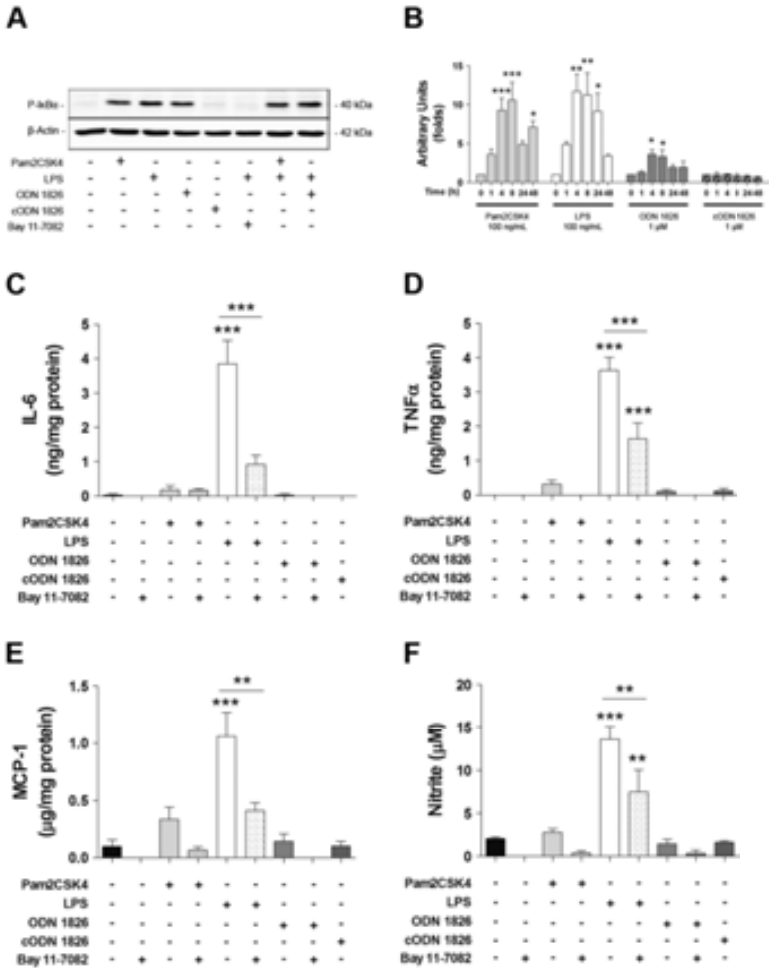


Figure 4 – ENS culture activation and response to TLR stimulation with MAMPs. A) Rat ENS culture was stimulated for 8 hours with the selected MAMPs or the induced-IκB phosphorylation inhibitor Bay 11-7082, and then cell protein extraction and western blot were performed to determine phosphorylated IκB (P-IκBα) as an indicator of NF-κB p65 translocation to the nucleus. β-actin was used as a loading control. **B)** Time-course densitometric quantification of P-IκBα bands corrected to β-actin and related to basal activation levels; representative membranes are shown in Suppl. Fig. 2. Rat ENS culture was incubated with MAMPs during specified time lapses and time-course western blots were performed. Statistical analysis was performed independently for each ligand, using one-way ANOVA followed by Dunnett's test (n=4 for each ligand and time point; **P*<0.05, ***P*<0.01 and ****P*<0.001). **C-F)** Rat ENS culture was stimulated for 24 hours and culture supernatants were collected and centrifuged prior to measuring cytokine and chemokine secretion. **C)** IL-6 production (n=4-8; LPS vs. control and Bay 11-7082 + LPS, ****P*<0.001). **D)** TNF-α production (n=4-8; LPS vs. control and Bay 11-7082 + LPS, ****P*<0.001; Bay 11-7082 + LPS vs. control, ****P*<0.001). **E)** MCP-1 production (n=4-8; LPS vs. control, ****P*<0.001; LPS vs. Bay 11-7082 + LPS, ***P*<0.01). **F)** NO production (n=4-8; LPS vs. control, ****P*<0.001; LPS vs. Bay 11-7082 + LPS, ***P*<0.01; Bay 11-7082 + LPS vs. control, ***P*<0.01).

P<0.01 vs. MCP-1_{LPS}, Fig. 4E; $NO_{LPS+Bay}$= 7.5±2.56 µM, n=4, *P*<0.01 vs. NO_{LPS}, Fig. 4F). This observation suggests that other signalling pathways might also be involved in responses to such MAMPs.

ODN 1826 synergises with LPS amplifying the inflammatory response of ENS culture

We next aimed to evaluate to what extent stimulation with one particular MAMP may result in altered response to another one as a result of a cross-regulation between TLRs. Quantitative RT-PCR assays of ENS culture showed up-regulation of TLR2 after an 8 hour-exposure to tested MAMPs ($TLR2_{Pam2CSK4}$= 18.78±3.02, $TLR2_{LPS}$= 40.78±7.84 and $TLR2_{ODN\ 1826}$= 8.29±2 vs. $TLR2_{control}$= 1.23±0.33 folds; Fig. 5A). A slight increase was also observed for TLR4 expression after LPS challenge ($TLR4_{LPS}$= 1.78±0.28 vs. $TLR4_{control}$= 1.06±0.13 folds, n=3-10, *P*<0.05; Fig. 5B), as well as a trend to increase in TLR9 after ODN 1826 stimulation ($TLR9_{ODN\ 1826}$= 2.28±0.43 vs. $TLR9_{control}$= 1.1±0.17 folds, n=3-10, *P*=0.08; Fig. 5C).

In further cross-regulation studies, we assessed whether combinations of the selected MAMPs can influence the response to LPS, as inhibitory [37], additive [38] and synergistic [32, 39] cross-regulation has been described in the literature for different cell types. We evaluated two different situations: Gram-positive + Gram-negative interaction (Pam2CSK4 + LPS, represented by grey bars in Fig. 5D-G), and Gram-negative + general bacterial stimulus (LPS + ODN 1826, represented by black bars in Fig. 5D-G). In order to compare to single LPS stimulation, an additional group of culture medium (Ctrl) + LPS was included in the experiment (white bars in Fig. 5D-G).

In ENS culture, addition of ODN 1826 4 hours after LPS exposure led to a significant increase in IL-6 production when compared to control group (IL-$6_{ODN\ 1826\ Post\text{-}LPS}$= 8.1±1.36 vs. IL-$6_{Ctrl\ Post\text{-}LPS}$= 3.35±0.48 ng/mg of protein, n=5, *P*<0.01; Fig. 5D). On the other hand, challenge with ODN 1826 4 hours before LPS stimulation significantly increased secretion of IL-6 and TNF-α (IL-$6_{ODN\ 1826\ Pre\text{-}LPS}$= 7.85±1.82 vs. IL-$6_{Ctrl\ Pre\text{-}LPS}$= 3.69±0.67 ng/mg of protein, n=5, *P*<0.05, Fig. 5D; TNF-$\alpha_{ODN\ 1826}$= 5.33±0.82 vs. TNF-$\alpha_{Ctrl\ Pre\text{-}LPS}$= 3.31±0.35 ng/mg of protein, n=5, *P*<0.01, Fig. 5E), and caused a trend to increase MCP-1 production (MCP-$1_{ODN\ 1826\ Pre\text{-}LPS}$= 1.27±0.12 vs. MCP-$1_{Ctrl\ Pre\text{-}LPS}$= 0.86±0.11 µg/mg of protein, n=5, *P*=0.08; Fig. 5F). Finally, simultaneous addition of TLR4 and TLR9 ligands LPS and ODN 1826 significantly increased production of IL-6, TNF-α and NO (IL-$6_{ODN\ 1826\ Co\text{-}LPS}$= 8.01±1.99 vs. IL-$6_{Ctrl\ Co\text{-}LPS}$= 3.35±0.48 ng/mg of protein, n=5, *P*<0.01, Fig. 5D;

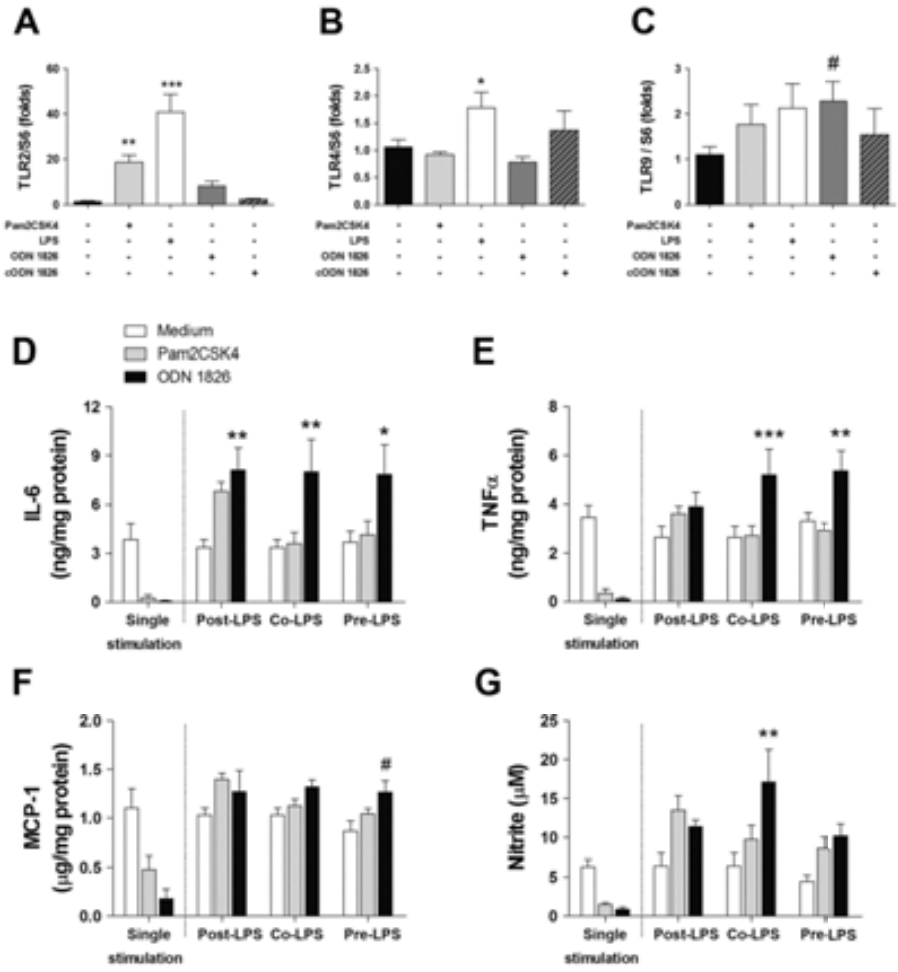


Figure 5 – TLR expression cross-regulation and ENS primary culture synergistic response after combined challenge with TLR agonists. TLR cross-regulation was assessed in rat ENS culture stimulated for 8 hours with the indicated MAMPs. **A)** TLR2 mRNA levels (n= 3-10; Pam2CSK4 vs. control, ***P*<0.01; LPS vs. control, ****P*<0.001). **B)** TLR4 mRNA levels (n=3-10; LPS vs. control, **P*<0.05). **C)** TLR9 mRNA levels (n=3-10; ODN 1826 vs. control, *P*=0.08). **D-G)** For combined challenge experiments, rat ENS culture was stimulated with combinations of MAMPs for a total period of 24 hours. Culture medium control (white bars), Pam2CSK4 (grey bars) or ODN 1826 (black bars) ligands were added to culture medium 4 hours before (Pre-LPS), simultaneously (Co-LPS) or 4 hours after (Post-LPS) LPS challenge. Results were analysed using randomised block design in order to minimize variability between different ENS cultures. **D)** IL-6 production (n=5; ODN 1826 + LPS vs. control + LPS, ***P*<0.01 in post- and costimulation with LPS, and **P*<0.05 in prestimulation). **E)** TNF-α production (n=5; ODN 1826 + LPS vs. control + LPS, ***P*<0.01 for pre-LPS stimulation, and ****P*<0.001 for costimulation). **F)** MCP-1 production (n=5; ODN 1826 + LPS vs. control + LPS, # p=0.08 only when ODN 1826 was added 4 hours before LPS). **G)** NO production (n=5; ODN 1826 + LPS vs. control + LPS, ***P*<0.01 when costimulating with both MAMPs).

TNF-$\alpha_{ODN\ 1826\ Co\text{-}LPS}$= 5.2±1.06 vs. TNF-$\alpha_{Ctrl\ Co\text{-}LPS}$= 2.63±0.46 ng/mg of protein, n=5, *P*<0.001, Fig. 5E; $NO_{ODN\ 1826\ Co\text{-}LPS}$= 17.09±4.24 vs. $NO_{Ctrl\ Co\text{-}LPS}$= 6.42±1.61 µM, n=5, *P*<0.01, Fig. 5G).

Measured effects of combined stimulations exceeded twice the predicted effect to be expected for two ligands interacting additively (summarised in Fig. 5D-G, left panels), demonstrating a synergistic interaction between TLR4 and TLR9 ligands (Table 2). In contrast to expression cross-regulation results, no significant interactions could be seen for Pam2CSK4 and LPS combinations, suggesting that regulation of TLR expression by other TLR ligands is not mechanistically relevant to the synergistic effect observed. In addition, this result might also indicate that motifs from Gram-positive bacteria do not modify ENS responses to Gram-negative bacteria.

Cytokine	TLR ligand combination	Expected additive effect (%)	Measured effect (%)	*P* value
IL-6	ODN 1826 Post-LPS	42.3 ± 5.9	101.2 ± 17	< 0.01
	ODN 1826 Co-LPS	42.3 ± 5.9	100 ± 24.8	< 0.05
	ODN 1826 Pre-LPS	46.5 ± 8.1	98 ± 22.7	< 0.05
TNF-α	ODN 1826 Co-LPS	51.9 ± 8.5	100 ± 20.4	< 0.05
	ODN 1826 Pre-LPS	64.6 ± 6	102.5 ± 15.8	< 0.05
Nitrite	ODN 1826 Co-LPS	40.6 ± 9.2	100 ± 24.8	< 0.05

Table 2 – Comparison between expected additive effects and measured effects of TLR ligand interactions from Figure 5. To calculate the expected additive effect of TLR ligand combinations, the mean values for IL-6, TNF-α, MCP-1 and nitrite production from simultaneous ODN 1826 + LPS combined stimulation (Co-LPS) were considered the 100% of the effect. All other values were expressed as a percentage of this maximum effect. Expected additive effects of ligand combinations were calculated according to the model of functional interaction, represented by the equation $E_{(ODN+LPS)expected} = E_{(ODN)measured} + E_{(LPS)measured} - E_{(ODN)measured} * E_{(LPS)measured}$. Statistical comparisons were performed by means of randomized block design analysis to minimize the random effects due to ENS culture differences. Statistics were only performed for treatments that were significantly different from single LPS stimulation, as shown in Figure 5.

MAMP-stimulated ENS culture promotes chemoattraction of RAW 264.7 cells.

Taking into account the increased MCP-1 production after exposure of ENS cultures to LPS, we studied whether stimulated ENS primary culture was capable of attracting immune cells.

MAMP-enriched DMEM culture medium did not induce RAW 264.7 cell migration to the lower compartment of the transwell inserts (data not shown). Similarly, conditioned medium from non-stimulated ENS culture did not increase migration when compared to DMEM culture medium (Fig. 6B). However, conditioned medium from LPS-stimulated ENS culture showed a tendency to increase the number of cells migrating to the lower chamber (59% increase: LPS= 114.3±35.56 vs. Ctrl= 71.83±17.24 cells per field, n=4, *P*=0.08; Fig. 6Ai, Aii and B). This chemotactic effect was further enhanced to a 107% and 112% increase by combination of Pam2CSK4 or ODN 1826 with LPS, respectively (Pam2CSK4+LPS= 149.3±36.03 and ODN 1826+LPS= 152.6±34.77 vs. Ctrl= 71.83±17.24 cells per field, n=4, *P*<0.001 for both, Fig. 6Aiii and B). Taken together, these data suggest that MAMPs themselves are not chemoattractant, but LPS

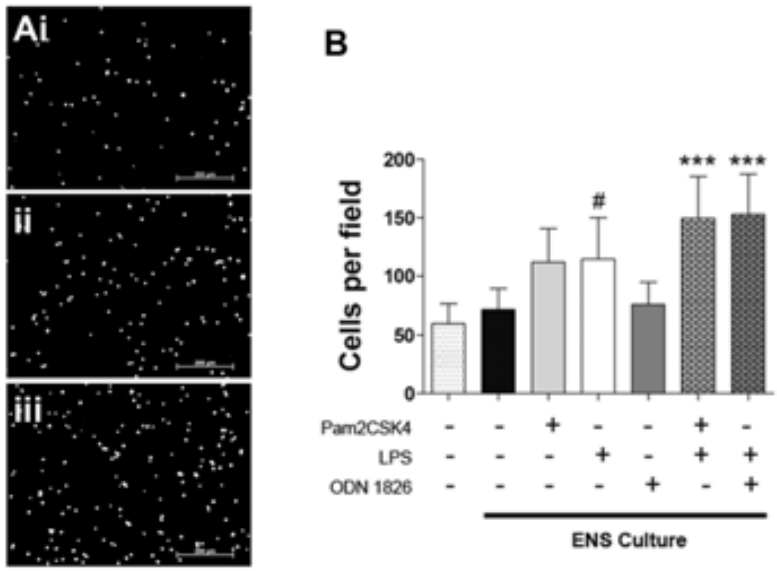


Figure 6 – RAW 264.7 migration elicited by ENS culture conditioned media. RAW 264.7 macrophages seeded in the upper chamber of 8 µm-pore transwell inserts were left to migrate for 4 hours towards lower chambers filled with culture medium (white dotted bar) or stimulated ENS culture conditioned media. Scale bars correspond to 200 µm. **A)** Representative pictures of one of the fields counted in the lower chamber of a transwell insert mounted upon **(Ai)** Control (unstimulated) ENS culture medium, **(ii)** LPS-stimulated ENS culture medium and **(iii)** ODN 1826 + LPS-stimulated ENS culture medium. **B)** Number of migrating cells per field (n=4, with 2 replicates for experiment; LPS vs. unstimulated ENS culture supernatant, *#P*=0.08; Pam2CSK4 + LPS vs. unstimulated ENS culture supernatant, ****P*<0.001; ODN 1826 + LPS vs. unstimulated ENS culture supernatant, ****P*<0.001; randomised block design analysis was performed to minimize the variability in migration due to RAW 264.7 passage, followed by Bonferroni's post-hoc test).

alone or in combination with other TLR ligands elicit secretion of chemotactic substances by the ENS culture that promote migration of RAW 264.7 macrophages.

EGCs participate in responses to Gram-negative bacteria and chemoattraction of RAW 264.7 macrophages.

In order to evaluate the contribution of EGCs to the MAMP-elicited responses observed in the ENS primary cultures, the JUG2 EGC line was challenged in parallel with TLR2/4/9 ligands and its activation and cytokine production responses were evaluated as done for ENS culture.

In contrast to what we observed in ENS cultures, activation of the NF-κB signalling pathway in JUG2 EGCs was only induced by LPS (Fig. 7A and Suppl. Fig. 2F), and showed faster kinetics, starting at 10 minutes and peaking 1 hour after challenge (Fig. 7B). Hence, LPS was the only MAMP which triggered a pro-inflammatory response in JUG2 cells, which was characterised by significant increases in IL-6 ($IL\text{-}6_{LPS}$= 78.42±14.8 vs. $IL\text{-}6_{Ctrl}$= 21.43±4.95 pg/mg of protein, n=6, *P*<0.01; Fig. 7D) and MCP-1 ($MCP\text{-}1_{LPS}$= 39.53±8.6 vs. $MCP\text{-}1_{Ctrl}$= 8.05±1.99 ng/mg of protein, n=6, *P*<0.01; Fig. 7E) secretion at 24 hours. Such results confirm that EGCs are only capable of responding to Gram-negative bacteria.

We did not detect NO or TNF-α production in our JUG2 EGC cultures. Indeed, immunolabeling of iNOS in ENS cultures revealed that iNOS-producing cells colocalised with IBA-1-expressing macrophages (Suppl. Fig. 3iii), demonstrating that EGCs do not produce NO in our culture models.

Although LPS treatment induced TLR2 significant up-regulation after a 4 hour-stimulation ($TLR2_{LPS}$= 15.14±2.12 vs. $TLR2_{Ctrl}$= 1.05±0.13 folds, n=4-7, *P*<0.001, Fig. 7C), combined MAMP challenge did not elicit any response (data not shown), suggesting that TLR2 up-regulation does not increase sensibility to TLR2 ligands, and is probably involved in other processes.

Finally, and consistent with higher MCP-1 secretion after LPS challenge, randomised block design analysis demonstrated that, after eliminating the RAW 264.7 passage random effects (*P*<0.001), LPS-treated JUG2 EGCs elicited a 10% increase in the number of migrating cells to the lower chamber of cell transwell inserts (LPS= 170.4±19.63 vs. Ctrl= 154.8±17.18 cells per field, n=9, *P*<0.01; Fig. 7F). This finding demonstrates that MAMP-activated EGCs can also chemoattract RAW 264.7 macrophages.

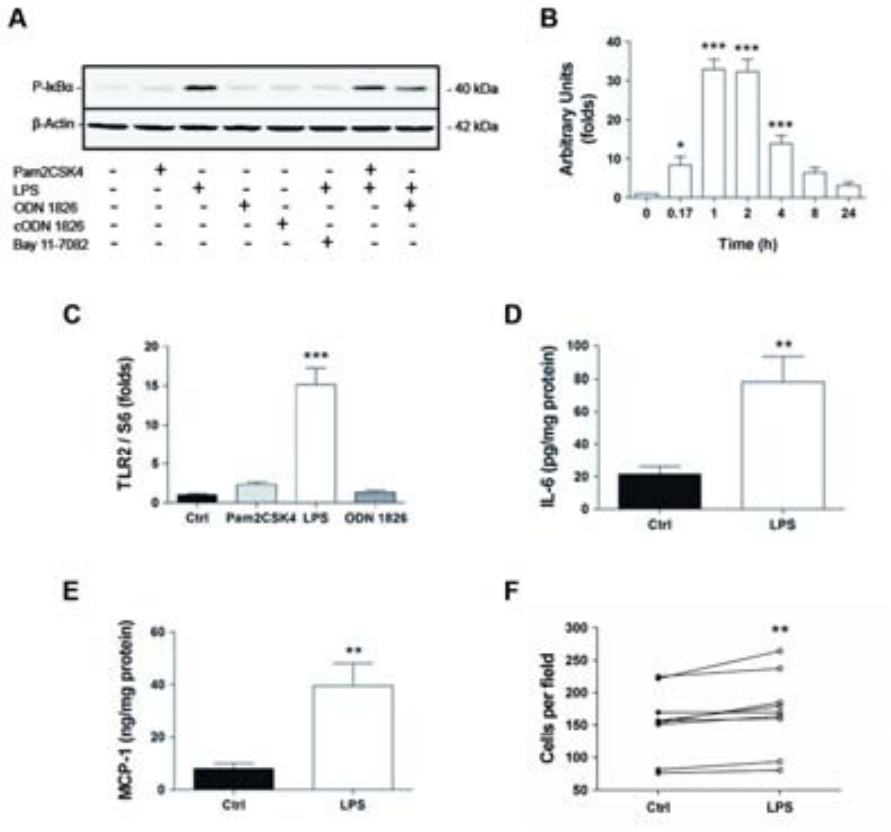


Figure 7 – JUG2 EGC responses to TLR ligands. A) Phosphorylated IκB from JUG2 EGCs stimulated for 1 hour with the selected MAMPs or the induced-IκB phosphorylation inhibitor Bay 11-7082. β-actin was used to ensure equal loading of all samples. **B)** Densitometric quantification of P-IκBα bands in time-course stimulations with LPS (n=6-8; 0.17 hours vs. 0 hours, **P*<0.05; 1, 2 and 4 hours vs. 0 hours, ****P*<0.001; one-way ANOVA followed by Dunnett's post-hoc test; representative bands are shown in Suppl. Fig. 2F). **C)** TLR2 mRNA levels after 4 hours of stimulation with MAMPs (n=4-7; LPS vs. unstimulated control, ****P*<0.001). **D)** IL-6 production after 24 hour stimulation with LPS (n=6; LPS vs. unstimulated control, ***P*<0.01). **E)** MCP-1 production after 24 hour stimulation with LPS (n=6; LPS vs. unstimulated control, ***P*<0.01). **F)** RAW 264.7 macrophages seeded in the upper chamber of 8 µm-pore transwell inserts were left to migrate for 4 hours towards lower chambers filled with supernatants from untreated (Ctrl) or LPS-treated EGC cultures and the number of migrating cells per field was counted (n=9, ***P*<0.01; randomised block design analysis was performed to minimize the variability in migration values due to RAW 264.7 passage, followed by Bonferroni's post-hoc test).

Taken together, obtained results suggest that EGCs recognise Gram-negative bacteria through TLR4, which subsequently activates the NF-κB signalling pathway and induces production of cytokines and chemokines such as IL-6 and MCP-1. Through release of these molecules, EGCs may probably exert chemoattractant functions on immune cells, facilitating their homing to areas challenged by microorganisms.

Discussion

Since their initial description in human in the late 90's [19], TLRs have been widely studied and described in several tissues and cells, including the CNS and the peripheral nervous system [8, 21]. In this study, we report the presence of TLR2/4/9 in the ENS plexuses of healthy mice, emphasising changes observed in TLR4 expression in areas displaying reactive gliosis in an experimental model of colitis. To assess the functionality of such receptors and to overcome the response of homing immune cells during inflammation, we have worked on a simplified *in vitro* model of ENS primary culture, which is however not devoid of resident *muscularis* macrophages [40]. Therefore, in an attempt to characterise the contribution of EGCs, we have additionally evaluated TLR-mediated responses in the JUG2 EGC line, which was initially isolated from this ENS culture [31]. Our results show that both ENS and EGCs cultures can directly sense LPS and respond to Gram-negative stimuli by secreting inflammatory mediators. Additionally, we report some evidence regarding a putative role for the ENS and EGCs in expanding inflammation by chemoattraction of macrophages to compartments that have been challenged with MAMPs, further supporting the idea that the ENS is immunologically active in resting and inflammatory conditions.

Expression of TLRs within the ENS has been demonstrated by other groups [26, 27]. Our observations corroborate previous descriptions of TLR4 immunoreactivity in smooth muscle cells and neurons from the myenteric plexus [27], and show minor but existing expression of TLR4 in EGCs from the submucosal plexus. Additionally, we provide new evidence concerning the expression of TLR2/9 in neuronal somata, nerve bundles and EGCs from the submucosal plexus of the murine colon. Although TLR2/4/9 have been classically described as membrane-bound proteins, different reports have shown expression of these receptors in cytoplasmic compartments of neurons from the CNS and dorsal root ganglia [25, 41], CNS astrocytes [42] and EGCs [18], in accordance with our observations. In parallel, our findings also indicate that, in EGCs, TLR4 is the most expressed TLR among the studied ones, and that it is located in both cytosolic and nuclear compartments, in agreement with previous reported results in human EGCs [18]. The differences in distribution patterns between submucosal plexuses and EGC cultures may perhaps be due to distinct states of maturation of the EGCs in each preparation,

supporting the idea that local microenvironment influences glial morphology and function [10].

Functionality of TLR2/4/9 was only observed in ENS culture, where addition of the different selected MAMPs activated in all cases the NF-κB pathway. Activation of this signalling cascade accounted for an important part of the response, since inhibition with Bay 11-7082 resulted in a substantial decrease in cytokine production, but did not completely abrogate it. Thus, it is likely that other signalling pathways like AP-1 and the myeloid differentiation primary response gene 88 (MyD88)-independent pathway may contribute to some extent to the TLR4-dependent production of cytokines in ENS cultures [11, 20, 29]. However, although all TLRs assayed in ENS culture were functional, significant increases in production of inflammatory mediators were only observed after stimulation of TLR4, which was interestingly the sole TLR inducing NF-κB activation and cytokine production in the JUG2 EGC culture. In the same vein, translocation of the NF-κB p50 subunit into the nucleus of human EGCs has been described after exposure to enteropathogenic bacteria, but not to probiotic strains [18]. These observations indicate that the ENS could be able to sense microbial penetration across the GI mucosa through EGCs, but would only trigger inflammation in response to Gram-negative bacteria. Indeed, accumulating evidence support an important role for enteric glia during inflammation through activation of TLR4. First, human EGCs challenged with enteropathogenic bacteria show increased S100β and NO release in a MyD88-dependent fashion [18]. Second, isolated EGCs from mouse experimental models of colitis and UC patients over-express TLR4, GFAP and S100β, display activated phenotypes and produce pro-inflammatory mediators such as NO and TNF-α [14, 29]. Third, drugs targeting reactive astrogliosis are useful in improving the signs of experimental colitis through reduction of TLR4 [29]. And fourth, we have shown that TLR4 up-regulation is associated to gliosis in areas devoid of any other sign of inflammation *in vivo*, and that LPS-stimulated JUG2 EGCs release IL-6, increase MCP-1 production and have chemoattractant effects on macrophages *in vitro*. All these findings, taken together with results obtained by Rumio and colleagues, who demonstrated IL-8 secretion in muscular layers from human jejunum biopsies upon LPS stimulation [26], suggest that the ENS and its surrounding muscle layers might have the capability to recognise MAMPs

and DAMPs through TLR4, participating in the inflammatory response and amplifying it by active recruitment of immune cells.

The presence of resident macrophages in ENS cultures [40], and potentially in EGC cultures, represents an important drawback in the study of the immune functions of the isolated ENS. Our findings corroborate the need of pure cultures to address EGC immune functions, since important differences were observed between both cultures. Indeed, despite EGCs have been reported to release all the evaluated mediators [16-18, 29, 36], neither TNF-α nor NO were produced by the EGC line JUG2 upon LPS stimulation. Furthermore, iNOS reactivity in ENS culture was exclusively shown to colocalise with IBA-1-expressing macrophages. Such results are in accordance with those from Jack and colleagues, who described TNF-α production in response to LPS in microglia, but not astrocytes [11]. Moreover, our findings confirm that in ENS culture myeloid-derived contaminating cells are the main source of NO production after LPS single stimulation, as reported in astrocyte-enriched cultures [43]. Therefore, although induction of iNOS in EGCs and astrocytes is achievable through combined stimulation with LPS and IFN-γ [17, 18, 43], special care must be also taken when working with EGC cultures in order to avoid misleading assumptions concerning their immune functions. In the same regard, EGCs do probably contribute to the total amount of IL-6 and MCP-1 produced in ENS culture, but may not account for the most of it, since stimulated ENS culture secreted 50 folds more IL-6 and 25 folds more MCP-1 than LPS-challenged EGC culture, in parallel with previous work in microglial and astroglial cells [11].

The chemotactic effects of LPS-treated cultures do also support a major involvement of macrophages in eliciting immune cell attraction *in vitro*, as LPS-treated ENS cultures caused a 59% increase in the number of migrating cells, whereas EGCs induced a 10% increase in migration. However, to our knowledge, this is the first study demonstrating a chemoattractant role for EGCs. The putative implications of these findings must still be addressed, but different works indicate that EGCs could recruit leukocytes *in vivo*. On one hand, MHC class II expression in EGCs is correlated with higher inflammatory infiltrates surrounding the ENS in IBD patient specimens [7]. On the other, MHC class II is expressed in EGCs challenged with enteropathogenic bacteria or LPS [17, 18]. Therefore, it is conceivable that MAMP-or DAMP-stimulated EGCs liberate chemokines such as MCP-

1 while up-regulating MHC class II, inducing migration of leukocytes and performing specific antigen presentation.

Different works have documented interactions after combined TLR ligand challenges. Macrophages and microglia show additive or supra-additive effects when costimulated with two different MAMPs [32, 38, 39]. However, phenomena of cross-tolerance have been described in which prestimulation with a TLR2 ligand induces hyporesponsiveness to a subsequent LPS stimulation [37, 39]. Our experiments show that the Gram-negative stimulus elicited by ODN 1826 and LPS induces a synergistic response in terms of cytokine and chemokine secretion, as IL-6, TNF-α, NO and the number of chemoattracted cells were increased in different culture conditions (especially during simultaneous costimulation). Such interaction is not explained by cross-regulation changes in TLR expression, since neither ODN 1826 induced TLR4, nor did LPS up-regulate TLR9. Mechanisms involved in this effect might be redistribution of receptors from cytoplasm to membrane, changes in molecular components of the TLR signalling cascade [37] or interactions between responding cell types. In fact, ENS culture macrophages are possibly the major cells involved in such effect [32, 38, 39], since EGCs showed neither ligand additive responses nor TLR2/9 mediated activation of NF-κB. Nevertheless, microglial interactions have been shown to promote astrocyte hyper-sensitivity to MAMPs [44], and therefore it is feasible that upon macrophage influence, EGCs may display hyper-reactive TLR4/9. Actually, in ENS cultures TLR9 staining was largely found in EGCs.

Conversely, the TLR2 ligand Pam2CSK4 did not interact with LPS to generate supra-additive effects in production of the inflammatory mediators evaluated, even though TLR2 was preferentially expressed in neurons, which have been shown to enhance pro-inflammatory responses [6], and macrophages. Furthermore, although consistent up-regulation of TLR2 after MAMP-treatment was observed, no synergisms were identified. TLR2 has been reported to be up-regulated in astrocytes, neurons and other cell types after stimulation with different MAMPs [11, 12, 25, 42]. However, this receptor appears to exert an autocrine/paracrine feedback on NF-κB, as it is believed to be involved in signalling the formation of lipoproteins bearing lipid oxidation end products [45, 46]. Hence, TLR2 up-regulation did not alter the production of cytokines in costimulation experiments. Nevertheless, an additive release of chemokines during

TLR2/4 costimulation cannot be dismissed, since RAW 264.7 macrophages were also significantly attracted by these supernatants, and neurons from submucosa have been shown to produce IL-8 in response to pro-inflammatory challenges [47].

Taken together, our data suggest that the ENS is capable of recognising bacteria and triggering an inflammatory response in the presence of Gram-negative stimuli, while simultaneously promoting the homing of macrophages. EGCs, which play an important role in the immune response through production of cytokines and chemokines, would partially mediate these effects through activation of TLR4. However, their participation in the inflammatory response must not be over-estimated, since surrounding cells such as resident macrophages have been demonstrated to be immunologically active [3], and might account for most of the responses reported in ENS culture.

The potential implications of TLR expression and functionality in the ENS and EGCs besides promotion of homing in immune cells are still to be determined. In inflammatory diseases such as IBD, TLR activation might be involved in shaping the ENS plasticity through different mechanisms: 1) Priming of the subsequent adaptive immune response through cytokine secretion and up-regulation of MHC class II molecules in EGCs [15]; 2) Controlling permeability of the epithelial barrier [4, 48]; 3) Mediating apoptosis and cell survival [25, 28]; and 4) Signalling pain through expression of different nociceptors [41] after bacterial recognition. Their understanding can be therefore of great value to delineate strategies of intervention to address functional impairment and discomfort and to prevent systemic complications due to immune system imbalance.

Acknowledgements

This work, JFB and CR were supported by grants from the Spanish Ministry of Education & Science (BFU2006-15063-C03-01/BFI, AP2005-1160 and AP2006-02705, respectively) and the Spanish Ministry of Health (PS09/01127). Work in EF's group is also supported by Generalitat de Catalunya (SGR-2009-997). Authors would like to thank J. Chevalier, M. Biraud and M. Ribeiro-Neunlist for providing training and technical support on ENS culture preparation, and N. Barba from the Institut de Neurociències for technical assistance in confocal microscopy imaging.

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Supplementary materials and methods

Antibodies

For immunofluorescence, primary antibodies used were rabbit polyclonal anti-IBA-1 (1:500; Wako Chemicals, Neuss, Germany) and mouse monoclonal anti-iNOS (1:500; Santa Cruz Biotechnology, Santa Cruz, USA). Secondary antibodies used were Alexa Fluor 568 goat anti-rabbit IgG (1:500; Life Technologies) and CF488A donkey anti-mouse IgG (1:500, Biotium). For western blot, mouse monoclonal anti-iNOS (1:1000, Santa Cruz Biotechnology) and rabbit polyclonal anti-COX-2 (1:5000; Abcam) were detected with HRP-linked sheep anti-mouse IgG (1:5000; GE Healthcare) and HRP-linked goat anti-rabbit IgG (1:10,000; Cell Signaling Technology), respectively. Protocols used for both types of immunodetection were performed as described in material and methods paragraphs from Chapter 3.

Disease Activity Index

Body weight, stool consistency, faecal blood and general aspect of every animal were determined daily in order to build up a disease activity index (DAI) as previously described [1]. Briefly, every parameter was given a numeric score as follows: for weight: 0, no loss; 1, up to 5%; 2, 5%–10%; 3, 10%–15%; and 4, >15% weight loss; for stool: 0, normal; 1, soft stool; 2, semi-liquid; 3, diarrhoea; and 4, gross diarrhoea; and for bleeding: 0, no blood; 1, slight presence in stool; 2, apparent presence in stool; and 3, gross blood. Global DAI was calculated at the end of experiments by representing daily DAI time-course for each animal and calculating the area under the curve (AUC).

Histologic score

Histologic assessment of colitis was performed by an investigator blinded to the study design. Haematoxylin & eosin sections from mid-distal colons of each animal were evaluated as previously described [2]. Briefly, five different areas were evaluated as follows: for inflammation: 0, none; 1, slight; 2, moderate; and 3, severe; for extent of inflammation: 0, none; 1, mucosa; 2, mucosa and submucosa; and 3, transmural; for crypt damage: 0, none; 1, basal 1/3 damaged; 2, basal 2/3 damaged; 3, only surface epithelium intact; and 4, entire crypt and epithelium loss. Each of these values was

multiplied for the corresponding percentage of involvement of the studied area: 1, 1-25%; 2, 26-50%; 3, 51-75%; and 4, 76-100%.

Colonic Myeloperoxidase Activity

Leukocyte infiltration into submucosal or muscular layers, dissected fresh in 50 mM hexadecyltrimethylammonium bromide solution, was determined as previously described for use in a 96-well plate [3]. Changes in optical density at 450 nm were measured at 3-minutes intervals for 15 minutes. One unit of myeloperoxidase (MPO) activity was defined as the amount that degraded 1.0 µmol of peroxide per minute at 25 °C.

MAMP NF-κB total activation quantification

In order to quantify the overall NF-κB activation after challenging ENS cultures with different MAMPs, densitometric measurements for each experiment were represented as time-course curves by means of the GraphPad Prism 5.0 software (GraphPad Software Inc.). Subsequently, the AUC was calculated for each replicate. The resulting values were used to compare the NF-κB inducing strength of each TLR ligand.

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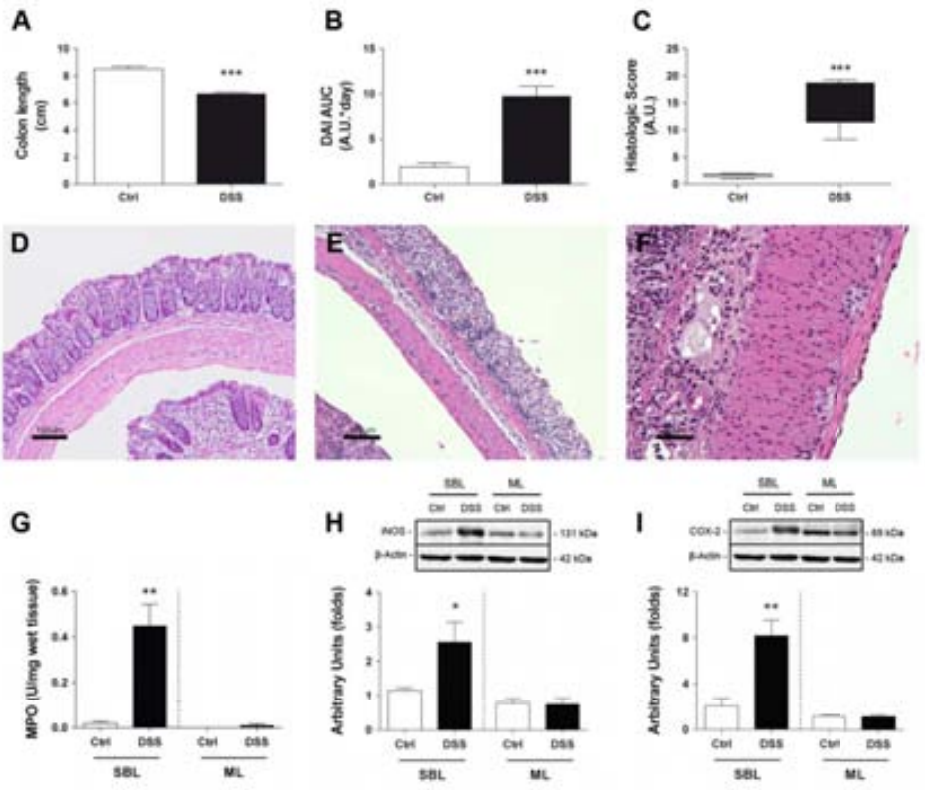
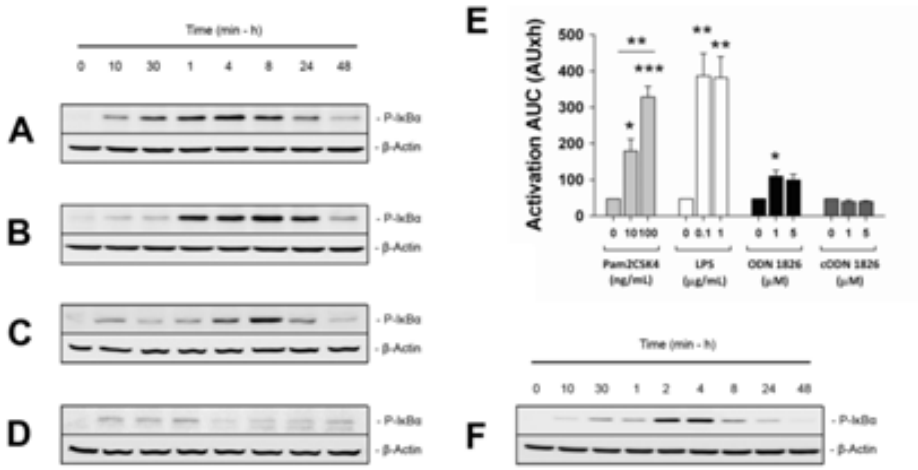
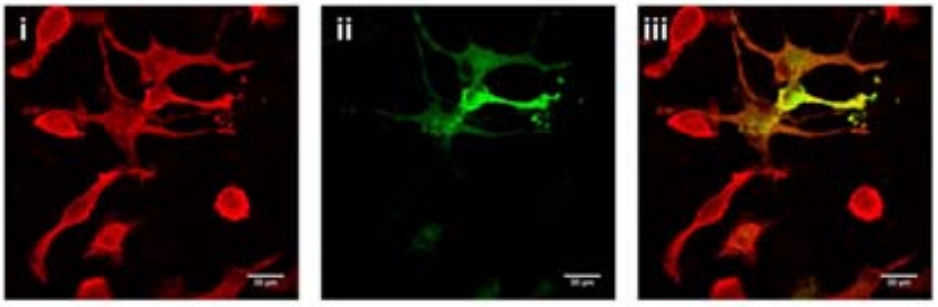
Supplementary figures



Supplementary Figure 1 – Colitis characterisation and assessment of leukocyte infiltration and inflammation. A) Colon length (n=8; DSS-treated (DSS) vs. control (Ctrl) mice, ***P<0.001). **B)** Global DAI from Ctrl and DSS-treated mice (n=8; ***P<0.001). **C)** Histologic score of evaluated colons (n=6; ***P<0.001). **(D-F)** Examples of haematoxylin & eosin micrographs evaluated for histologic scoring of colons from **(D)** control or **(E, F)** DSS-treated mice. Scale bars correspond to 100 µm in **(A, B)** and to 50 µm in **(F)**. **G)** MPO activity in submucosal (SBL) and muscular (ML) layers of Ctrl and DSS-treated mice (n=4; SBL-DSS vs. SBL-Ctrl, **P<0.01, one-tailed t-test). **H)** iNOS protein expression in SBL and ML of Ctrl and DSS-treated mice (n=4; SBL-DSS vs. SBL-Ctrl, *P<0.05, one-tailed t-test). **I)** COX-2 protein expression (n=4; SBL-DSS vs. SBL-Ctrl, **P<0.01, one-tailed t-test).



Supplementary Figure 2 – NF-κB activation upon stimulation with different doses of selected TLR ligands. Time-course activation after stimulation of ENS cultures with **(A)** 100 ng/mL Pam2CSK4, **(B)** 100 ng/mL LPS, **(C)** 1 µM ODN 1826 or (**D**) 1 µM control ODN 1826. **E)** Quantification of the overall NF-κB activation after treatment with different doses of the indicated MAMPs (n=4; **P*<0.05, ***P*<0.01 and ****P*<0.001, one-way ANOVA followed by Dunnett's post-hoc test). Statistics were performed independently for each ligand. **F)** Time-course activation of JUG2 EGCs after challenge with 100 ng/mL LPS.



Supplementary Figure 3 – IBA-1 and iNOS colocalisation after LPS treatment in ENS culture. Immunostaining of IBA-1 **(i)**, iNOS **(ii)** and merged images showing colocalisation of both markers **(iii)**. All scale bars correspond to 20 µm.

Chapter 4

Microbial challenge of enteric nervous system cultures elicits subsequent hyporesponsiveness in RAW 264.7 macrophages

Abstract

We have previously shown that enteric nervous system (ENS) cultures recognise bacterial motifs and respond to them by releasing cytokines and chemokines to the medium and increasing macrophage chemoattraction. To assess whether this conditioned medium can prime the response of recruited cells, tumour necrosis factor (TNF)-α release upon Toll-like receptor (TLR) stimulation was analysed in differently conditioned RAW 264.7 macrophages. Our results indicate that incubation of macrophages with media from unstimulated ENS culture elicits increased responses to lipopolysaccharide (LPS), whereas conditioning with LPS-challenged ENS supernatants induces marked inhibition in TNF-α secretion by macrophages. Such effect, which was not seen after fibroblast or EGC conditioning, was enhanced following combined TLR4/9 ligand challenge and lasted at least for 24 hours after withdrawal of the stimuli in ENS cultures. Hyporesponsiveness was neither due to neuronal cell death nor mediated by nitric oxide or interleukin-10 release, or mediated by cholinergic or adrenergic receptors. Further studies are needed to disclose the precise nature of the mediators responsible for the effects described herein.

Keywords

Enteric nervous system, Toll-like receptor 4, Toll-like receptor 9, hyporesponsiveness, inflammation, macrophage.

Introduction

Chronic and recurrent inflammation of the gastrointestinal tract is a shared and characteristic feature of inflammatory bowel diseases (IBD). Such disorders, including Crohn's disease and ulcerative colitis, are usually accompanied by common symptoms such as diarrhoea and abdominal pain [1]. These symptoms, which are the final outcome of alterations involving secretion, motility and visceral sensitivity, are caused by plastic adaptions underwent by the enteric nervous system (ENS) in response to different interactions with inflammatory cells [2, 3]. The study of the crosstalk between immune cells and the ENS has revealed that both neurons and enteric glial cells (EGC) are not only bystanders, but important players in determining the resulting inflammatory response. For instance, increased enteric neuronal density has been associated to more severe inflammation in two different animal models of colitis [4]. In contrast, targeted glial depletion leads to fulminant enterocolitis that is associated to decreased epithelial barrier integrity [5-7]. Other findings supporting such role for the ENS are the following: 1) the inflammatory infiltrate is often dense around the plexuses of the ENS [2]; 2) there is a positive correlation between inflammation and expression of major histocompatibility complex (MHC) class II molecules in EGCs [8]; and 3) structural and phenotypic abnormalities are found in the ENS of inflamed and non-inflamed intestinal segments of IBD patients [2, 9].

There are several mechanisms through which the ENS might influence the behaviour of immune cells. On one hand, neurons can release different neurotransmitters depending on their neurochemical coding. Immunomodulatory properties have been described for neurotransmitters such as acetylcholine (ACh) [10], norepinephrine (NE) [11], vasoactive intestinal peptide (VIP) [12], serotonin [13] or nitric oxide (NO) [14, 15]. Conversely, substance P (SP), which has pro-inflammatory effects [9], can be also released by neurons under inflammatory conditions. On the other hand, EGCs have been shown to secrete interleukin (IL)-6 [16] and up-regulate the inducible form of the NO synthase (iNOS) in response to different stimuli [17, 18]. Such observations, in addition to expression of MHC class II, might further link EGCs with recruitment of inflammatory cells [2, 19], whereas the production and secretion of neurotrophins by these cells might exert protective functions [20].

Previous work by our group and others has demonstrated that neurons and EGCs express different Toll-like receptors (TLR) [18, 21-23]. TLRs play important roles in recognition of damage- and microorganism-associated molecular patterns (MAMP), activating different signalling pathways such as the nuclear factor (NF)-κB cascade, which terminate in production of inflammatory mediators [24, 25]. Since inflammation modifies the neurochemical coding of neurons [2, 7, 9] and alter the phenotype of EGCs [26], it is reasonable to hypothesise that TLR signalling might be involved in ENS plasticity. Indeed, TLR-mediated recognition of MAMPs by neurons and EGCs could modulate their phenotypes, promoting plasticity and orchestrating their interactions with surrounding and recruited immune cells, influencing their inflammatory status and responses to microbes.

We have described that MAMP-challenged ENS cultures secrete different cytokines and chemokines that promote migration of the RAW 264.7 macrophage cell line [23]. Such substances, which are mainly released by resident *muscularis* macrophages [27] and EGCs, might additionally play important roles in priming subsequent RAW 264.7 pro-inflammatory responses. Therefore, the aim of this study was to evaluate whether neuro-inflammation elicited by TLR ligands in ENS cultures is capable of modulating the production of inflammatory cytokines such as tumour necrosis factor (TNF)-α in RAW 264.7 macrophage cultures. Our results indicate that, although ENS cultures produce increased levels of pro-inflammatory mediators following TLR stimulation, RAW 264.7 macrophage cultures conditioned with such media become hyporesponsive in terms of cytokine production upon recognition of MAMPs. This tolerogenic effect is not associated to a loss of neurons, but possibly to alterations in production of a soluble molecule whose nature remains elusive.

Materials and Methods

Reagents and antibodies

All culture media, foetal bovine serum (FBS), antibiotics, N-2 supplement and 4',6-diamidino-2-phenylindole (DAPI) were from Life Technologies (el Prat de Llobregat, Spain). Trypsin, DNase I and gelatine were from Sigma (Madrid, Spain). Lipopolysaccharide (LPS) stimulation of TLR4 was performed with a mixture 1:1 of LPS from E. coli O55:B5 and S.typhosa, both purchased from Sigma. TLR9 was stimulated by means of the phosphorothioate-modified type B CpG oligonucleotide (ODN) 1826 (5'-TCCATGACGTTCCTGACGTT-3'), which was synthesised by Isogen (De Meern, The Netherlands). The antibodies used for immunofluorescence were mouse monoclonal anti-Hu C/D (1:200; Life technologies) and CF488A-conjugated donkey anti-mouse IgG (1:500; Biotium, Hayward, USA). Drugs used to block the effects of different molecules potentially secreted by the ENS cells and/or *muscularis* macrophages are summarised in Table 1.

Drug	Concentration	Abbreviation	Function	Manufacturer
Nω-nitro-L-arginine	1 mM	LNNA	Inhibitor of the nitric oxide synthases (unselective)	Sigma Aldrich
ODQ	10 μM	ODQ	Selective inhibitor of the guanylyl cyclase	Tocris
Atropine	1 μM	NANC	Muscarinic ACh receptor antagonist	Sigma Aldrich
Hexamethonium	1 μM		Nicotinic ACh receptor antagonist	Sigma Aldrich
Phentolamine	1 μM		α-adrenergic receptor antagonist	Sigma Aldrich
Propranolol	1 μM		β-adrenergic receptor antagonist	Tocris
IL-10 neutralising antibody	1 μg/mL	αIL-10	Neutralisation of the effects of IL-10	R&D Systems

Table 1 – List of drugs used in macrophage conditioning experiments.

Cell cultures

Isolation and culture of rat ENS primary culture was performed as described elsewhere [28]. Briefly, intestines of rat embryos (E16) were removed and finely diced in PBS. Tissue fragments were digested with trypsin and DNase I, and cells obtained were counted and seeded at a density of $2.4x10^5$ cells cm^{-2} on 48-well plates (Falcon, BD, San Agustín de Guadalix, Spain), previously coated with a 0.5% gelatine solution in sterile phosphate buffered saline (PBS). Stimulation was performed for 24 hours after 15 day-culture in FBS-free Dulbecco's modified Eagle's medium (DMEM)-F12 containing 1% of N-2 supplement.

JUG2 EGCs were kindly donated by Dr. Michel Neunlist. This cell line was isolated from rat ENS culture, and after 1 month of sub-culture, cells were immunoreactive for glial but not for neuronal or myofibroblast markers [29].

RAW 264.7 macrophages and 3T6 fibroblasts were purchased from the American Type Culture Collection. Both cell lines were cultured in DMEM supplemented with 10% heat-inactivated FBS. Two days before stimulation experiments, JUG2 EGCs and 3T6 fibroblasts were seeded at a density of 50,000 cells cm^{-2} in 24 well plates and grown to confluence.

Both stimulation and conditioning experiments were carried out in FBS-free media, to avoid interactions with uncontrolled substances. Stimulation experiments were performed in ENS culture, JUG2 EGCs or 3T6 fibroblasts with 100 ng/mL LPS or combinations of 100 ng/mL LPS and 1 µM ODN 1826. Supernatants were harvested after 24 hours, centrifuged, aliquoted and kept at -80^{o}C until their use as conditioning media.

Macrophage conditioning

RAW 264.7 macrophages were seeded in 96-well plates at a density of $1,5x10^5$ cells cm^{-2} in 10% heat-inactivated FBS supplemented DMEM medium, and grown to confluence for 2 days. Conditioned supernatants from unstimulated, LPS- or ODN 1826+LPS-stimulated ENS cultures, JUG2 EGCs or 3T6 fibroblasts were transferred to confluent RAW 264.7 cultures. Macrophage cultures receiving conditioned media from unstimulated ENS (uENS), JUG2 (uJUG2) or 3T6 (u3T6) cultures were subsequently stimulated with LPS or ODN 1826+LPS combinations of ligands, whereas those receiving supernatants from already stimulated ENS (sENS) cultures, JUG2 EGCs (sJUG2) or 3T6

(s3T6) fibroblasts were not (Fig. 1). Nevertheless, some macrophage cultures were restimulated to confirm that the obtained effect was not due to ligand degradation in ENS culture conditions (Fig. 1). An additional experiment was performed to evaluate whether the ENS-induced effect was conserved after withdrawal of the pro-inflammatory stimulus. ENS cultures were stimulated with ODN 1826+LPS for 24 hours, MAMP-enriched medium was removed, cultures were washed with PBS and fresh MAMP-free medium was added. ENS cultures were incubated for an additional period of 24 hours.

In either case, 4 hours after beginning conditioning experiments, RAW 264.7 macrophage supernatants were harvested, centrifuged and frozen in working aliquots to evaluate the response of these cells under the influence of the different transferred conditioned media.

Drug pre-treatment of cultures

In order to identify the substance that might potentially drive the observed effects, the different cell cultures were treated as indicated:

1) ENS cultures were pre-treated 1 hour before MAMP challenge with the inhibitor LNNA to block NO production; this drug was maintained in the supernatant during the 24 hour stimulation period. Unstimulated ENS supernatants were supplemented with the same LNNA concentration prior to their addition to macrophage cultures to perform TLR ligands challenge experiments.

2) RAW 264.7 macrophage target cultures were pre-incubated for 1 hour with ODQ or equivalent volume of its vehicle (ethanol), NANC drugs or an IL-10 neutralising antibody in FBS-free DMEM. Drugs were also added to transferred supernatants to maintain their effects in macrophage culture until harvesting of media.

Immunofluorescence and neuron counts

ENS cultures grown on cover-slips were fixed in Lana's fixative (4% paraformaldehyde and 14% picric acid in 0.35 M phosphate buffer) for 30 minutes before a one-hour blocking step in PBS containing 4% horse serum and 1% Triton X-100. Samples were then incubated overnight at 4°C with the anti-Hu C/D antibody, followed by extensive washing and incubation with secondary antibody to mouse IgG for 1 hour. Sample

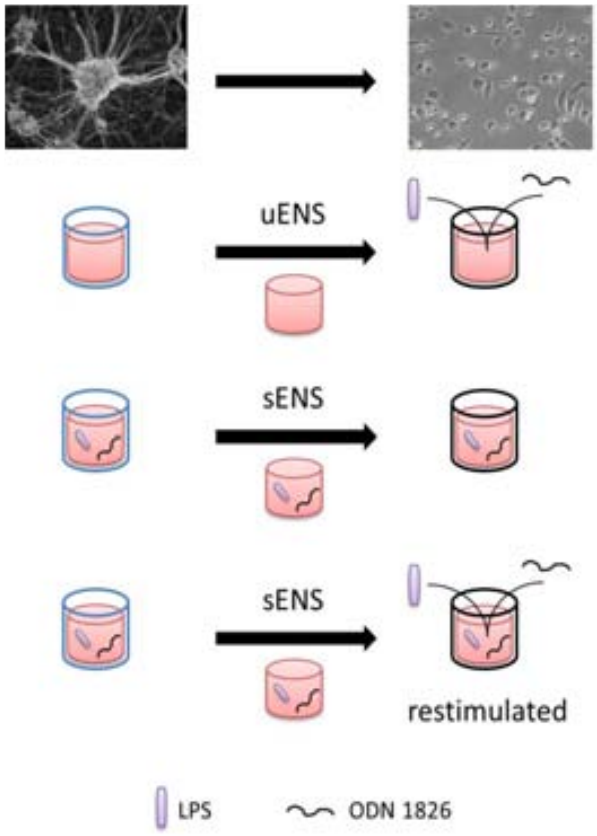


Figure 1 – Illustrating scheme of the study design. (Only ENS culture supernatants are represented). ENS cultures were stimulated (sENS) or not (uENS) with LPS or combinations of LPS and ODN 1826 for 24 hours. Conditioned supernatants were then transferred to RAW 264.7 macrophage cultures, which were subsequently stimulated or not, as illustrated. RAW 264.7 cells were incubated for 4 hours before harvesting of supernatants and determination of TNF-α.

washing was followed by addition of a 300 nM DAPI solution for 1-2 minutes before mounting the cover-slips in Vectashield aqueous anti-fading mounting medium (Vector Laboratories, Burlingame, CA, USA). Eleven to twenty ganglia from each ENS culture preparation were analysed under a Zeiss LSM 700 confocal laser microscope (Carl Zeiss, Madrid, Spain). Merged images from Hu C/D and DAPI staining were used to count the number of neurons per ganglion.

Calculation of the Nuclear Area Factor

The Nuclear Area Factor (NAF) was calculated as previously described [30, 31]. Briefly, eleven micrographs from each preparation of DAPI-stained ENS culture were analysed by means of the ImageJ software (National Institutes of Health, Bethesda, USA). DAPI staining was automatically thresholded to determine the neuronal area and perimeter (in pixel units, as indicated elsewhere [31]), and circularity was calculated by applying the

formula $4*\pi*(area/perimeter^2)$. Nuclei roundness was calculated as the inverse of circularity, and NAF was obtained as the product of the object area (in square pixels)*roundness. An apoptotic cell has an almost perfectly round nucleus with reduced area; as roundness is given a value of 1 (the lowest possible), its product with the area results in smaller values for apoptotic cells [30].

TNF-α ELISA

Culture supernatants were thawed and assayed with the TNF-α BD OptEIATM ELISA Set (BD), following manufacturer's instructions. Final cytokine values were related to the total protein amount of the sample, which was determined by means of the BCA protein assay kit (Pierce, Rockford, USA).

Nitrite quantification assay

NO release to culture supernatants and its inhibition by LNNA was quantified through measurement of nitrite, one of its stable reaction products, by means of the Griess reagent. Equal volumes of supernatant and Griess reagent (Sigma) were mixed in a 96-well plate and incubated for 10 min in the dark. Optical density of the reaction was read at 550 nm in an iEMS MF plate reader (Labsystems, Helsinki, Finland). NO concentrations in experimental samples were calculated from a standard concentration-absorbance curve.

Real-time RT-PCR analysis

Total RNA from ENS culture was extracted using the RNeasy Mini Kit (Qiagen, Las Matas, Spain) and quantified by optical densitometry. 100 ng of RNA were retro-transcribed by using the Transcriptor First-strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany) for reverse-transcriptase polymerase chain reaction (RT-PCR). Primer sequences listed in Table 2 were previously described elsewhere [32]. PCR amplifications were performed using the LC480 SYBRGreen I Mastermix (Roche Applied Science) according to manufacturer's protocol, and run on a LightCycler 480 II instrument (Roche Applied Science). mRNA level of expression of the genes of interest was corrected to that of the housekeeping gene S6 and calculated by the ΔΔCt method[33].

Gene	Sense primer	Antisense primer	Reference
rS6	CCAAGCTTATTCAGCGTCTTGTTACTCC	CCCTCGAGTCCTTCATTCTCTTGGC	NM_017160
rIL-10	GCAACAACGCAATCTATGACA	GAAAGCCCTGTATTCCGTCTC	(32)
rTGF-β	ATTTTGGAGAGAGGTACAAACGAG	TAGTGCTGACAGATTCCTTACTGC	(32)

Table 2 – List of primers used.

Statistical analysis

Results are presented as mean values ± S.E.M. of at least three independent experiments. All data were compared using Student's t-test for comparisons of two means, and one-way or two-way ANOVA when comparing more than two groups, followed by Tukey's post hoc test (unless otherwise stated). Where stated, randomised block design analysis was performed to minimize the variability due to differences between individual culture responses. Data analysis and plot were performed with GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, USA). Randomised block design analyses were performed with Minitab 15 Statistical Software (Minitab Inc., Pennsylvania, USA). A P value < 0.05 was considered to be significant.

Results and discussion

MAMP-stimulated ENS culture modulates macrophage TNF-α production upon TLR stimulation

Previous work by our group demonstrated that supernatants from MAMP-stimulated ENS culture and EGCs have increased chemoattractant properties [23]. Migrating immune cells might thus encounter a conditioned milieu enriched in cytokines and chemokines, which may therefore prime their subsequent responses.

To evaluate how MAMP-stimulated ENS cultures can modulate macrophage responses, we studied the effects of the LPS- or ODN 1826+LPS-challenged ENS on macrophage activation after MAMP recognition. RAW 264.7 macrophages were initially incubated in conditioned media from ENS or 3T6 fibroblast cultures that had been previously stimulated or not with TLR4 or TLR4/9 ligands, and TNF-α production by macrophages was determined as an indicator of their pro-inflammatory response to MAMPs.

In basal conditions, incubation of RAW 264.7 cells with supernatants from uENS culture or DMEM medium did not induce TNF-α release, in contrast to u3T6 supernatants (DMEM= 2.74±1.05 and uENS= 1.45±0.68 vs. u3T6= 54.97±3.46 ng TNF-α/mg of protein, n=4-7, $P<0.001$ for 3T6 cells; Fig. 2A). Upon TLR4 stimulation with LPS, RAW 264.7 macrophages conditioned with uENS culture supernatants significantly increased their production of TNF-α when compared to DMEM-conditioned macrophages (uENS= 265.7±75.71 vs. DMEM= 51.13±30.19 ng TNF-α/mg of protein, n=5, $P<0.001$; Fig. 2B), demonstrating that factors secreted in ENS culture in basal conditions may enhance macrophage pro-inflammatory responses upon TLR4 signalling. In contrast, conditioning of RAW 264.7 cells with supernatants from LPS-sENS culture resulted in a less pronounced increase in cytokine production (sENS= 151.1±38.12 vs. uENS= 265.7±75.71 ng TNF-α/mg of protein, n=5, $P<0.05$; Fig. 2B), even though such supernatants contained pro-inflammatory cytokines such as IL-6 and TNF-α [23]. This attenuated response (40% of inhibition) was not reproduced by s3T6 supernatants (Fig. 2B). These observations might either indicate that MAMP-stimulated ENS cultures have tolerogenic effects on immunocytes or that, in basal conditions, ENS cultures secrete factors that favour pro-inflammatory responses by immunocytes; such factors would be lost or down-regulated after MAMP-challenge, perhaps as a result of cell death.

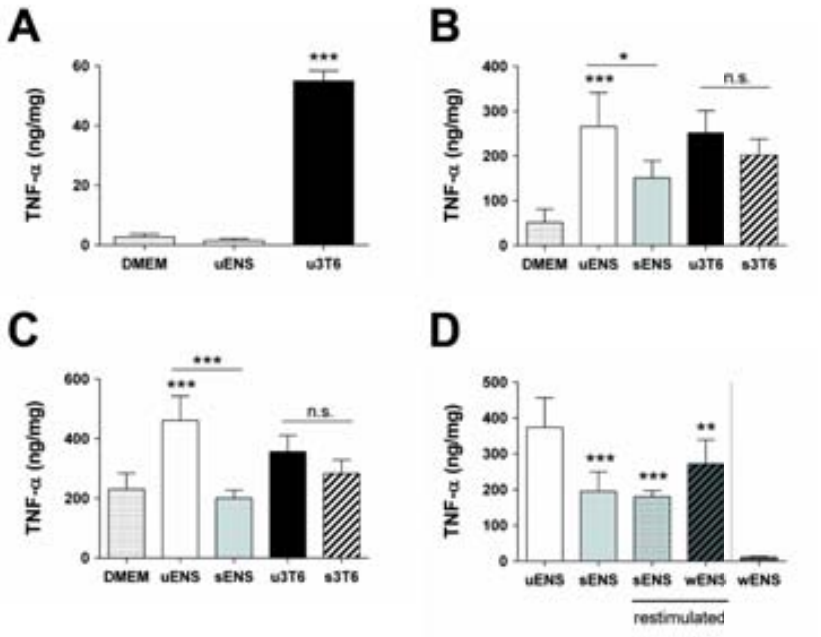


Figure 2 – Effects of conditioned media from ENS and 3T6 fibroblast in RAW 264.7 macrophage production of TNF-α. A) Basal conditions (n=4-7; u3T6 vs. DMEM and uENS, ***P<0.001). **B)** LPS challenge (n=5; uENS vs. DMEM, ***P<0.001; sENS vs. uENS, *P<0.05; s3T6 vs. u3T6, not significant (n.s.); randomised block design analysis followed by Tukey's post-hoc test). **C)** ODN 1826+LPS combined challenge (n=7; uENS vs. DMEM, sENS vs. uENS, ***P<0.001; s3T6 vs. u3T6, not significant (n.s.); randomised block design analysis followed by Tukey's post-hoc test). **D)** ODN 1826+LPS combined stimulation of ENS culture followed by stimulus washing (wENS) and/or restimulation in RAW 264.7 macrophages (n=4-6; sENS and restimulated sENS vs. uENS, ***P<0.001; restimulated wENS vs. uENS, **P<0.01; randomised block design analysis followed by Tukey's post-hoc test).

The observed inhibitory effect was also tested after combined challenge with ODN 1826 and LPS, which had already shown synergic effects in cytokine release in ENS culture [23].

Costimulation with TLR4/9 ligands enhanced TNF-α production in the macrophage cell line, which was further increased by incubating RAW 264.7 cells in uENS conditioned media (uENS= 461.8±81.11 vs. DMEM= 230.6±53.48 ng TNF-α/mg of protein, n=7, P<0.001; Fig. 2C). However, combined challenge of TLR4/9 in ENS culture elicited an increased inhibitory effect in the release of TNF-α by RAW264.7: in ODN 1826+LPS-sENS-conditioned macrophages the inhibitory effect was 50-55% (sENS= 200.5±27.1 vs. uENS= 461.8±81.11 ng TNF-α/mg of protein, n=7, P<0.001; Fig. 2C), compared to the

40% inhibition reported when macrophages were exposed to LPS-sENS supernatants. Once again, such inhibitory effect was not mimicked by 3T6 fibroblast supernatants (Fig. 2C), suggesting that the observed response was not dependant on myofibroblast-secreted factors.

TLR4/9 ligands were added fresh in uENS-conditioned macrophages, whereas challenge of sENS-conditioned macrophages was performed by remaining ligands from previous 24-hour stimulation in ENS cultures. Therefore, inhibition observed in sENS-conditioned macrophages could be secondary to degradation of the ligands in ENS cultures. To discard this possibility, fresh TLR4/9 ligands were added in parallel to sENS- and uENS-supernatants. Of note, the inhibitory effect elicited by sENS supernatants on TLR4/9-induced macrophage cytokine release was preserved in restimulated sENS-supernatants (restimulated sENS= 180.3±16.61 vs. uENS= 373.8±82.75 ng TNF-α/mg of protein, n=5-6, $P<0.001$; Fig. 2D), indicating that the inhibition observed was not due to a decay in the concentration of ligands.

Finally, we assessed whether withdrawal of the inflammatory stimuli in ENS culture resulted in loss of the hyporesponsive effect in macrophages. ENS stimulated for 24 hours with TLR4/9 ligand combinations were washed with PBS and fresh medium was added for an additional period of 24 hours. Supernatants were then harvested and used to condition RAW 264.7 cells. Washed ENS (wENS) supernatants did not induce important TNF-α production in conditioned macrophages (wENS= 10.93±2.13 ng TNF-α/mg of protein, n=4; Fig. 2D). Addition of fresh ODN 1826+LPS to wENS supernatant induced TNF-α production in conditioned macrophages, but 27% inhibition was still preserved when compared to uENS (restimulated wENS= 271.9±67.36 vs. uENS= 373.8±82.75 ng TNF-α/mg of protein, n=5-6, $P<0.01$; Fig. 2D). Thence, the hyporesponsiveness elicited by the ENS is still present 24 hours after withdrawal of the stimulus, pointing out that such finding is dependent on the plastic changes underwent by the ENS, and not on the presence of the ligands.

Isolated EGCs are not involved in the observed hyporesponsiveness

EGCs in culture conditions produce neurotrophins that are up-regulated after LPS challenge [34]. Since neurotrophins can play both pro-inflammatory and immune-modulatory roles [9, 20], JUG2 EGC cultures were treated like ENS and 3T6 fibroblast cultures, and their supernatants were then transferred to macrophage cultures. The

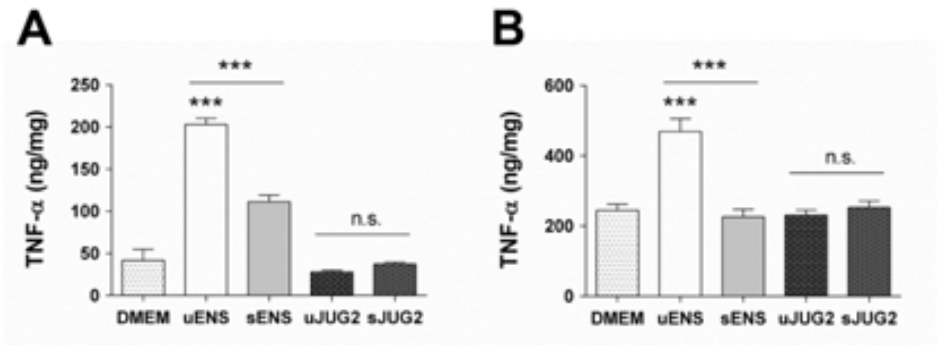


Figure 3 – Effects of JUG2 EGC conditioned media in RAW 264.7 macrophage production of TNF-α. A) LPS challenge (n=3; uENS vs. DMEM, sENS vs. uENS, ***P<0.001). **B)** ODN 1826+LPS combined challenge (n=3; uENS vs. DMEM, sENS vs. uENS, ***P<0.001).

responses obtained after transferring ENS conditioned media were not mimicked by EGC supernatants, as TNF-α production values after LPS conditioning were almost the same as DMEM values (uJUG2= 28.11±1.81 and sJUG2= 37.39±2.03 vs. DMEM= 41.75±13.02 ng TNF-α/mg of protein, n=3; Fig. 3A). Furthermore, according to previous observations demonstrating that JUG2 cells do not respond to ODN 1826 in TLR4/9 combined challenges [23], EGC costimulation with such MAMPs did not alter the macrophage response (uJUG2= 230.7±15.03 and sJUG2= 252.6±18.57 vs. DMEM= 244.8±17.52 ng TNF-α/mg of protein, n=3; Fig. 3B). Therefore, we concluded that EGCs are not involved in the inhibitory effect promoted by stimulated ENS medium, at least when they are cultured in the absence of other cell types.

ENS supernatant-induced hyporesponsiveness is not due to neuronal apoptosis

ENS neurons have been shown to play crucial roles in determining inflammation severity. Transgenic mice bearing 150% enteric neurons display increased inflammation severity in two different chemically-induced models of colitis, whereas mice that have decreased density of neurons (around 50%) exhibit milder inflammatory processes [4]. In view of such evidence, it is reasonable to think that enteric neurons might have pro-inflammatory properties.

One possible explanation to the fact that uENS-conditioned macrophages had increased responses to LPS and ODN 1826+LPS when compared to DMEM-conditioned macrophages (Fig. 2B and C), is that ENS cultures in basal conditions have some pro-

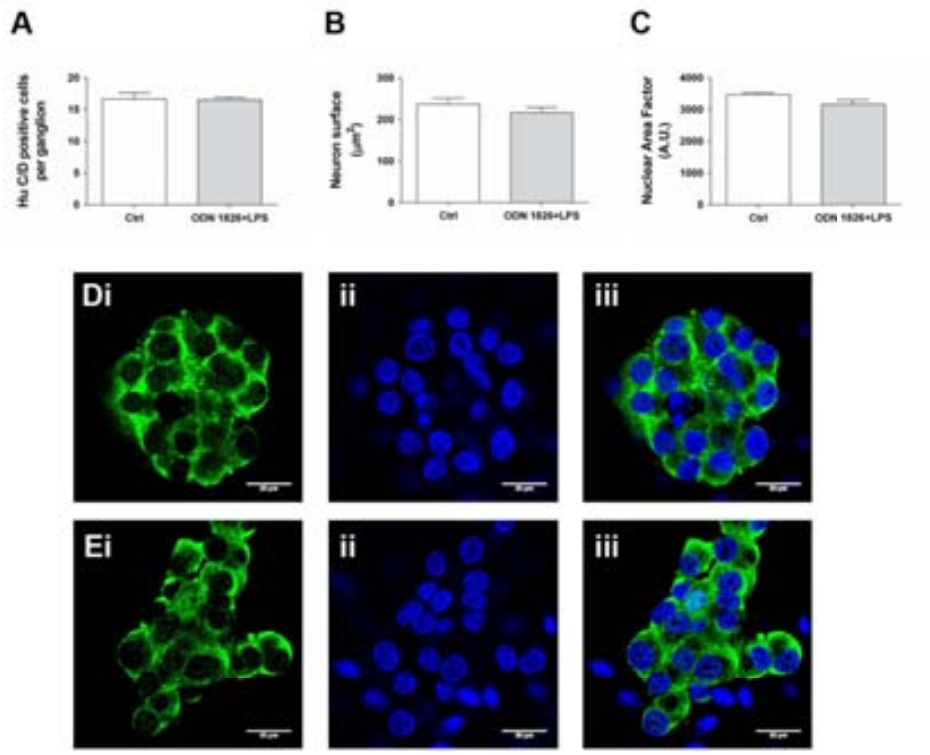


Figure 4 – Assessment of morphometric parameters in enteric neurons. A) Neuronal counts expressed as Hu C/D positive cells per ganglion (n=3). **B)** Neuronal size (obtained as the ratio between the ganglion total area and the number of neurons; n=3). **C)** NAF values (n=3). (**D, E)** Representative micrographs corresponding to untreated **(D)** or ODN 1826+LPS-treated **(E)** ENS cultures stained with Hu C/D **(i)** or DAPI **(ii)**. Merged channel pictures **(iii)** were used to count neurons. Scale bars correspond to 20 µm.

inflammatory priming capacity in RAW 264.7 cells, facilitating enhanced TNF-α production upon TLR stimulation. During inflammation, however, an event such as neuronal death would terminate in a decrease of priming factor secretion. To test this possibility, we compared the number of neurons per ganglion in untreated and ODN 1826+LPS-treated ENS cultures, as well as nuclear morphology of enteric neurons. Though calculation of the NAF on DAPI staining is not the best method to estimate apoptosis, the determination of the NAF in neurons is relatively straightforward, and nuclear morphology changes are found as soon as 4 hours after the addition of the apoptotic stimulus [30].

24 hours after MAMP addition, morphologic alterations were evident in EGCs (not shown), whereas no apparent changes were observed in neurons (Fig. 4Di and Ei). Indeed, the number of enteric neurons per ganglion was preserved in challenged ENS cultures (ODN 1826+LPS= 16.52±0.42 vs. Ctrl= 16.66±0.98 neurons/ganglion, n=3; Fig. 4A) and no significant changes were observed in their size (ODN 1826+LPS= 216.6±7.4 vs. Ctrl= 236.8±8.6 μm^2/neuron, n=3; Fig. 4B) and NAF (ODN 1826+LPS= 3162±138.8 vs. Ctrl= 3469±58.14 A.U., n=3; Fig. 4C).

These results point out that there is no neuronal loss or hyperplasia associated to TLR-induced inflammation. Such findings are in accordance with a report by Anitha and collaborators, where stimulation of ENS cultures with the same doses of LPS resulted in reduced apoptosis compared to control cultures [22]. Furthermore, inflammatory pathologies such as Crohn's disease and ulcerative colitis are not characterised by apoptotic but hyperplastic neurons [2].

In view of these observations, we considered that neuronal impairment or apoptosis was not the causative of the reported effect.

Nitric oxide does not participate in macrophage hyporesponsiveness

We next focused on the possibility that, following TLR stimulation, ENS cultures might produce immunomodulatory molecules that would promote tolerance in macrophages. Several molecules released by the ENS might mediate such response, but we first got interested in NO for two reasons. First, previous reports in ENS cultures show that 68±6% of neurons are immunoreactive for the neuronal form of the NOS (nNOS) [28], and second, iNOS is up-regulated and NO production is increased after TLR4/9 stimulation in these cultures [23]. Furthermore, addition of NO donors to RAW 264.7 cell cultures has been shown to attenuate LPS-induced cytokine production through different mechanisms such as inhibition of the NF-κB signalling pathway [35] or activation of the soluble guanylyl cyclase, which increases the expression of the immunomodulatory molecule transforming growth factor (TGF)-β [36, 37]. In the same vein, nNOS and iNOS have been shown to decrease NF-κB activity through S-nitrosylation of its subunits [14, 38]. Taken together, these evidences supported the involvement of NO as a released mediator potentially responsible for the conditioning effects observed in RAW 264.7 cells.

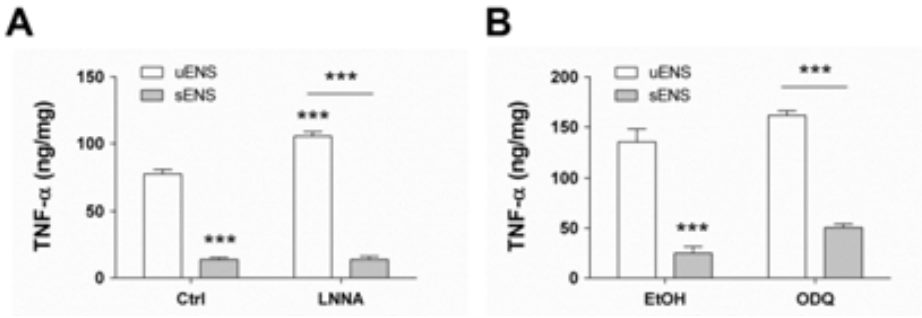


Figure 5 – TNF-α production in conditioned macrophages after blockade of NO synthesis and signalling pathways. A) Pre-treatment of ODN 1826+LPS-stimulated ENS cultures with 1 mM LNNA followed by supernatant transferring to RAW 264.7 cells (n=3; sENS vs. uENS, sENS-LNNA vs. uENS-LNNA, uENS-LNNA vs. uENS-Ctrl, ***P<0.001). **B)** ODN 1826+LPS-challenged macrophages pre-treated with 10 µM ODQ or the same amount of its vehicle, ethanol (EtOH) (n=3; sENS-EtOH vs. uENS-EtOH, sENS-ODQ vs. uENS-ODQ, ***P<0.001).

We followed two distinct strategies to evaluate the effects of NO in our system. First, we non-selectively blocked NO synthases by using 1 mM LNNA, and second, we selectively inhibited guanylyl cyclase activation through incubation with 10µM ODQ, targeting the downstream effects of NO driven by this enzyme. Inhibition of nNOS and iNOS activity in sENS cultures with LNNA caused a marked decrease in NO production (sENS-LNNA= 2.85±1.15 vs. sENS= 15±2.8 µM, n=3, P<0.01; not shown). However, addition of these NO-depleted sENS supernatants to RAW 264.7 cells did not reverse their hyporesponsiveness to TLR4/9 ligands (Fig. 5A). In contrast, incubation of macrophages with uENS supernatants in the presence of LNNA enhanced their response to combined TLR4/9 ligands (uENS-LNNA= 105.45±3.3 vs. uENS-Ctrl= 78.1±3.19 ng TNF-α/mg of protein, n=3, P<0.001; Fig. 5A), indicating that blockade of iNOS in these cells is associated with increased NF-κB activation, as previously described [38].

In the same vein, pre-incubation of macrophages with ODQ to block guanylyl cyclase did not modify the inhibitory response induced by sENS cultures, either (Fig. 5B). So, we concluded that NO was not the molecule of interest we were seeking.

Blocking the effects of other molecules such as ACh, NE and IL-10 does not reverse the sENS-induced tolerogenic response

Immunodetection of iNOS in ENS cultures revealed the presence of resident macrophages around the ganglia [23]. Since these cells are known to produce immunomodulatory substances such as IL-10 and TGF-β, we performed RT-qPCR for these cytokines to check their mRNA expression levels in LPS-treated ENS cultures at 8 hours post-stimulation. Transcripts for IL-10 were increased from undetectable levels (more than 40 cycles) in control cultures to crossing points of 32 cycles in LPS-challenged cultures (n=3; not shown). Assuming that untreated cultures had a crossing point value of 40 cycles, this could represent at least a 250-fold increase in IL-10 expression. On the other hand, TGF-β mRNA was also increased (LPS= 2.19±0.41 vs. Ctrl= 1.03±0.17 folds, n=3, *P*=0.058) but not so strikingly, so we decided to block IL-10 effects with a neutralising antibody (αIL-10). Following manufacturer's instructions, a dose of 1 µg/mL was used to pre-incubate macrophages with αIL-10. Although αIL-10 was maintained during the whole conditioning period, no differences in production of TNF-α between sENS- and sENS+ αIL-10-conditioned macrophages were observed (Fig. 6A).

In parallel, we additionally blocked cholinergic and adrenergic receptors in macrophages with a combination of nicotinic, muscarinic, α-adrenergic and β-adrenergic (NANC) antagonists. Indeed, activation of α7 nicotinic ACh receptors in macrophages after vagal stimulation exerts a potent anti-inflammatory effect that can

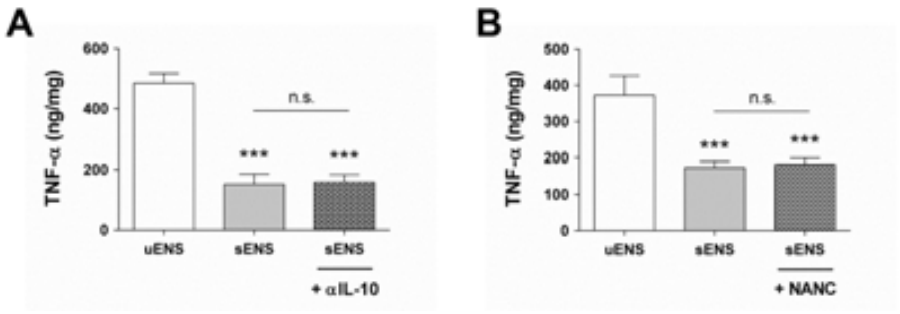


Figure 6 – Neutralisation of IL-10 and cholinergic/adrenergic receptors in conditioned RAW 264.7 cells. A) ODN 1826+LPS-conditioned macrophages pre-treated with 1 µg/mL of IL-10 neutralisation antibody (n=3; sENS and sENS+αIL-10 vs. uENS, ****P*<0.001). **B)** ODN 1826+LPS-challenged macrophages pre-treated with 1 µM of different cholinergic and adrenergic antagonists (n=3; sENS and sENS-NANC vs. uENS, ****P*<0.001).

be inhibited by hexamethonium pre-treatment [10], and β2-adrenergic receptor stimulation with salbutamol reduces the inflammatory responses after LPS challenge in RAW 264.7 macrophages [11, 39]. However, in our culture system, blockade of these receptors had no effects in sENS-induced tolerance (Fig. 6B), suggesting that ACh and NE could be discarded as candidate molecules to exert such effect.

Final discussion and perspectives

In this study, we report a remarkable decrease in cytokine production after conditioning of macrophages with supernatants from MAMP-challenged ENS cultures. As stimulation of TLR4 in ENS cultures had previously resulted in cytokine and chemokine secretion [23], we initially expected ENS-conditioned media to prime subsequent macrophage response, enhancing TNF-α production after LPS-challenge. Contrastingly, our experimental results demonstrate that inflamed ENS cultures induce important hyporesponsiveness in RAW 264.7 cells, with inhibition percentages of 40-55% depending on the ligand combination used. Although the search of the causative mechanism lured our attention, we were not able to unveil it. Identification of the cell type/s and the molecule/s responsible for such effects might be of enormous interest in designing novel therapeutic strategies to treat inflammatory processes, and so, further efforts should be devoted to the characterisation of this response. Initially, the explanation for the obtained responses was sought in the design of the study: uENS-conditioned macrophages were stimulated with fresh TLR ligands, whereas sENS-conditioned cells might be receiving degraded MAMPs. Restimulation experiments demonstrated that such effect was not dependent on ligand degradation, and that even after withdrawal of the TLR stimulus, ENS cultures retained part of their inhibitory effect. Henceforth, two different hypotheses might explain the observed effect. On one hand, uENS could be releasing one or more substances that would facilitate macrophage TNF-α release upon TLR4/9 stimulation; production of such substances would be lost or down-regulated after MAMP-treatment, perhaps associated to cellular apoptosis. This hypothesis was supported by the similar responses elicited by sENS supernatants and DMEM in macrophages. However, increased TNF-α production in conditioned macrophages might be due to enrichment of media with different factors. Such factors would facilitate and enhance their responses (similar effects were observed after ENS and 3T6 fibroblast conditioning), as it is observed when they are

cultured in FBS-supplemented medium (unpublished observations). Furthermore, the number, size and NAF of enteric neurons, which may play such facilitating role [4], were unaltered in sENS cultures. Therefore, such hypothesis was soon dismissed.

On the other hand, the most likely explanation for the observed inhibition of cytokine production was that MAMP-stimulated ENS cultures, in addition to pro-inflammatory cytokines such as IL-6 or TNF-α [23], were also releasing immunomodulatory molecules. In fact, combination of TLR4/9 ligands caused a marked increase in the percentage of inhibition when compared to single activation of TLR4. This response, similar to what we observed with IL-6, TNF-α and NO [23], is compatible with the enhanced production of a modulatory substance. In this regard, an important drawback of these ENS cultures is that they also harbour resident macrophages from the *muscularis externa* [27]. Thus, in addition to neurons, glia and myofibroblasts, these cells might additionally play a role in the observed effect. Participation of EGCs and myofibroblasts could be initially discarded, as well as NO, ACh, NE and IL-10 involvement. However, the possibilities are still numerous. Thence, the characterisation of the molecule responsible for such effects might benefit of an alternative approach, maybe similar to that described by Savidge and co-workers [40]. Supernatant fractioning by size-exclusion chromatography and determination of the fraction responsible for the effect should be accompanied by screening of up-regulated molecules in sENS supernatants.

Whether MAMPs are capable of evoking plastic changes in enteric neurones must still be addressed in cultures devoid of *muscularis* macrophages, to avoid the effects of pro-inflammatory cytokines. However, the present study shows that in the complex microenvironment generated within ENS primary cultures, MAMP-signalling through TLR4/9 elicits the production of immunomodulatory substances that might play important roles in attenuation of inflammation. Therefore, identification of such involved molecules, as well as of the cell type responsible for its release, could be of great value to develop new therapeutic approaches in the treatment of inflammatory bowel diseases. Potentiating the release of such molecules, or directly administrating them to patients suffering from these pathologies, could be useful in ameliorating and reducing severity of their symptoms.

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Discussion

Since the discovery of TLRs as PRRs which are able to sense the presence of MAMPs and to shape the immune response, a wide number of researchers have devoted their efforts to understand different aspects of their functional regulation. This has given rise to an overwhelming accumulation of data, but has also brought about conflicting data and puzzling views. Since the mucosa of the digestive system is the widest surface in contact with the external milieu, and since inflammatory events in the GI tract are a highly prevalent event, the aim of this work has been to approach some aspects of the function of TLRs in the GI tract, in an attempt to offer an integrated view on their role in different cell types and in particular situations. Specifically, our work has focused on the role of TLR2/4 during homeostasis and inflammation in different cell types that populate the lower GI tract.

Since IBDs are most likely linked to TLR dysregulation, we chose one of the most widely used animal models of IBD to evaluate the outcomes of TLR2/4 stimulation by specific ligands given locally, i.e. intracolonic administration. In addition, since strong TLR immunoreactivity was always observed in the intrinsic nervous plexuses when performing immunohistochemistry (IHC), we got interested on the putative role that these receptors might be playing in the ENS. Our results indicate that the intestinal barrier has a strong effectiveness in isolating the internal milieu from high MAMP loads in the lumen, but once it is broken, other mechanisms such as increased IEC proliferation might be activated in order to prevent invasion of the host internal milieu by microorganisms bearing such motifs. Taking advantage of these mechanisms may thus become a useful tool to reduce the severity of GI inflammation. In parallel, other cell types such as EGCs and resident macrophages can be activated in contact with MAMPs, secreting cytokines and chemokines to chemoattract immune cells. Such immune cells mediate the host protection against the invading microorganisms, but their activity is probably modulated by molecules secreted by resident cells that might prime the immune cell response depending on the stimulated TLRs.

To better understand how our findings might fit in what is known about TLR functioning in the GI tract, we would like to discuss them from a chronologic point of view, since the understanding of these receptors has rapidly evolved in recent years, in parallel to our work.

TLR discovery and description of LPS tolerance

The currently known TLR4 was first described by Medzhitov and collaborators [1] in 1997 as the human homologue to the *Drosophila* Toll receptor, which had been shown to mediate antifungal responses in this fly [2]. Such human homologue "Toll-like" receptor was expressed in monocytes, macrophages, DCs, γ/δ T cells, Th1 and Th2 α/β T cells, a small intestine epithelial cell line, and a B-cell line; however, the strongest expression was detected in spleen and peripheral blood cells [1]. Activation of this receptor induced IL-1, IL-6 and the chemokine IL-8 in a human monocyte cell line [1]. Henceforth, TLRs were mainly associated to immune cells, and subsequent studies revealed that their importance is not only circumscribed to cytokine and chemokine induction, since they are crucial in promoting maturation of APCs and up-regulating expression of costimulatory molecules [3, 4], as well as in providing additional costimulatory signals in T-cells, eliciting apoptosis inhibition and proliferation [5].

LPS was identified as the ligand of TLR4 when the phenotype of LPS-hyporesponsive mice was associated to mutations in the *Tlr4* gene [6]. Endotoxin (LPS) had been known for years as the causative of septic shock, and it had been also evidenced that, following repeated intravenous injections of this "antigen", tolerance was developed [7]. Therefore, TLR4 became not only related to inflammatory responses to Gram-negative bacteria, but also to tolerance induction. Currently, it is known that several mechanisms are involved in LPS tolerance development in peripheral blood leukocytes, from alterations in the signalling pathway to down-regulation of TLR4 surface expression [8].

Characterisation of other members of the TLR family increased diversity and complexity to the understanding of inflammatory and tolerogenic responses. The study of TLR2 gained importance since it was capable of recognising a number of different ligands, including Gram-positive and yeast molecules [9, 10]. Furthermore, TLR2 activation was able to induce both pro-inflammatory and immunomodulatory responses [9, 11], and showed cross-responsiveness with TLR4 [12-14]. Concerning immune tolerance, TLR2-mediated stimulation of DCs *in vitro* promoted increased IL-10 production that subsequently blocked TLR4-induced pro-inflammatory cytokines [14], as well as the antigen-specific proliferative responses of T-cells conditioned by LPS, both *in vitro* and *in vivo* [15].

TLRs, GI homeostasis and IBD: what we knew

Taking into consideration that 1) TLRs recognise microbial motifs [16]; 2) the GI tract harbours the highest microorganism burdens [17]; 3) the GI tract is considered an immune-privileged site in terms of tolerance [18]; and 4) IBDs are the outcome of a deregulated immune response to resident microbiota [19], it was reasonable to hypothesise that these receptors would be playing important roles in GI homeostasis and disease. Expression of TLRs in the GI tract was therefore addressed in both physiologic and inflammatory conditions, in both human and mice [20-22]. Initial descriptions indicated that TLR2 and TLR4 were only expressed by a few immune cells in the intestine and colon during resting conditions, but they increased in parallel to inflammation-associated macrophages [20, 21]. Furthermore, TLRs were also found in mouse colonic epithelial cells, especially in those populating the deeper layers of the crypts [22]. The lack of TLR expression in epithelial cells was interpreted as a mechanism to preserve mucosal tolerance to the lumen microbiota, whereas their increase during inflammatory conditions could be due to a deregulation in their function or to a secondary response to face the massive microbial challenge after barrier leakage. In this background, we hypothesised that challenge with different doses of TLR2 ligands could result in inflammation, but administration of a sub-inflammatory dose could lead to down-regulation of TLR2 expression and/or enhancement of Th2 cytokine production, which might counteract the Th1 inflammation observed in mouse experimental models of IBD. Such hypothesis was supported by the fact that different adaptions reported in absorptive enterocytes upon TLR2 signalling induced hyporesponsiveness after TLR challenges [23, 24]. Moreover, TLR2 activation in the follicle-associated epithelium enhanced particle uptake and transport by M cells, as well as subepithelial DC migration into this specialised epithelium [25], which could facilitate the generation of tolerance or Th2 responses by promoting IL-10 production in DC migrating to MLNs [14, 15, 26].

TLR2 was therefore initially chosen as our target TLR because it was someway associated to Th2 cytokine production [11, 14], which was known to inhibit development of Th1 responses [26, 27]. Additionally, stimulation of TLR2 in IECs could lead to inhibition and cross-regulation of TLR signalling [23, 24], which would also be beneficial in promoting hyporesponsiveness to luminal contents.

TLR2 expression during homeostasis and inflammation

As a first approach, we performed TLR2 IHC and qPCR in order to localise and quantify the expression of this protein in homeostatic conditions and inflammation of the mouse colon. In physiologic conditions, TLR2 was an intermediate expression gene ($\Delta Ct_{TLR2/\beta\text{-actin}}$= 7; Annex 1) [28] that was predominantly found in the crypt bottoms [29], although constitutive expression could be also observed in colonocytes from the apical parts of the crypt, discrete cells from the *lamina propria*, muscle fibres and in the intrinsic ENS plexuses (chapters 1 and 3). These observations were, to some extent, contradictory with what had already been described in human specimens, where expression was shown to be low and only in immune cells [20, 21]. In this regard, two possible interpretations might explain such reported differences. First, that there are marked differences in TLR expression between both species, since other work in mice corroborate their presence in IECs [22, 25, 29], Paneth cells [30], goblet cells [31] and enteroendocrine cells [32]. Or second, that the antibodies used in these first studies characterising expression of TLR2 in human tissues had a low sensitivity. Actually, the antibodies and antisera that were used [20, 21] have not been validated in other reports, as they are not commercially available. In contrast, our findings have been confirmed with three different antibodies (Imgenex IMG-5651, Biorbyt orb11487 and Abcam ab108998).

In either case, there was a common finding in our lab and elsewhere: TLR2 was up-regulated during colitis (chapters 1 and 2) [20-22, 29]. Such up-regulation was observed as an increased recruitment of positive inflammatory cells in the submucosa and a higher immunoreactivity in IECs positioned at the top of preserved crypts [29], especially in those acquiring hyperplastic morphology (chapter 1). These findings suggested that TLR2 expression was associated to the inflammatory infiltrate and the lesions observed. In the same vein, later studies addressing amelioration of colitis signs (colon length, DAI and histological score) through different approaches have been accompanied with significant TLR2 down-regulation in colon (chapter 2) [29, 33]. Furthermore, over-expression of this receptor caused by NF-κB activation or pro-inflammatory cytokine production can expand and perpetuate inflammation through positive feedback mechanisms on these factors and by signaling of reactive oxygen species [34-36]. Therefore, all these evidences point out that up-regulated expression of TLR2 during

experimental colitis is rather associated to the severity of disease than to its onset. However, this latter possibility cannot be dismissed, as TLR2 over-expression and altered responses to MAMPs have been described before the onset of inflammation in a spontaneous murine model of colitis [37]. Nevertheless, to date no polymorphisms in the *TLR2* gene have been associated to IBD risk, but to UC phenotype severity [31].

Zymosan intracolonic administration in homeostasis and inflammation

Our next objective was to achieve direct activation of TLR2 through administration of one of its ligands. The TLR2/6 and dectin-1 ligand zymosan, an insoluble carbohydrate from the yeasts' cell wall, was chosen for the intracolonic administration of mice. From an immunologic point of view, zymosan was a good candidate molecule to reach our aims, since it had been shown to induce pro-inflammatory and immunomodulatory cytokine expression in macrophages and DCs [9, 11, 15].

Selection of the doses to be given to mice was initially extrapolated from indications of the manufacturer (Invivogen). However, such amounts of product were the typically used to elicit macrophage responses in culture and, as already stated, this meant an important drawback in our research. In fact, the doses used in the first set of experiments (10 and 100 µg; chapter 1) are usually used to induce peritonitis [38], whereas sub-inflammatory responses in colon are observed after intracolonic administration of 3 mg of zymosan per mouse for 3 consecutive days [39]. Indeed, the only alteration we observed while working with zymosan was a 50% increase in the nNOS protein expression in CMC+Zym500-treated mice, but not in CMC+Zym200-treated animals (chapter 2; Annex 2). Of note, this latter batch of zymosan was purchased from Sigma, as described elsewhere [38, 39], whereas the one we used in the first trial studies (chapter 1) was from Invivogen. The batch from Invivogen is guaranteed to bear less than 0.001 endotoxin units per µg of product, a warrant that is not given for the Sigma batch. As shown in supplementary figure 1 from chapter 2, the responses of the RAW 264.7 macrophages to both batches of product in terms of pro-inflammatory mediator release are significantly different. This evidence points out that purified zymosan does not account for the whole response attributed to this product, and that other contaminating substances might be interfering in the results observed in several works [38, 39]. Overall, these data suggest that, in our studies, the selected doses

of zymosan were always too low to induce inflammatory or sub-inflammatory alterations, and that special care must be taken in selecting and purchasing the ligands in order to obtain pharmacologically strict selectivity and reliable data.

Repeated administration of 30% ethanol carrying 100 µg of zymosan provoked a tendency towards shortening of colon length, consistent increases in pro-inflammatory cytokines and a more severe histologic score. These findings initially suggested that a leakage in the epithelial barrier function would be necessary for zymosan to penetrate the outer mucus layer and run its effects. Later experiments performed in DSS-treated mice demonstrated that zymosan did not worsen colitis, but did not improve its signs either, although it decreased pro-inflammatory cytokine production and TLR2 transcription (chapter 2). Therefore, we concluded that both zymosan batches, at the doses tested, were unable to improve the signs of colitis, in spite of the fact that they significantly down-regulated TLR2 when given through intracolonic instillation. Perhaps alternative doses of zymosan administered in a different regime could improve such parameters, but currently better therapeutic approaches might be used to block TLR2 expression during acute inflammatory processes, such as neutralising antibodies [40].

New roles for TLRs: involvement in epithelial repair responses

As knowledge of TLRs evolved, it was clearly demonstrated that they were directly involved in the onset of inflammation, since microbial-dependent spontaneous colitis did not occur in IL-10$^{-/-}$ mice backcrossed to MyD88$^{-/-}$ mice [41]. However, publication of a set of studies concerning the effects of DSS administration in MyD88$^{-/-}$, TLR2$^{-/-}$ and TLR4$^{-/-}$ transgenic mice [42-44] drastically changed the understanding of TLR functions in colitis. Unexpectedly, these knock-out mice displayed increased susceptibility and mortality to DSS-induced colitis when compared to wild type littermates. The mechanisms accounting for such effects were more associated to alterations in the epithelial barrier function and wound repair [42-44] than to immune cell modulation [45]. Suddenly, from an initial paradigm in which TLR2 and TLR4 were only expressed in immune cells [20, 21], the general view changed into a new scene in which TLRs were expressed in the four epithelial lineages, where they played crucial roles in regulating all the epithelial barrier functions [24, 30-32, 43, 44]. TLRs were subsequently involved in 1) secretion of cytoprotective factors that improve epithelial recovery after inflammation

[42, 43]; 2) production of antimicrobial peptides, constitutive factors of the mucus layers and hormones [30-32]; 3) strengthening of the epithelial barrier through enhancement of TJ protein synthesis [23, 44]; 4) antiapoptotic functions in IECs and goblet cells [31, 44]; and 5) epithelial cell proliferation and differentiation [43, 46, 47]. Therefore, the study of TLR modulation in inflammatory cells was overshadowed by their new described functions in IECs.

From an integrative point of view, perhaps the work by Ungaro and co-workers might be illustrative of the involvement of TLRs in inflammation [47]. In this report, intraperitoneal administration of a TLR4 neutralising antibody during the development of DSS-induced colitis ameliorates the signs of colitis during the acute inflammatory phase (until day 7), but impairs subsequent mucosal repair in the wound healing phase (days 7 to 14). This defective healing caused increased histologic scores and higher mortality in the animal groups treated with the TLR4 neutralising antibody, which could be associated to decreased epithelial proliferation and reduced COX-2 and PGE2 production [47]. In the same vein, thorough interpretation of our findings in chapter 2 suggests that the final resulting inflammation in DSS-treated mice after TLR ligand administration is established as a dynamic interaction between activation of the hematopoietic and epithelial TLRs. However, evidences reported in our work must still be further complemented with additional experiments to find out the precise mechanisms implicated in IECs proliferation.

MAMP intracolonic administration: importance of the epithelial barrier

In the new described paradigm, we conceived the study design presented in chapter 2. Our aim was challenging the epithelial barrier and the immune system with higher amounts of a different zymosan batch and the potent pro-inflammatory ligand LPS. Either improving or worsening of colitis signs could be expected, since TLR2 and TLR4 had been already associated with pro-inflammatory responses in immune cells [48, 49], tolerance induction in APCs [8, 14, 50] and TLR cross-regulation, anti-apoptosis and proliferation in IECs [24, 42, 43, 47, 51].

The importance of the epithelial barrier in isolating the host from its external milieu and preserving hyporesponsiveness to microbial ligands was evidenced by intracolonic administration of zymosan and LPS. In physiologic conditions, high loads (200 and 500

μg) of these MAMPs added in the colon lumen of healthy untreated mice on two alternate days had no remarkable effects in colonic histology or expression of inflammatory transcripts (chapter 2). Indeed, although induction of colitis has been associated with increases in TLR2 and TLR4 ligand concentrations [52], an initial alteration of the inner mucus layer seems necessary to facilitate MAMP penetration and trigger an inflammatory response [53, 54]. Similar conclusions were obtained after our trial experiments in chapter 1, in which repeated administration of 30% ethanol as vehicle for zymosan particles was necessary to achieve an inflammatory phenotype. Such effect was probably observed because of the disrupting properties that ethanol has on the epithelial mucus layers [55]. Therefore, in homeostatic conditions, the mechanisms implicated in maintenance of the tolerance to the microbes dwelling the gut lumen are highly effective, as they avoid inflammation induction even after occasional instillation of high MAMP loads.

In addition, our experiments did also show that the epithelial barrier played crucial roles in protection of the internal milieu during inflammation. From an immunologic point of view, TLR4 activation has been generally associated to cytokine production [1], as we showed in RAW 264.7 macrophages. Moreover, the use of TLR4 antagonists has proven useful in attenuation of the signs of colitis during the acute inflammatory phase [47, 48], probably as a result of blocking activation of TLR4 in cells of hematopoietic origin. Therefore, if we only considered the immune system, it would be reasonable to hypothesise that, after DSS-treatment, administration of LPS into the lumen would easily reach the immune cells in the mucosa and submucosa. This would aggravate the existing inflammation, unless tolerogenic responses were raised [8, 50, 56]. Conversely, we reported significant reduction of colitis severity after LPS administration in DSS-treated mice. Interestingly, such findings were not associated to immune modulation or tolerance induction, but to preservation and proliferation of IECs. Therefore, activation of epithelial TLR4 elicited a proliferative drive in DSS-treated mice that was someway protective, perhaps by reducing exposure to luminal contents or by diminishing water and blood loss to the external milieu. Our findings suggest that enhancement of the epithelial barrier integrity might play beneficial roles during experimental colitis, as previously demonstrated [57-59]. Furthermore, the usefulness of TLR ligands in enhancing

epithelial barrier functions has emerged as an interesting therapeutic target with promising results in experimental models of IBD [31, 42, 44, 58].

TLR4 activation in IECs: potential therapeutic considerations

Tacking epithelial TLR4 activation during GI inflammation with therapeutic goals deserves special caution, since increased expression of TLR4 in IECs has been associated to UC and tumorigenesis [60, 61]. Indeed, we observed that DSS-treated animals had increased TLR4 expression (chapter 2), which was especially remarkable in dysplastic crypts (Annex 3).

TLR4$^{-/-}$ transgenic mice have higher susceptibility to DSS-induced colitis [42]. Diverse mechanisms have been proposed to account for this finding, such as lack of production of epithelial cytoprotective factors [42], deficient clearing of penetrating microbes [45], impaired epithelial repair pathways [43, 58] or reduced IEC proliferation [45]. Conversely, villin-TLR4 mice, which display sustained activation of TLR4 in IECs, have increased cytokine production, longer crypts with more proliferating IECs and do also show increased susceptibility to colitis and inflammation-induced neoplasia [60, 61]. Both transgenic models demonstrate that epithelial TLR4 activation must be tightly regulated in order to preserve homeostasis.

Our experiments point out that two alternate intracolonic instillations of LPS during the acute inflammatory phase of a DSS-induced colitis provoke a significant amelioration in the DAI and histologic score of treated mice. This response is not associated to alterations in COX-2 expression, but to enhanced preservation of the epithelial lining and increased colonocyte proliferation. These findings suggest that occasional stimulation of epithelial TLR4 might trigger an IEC proliferative response that can be helpful in management of acute inflammation. However, in order to achieve a better understanding of these preliminary results, it is still necessary to determine the proliferative pathway that accounts for the observed response and whether additional antiapoptotic signalling is involved in the preservation effect we reported.

Three different proliferative pathways emerge as candidates to continue our research: the Wnt/β-catenin [59, 62], the phosphoinositide 3-kinase/protein kinase B (PI3K/Akt)/β-catenin [61] and the epiregulin/epidermal growth factor receptor [63] pathways. Though the two latter pathways have been linked to TLR4 signalling [61, 63], the

Wnt/β-catenin cascade might better explain our results for different reasons. On one hand, the β-catenin transcriptional target cyclin D1 was clearly increased in DSS-treated mice. On the other, Nava and colleagues demonstrated that inhibition of the Akt/β-catenin signalling is associated with decreased colitis severity and increased IEC proliferation [62]. Although it might seem contradictory, pro-inflammatory cytokines produced during DSS-induced colitis activate this pathway, which subsequently elicits inhibition of the Wnt/β-catenin signalling, thereby inducing an almost complete arrest of proliferation in IECs from days 4 to 6 [62]. Of note, the LPS administration we performed at day 4 in both low- and high-dose experiments caused a change in the tendency of the daily DAI (chapter 2, Figs. 1C and 4C). This fact might support the idea that epithelial TLR4 activation on day 4 activates the Wnt/β-catenin pathway, causing a proliferative burst that protects the crypts from complete destruction. However, this rationale must still be corroborated.

TLR4 ligand administration had been previously addressed to treat acute colitis. Repeated oral and intrarectal administration of LPS had been used in a prophylactic regime to obtain a protective phenotype towards inflammation based in cytoprotective factor secretion [42] or hyporesponsiveness induction [64]. In this regard, our administration protocol offers different advantages. First, it has been proven to be useful with the need of only two intracolonic administrations. Indeed, given the fact that pro-inflammatory cytokines peak at day 5 [65] and inhibit IEC proliferation since day 4 [62], it is conceivable that a single LPS administration could have similar effects to those obtained with the regime we used. Second, the ameliorating effects we observed take place even during the acute inflammatory phase, though probably most differences would be more apparent during the recovery phase [59]. And third, our observed effects rely on an occasional activation of TLR4, in contrast to a tonic or sustained activation, which has been associated with colitis and tumorigenesis and seems detrimental to our purposes [60]. Preliminary data from other groups indicate that treatment of acute colitis through stimulation of the Wnt/β-catenin pathway does not increase susceptibility of mice to colitis-associated cancers [59]. Despite all these features, refinement of our protocol is still possible concerning administration timing, as already stated, and doses used. Indeed, LPS doses should be fine-tuned in order to reduce hematopoietic TLR4 activation while conserving epithelial TLR4-driven effects.

To summarise, though many question are still unanswered, single or occasional LPS intracolonic administration could become a valid therapeutic approach in the management of the acute inflammatory phase of DSS-induced colitis. Determination of the proliferative pathways involved in the development of this effect will become a key point to understand and continue evaluating the usefulness of epithelial TLR4 activation as a therapeutic tool.

TLR expression in the ENS: an unexplored field

A usual finding that centred our attention was that, when performing initial characterisation of TLR2 distribution through IHC studies, the ENS always displayed intense staining for this receptor. Subsequent studies with submucosal whole-mounts and paraffin-embedded samples confirmed that not only TLR2, but also TLR4/9 were clearly expressed in the ganglia of both the submucosal and the myenteric plexuses of the ENS (chapter 3). Despite there was no literature that could confirm such findings in the ENS, large work had been already performed evaluating the role of these receptors in the CNS [66]. In this tissue, TLRs were implicated in triggering innate immune responses towards virus and bacteria, and some evidence additionally suggested that they could be involved in autoimmune diseases and responses to aseptic injury [66]. The main cells expressing these receptors were microglia and astrocytes [28, 67, 68], although some TLRs could also be found in oligodendrocytes and neurons [69, 70]. Since participation of the ENS in GI inflammation had been previously suggested [71-75], we hypothesised that TLRs could be involved in recognition of microbial motifs and would facilitate the development of the ENS inflammatory phenotype.

Even though colocalisation studies in submucosal whole-mounts demonstrated that neurons were the main TLR-expressing cells, we focused our interest in studying EGCs for several reasons: 1) EGCs are the GI counterparts of CNS astrocytes, which had been shown to respond to MAMPs through TLRs [28, 68, 76]; 2) EGC function impairment had been associated to fulminant enterocolitis [77, 78]; 3) alterations in the number of EGCs have been described in CD patients [78]; 4) EGCs are located in close proximity to IECs [79], so they could contact penetrating microbiota after epithelial barrier leakage; 5) EGCs are activated after cytokine challenge and can secrete IL-6 [74, 75]; and 6) EGCs express MHC class II in IBD specimens, which might be related to increased recruitment of

inflammatory cells [71, 73]. As a first approach to assess enteroglial participation in inflammation, we determined GFAP expression during DSS-induced colitis. We found that reactive gliosis was seen in both mucosal-submucosal and muscle layers. Moreover, up-regulation of TLR4 in the muscle layers was associated to reactive gliosis in the absence of macrophage activation or inflammatory infiltrates (chapter 3). These results indicated that EGCs might be involved in inflammation through regulation of TLR4, and prompted us to investigate TLR2/4/9 functionality in cell culture models.

TLRs, EGCs and inflammation

The results we initially obtained concerning activation of the MyD88-dependent signalling pathway after stimulation with different MAMPs indicated that TLR2/4/9 receptors were functional in ENS cultures, whereas only TLR4 activated this cascade in EGCs. Production of cytokines and chemotactic factors after single and combined MAMP challenge were additionally determined, but unexpectedly, pure EGC cultures did not behave as ENS cultures. On one hand, although production of TNF-α and NO had been previously described in EGCs in response to pro-inflammatory stimuli [80, 81], such mediators were not found in the JUG2 EGC culture. On the other, the interactions observed in ENS cultures were not reproduced by EGCs. Determination of the iNOS reactivity rendered us the explanation for such controversial results: ENS cultures were contaminated with iba-1 expressing macrophages (chapter 3, supplementary data). Indeed, resident macrophages can be found in the gut of mouse embryos from E15 stage [82], and our cultures were derived from embryos harvested on day 16 of embryogenesis. Therefore, the responses we observed in EGCs cultures were genuine of EGCs, whereas the effects reported in ENS cultures were driven by both EGCs and resident macrophages.

In view of our results, we can say that EGCs express functional TLR4 that, upon LPS challenge, activates the NF-κB signalling pathway, inducing IL-6 and MCP-1 release and eliciting significant increase in chemoattraction of immunocytes *in vitro* (chapter 3). Hence, TLR4 confers EGCs the possibility to respond to Gram negative bacteria and, perhaps, to DAMPs such as S100β, which is increased during inflammation [83-85]. Indeed, the active participation of EGCs in amplifying inflammation in UC patients and DSS-treated mice has been recently confirmed [85], as well as MHC class II production after

challenge with enteropathogenic bacteria [84]. Moreover, expression of costimulatory molecules has been described in EGCs from patients with Chagas disease [86]. Taken together, these data suggest that EGCs have the appropriate machinery to recognise MAMPs and/or DAMPs, become activated, synthesise MHC class II and costimulatory molecules, chemoattract immune cells and perform antigen presentation in a stimulation microenvironment enriched with pro-inflammatory cytokines. Overall, EGCs might potentially act as professional APCs, enhancing the inflammatory response. Such idea is additionally supported by the fact that MHC class II expression in these cells is positively correlated with the presence of T CD8+ inflammatory infiltrate [73], but much work is still needed to corroborate that EGCs really play such roles.

Despite qualitative involvement of EGCs in expansion of inflammation has already been demonstrated [85], it is still unclear whether EGCs play indispensable roles during inflammation, whether they can initiate an inflammatory response or to which extent do they account for the inflammatory phenotypes observed in IBD. Currently, there is no answer for these questions, as there are no reliable data addressing them. Work by Esposito and collaborators demonstrated that administration of endocannabinoids to DSS-treated mice resulted in colitis amelioration that was accompanied with significant attenuation of EGC activation phenotypes [85], but since such molecules do not only target EGCs, but also mast cells, microglia and macrophages [87, 88], the reported results might be a consequence of an unselective immunomodulatory effect. In the absence of other studies, our findings in chapter 3 show that EGC cultures displayed low production of inflammatory mediators after TLR4 stimulation when compared to ENS cultures. In addition, and in contrast with previous observations [81, 83], purified cultures of EGCs did not express either TNF-α or NO (chapter 3). Such mediators were only detected in ENS cultures, as they are probably released by resident macrophages. Indeed, similar results have been reported in CNS astrocyte cultures: TLR4 challenge with LPS does not elicit TNF-α and NO production in purified cultures, but does in those contaminated with microglial cells [28, 89]. If we additionally take into account that experiments from chapter 4 demonstrated that there were no statistic differences in TNF-α production by RAW 264.7 macrophages after conditioning with uJUG2 and sJUG2 supernatants, it is reasonable to conclude that EGCs are probably not involved in the onset of inflammation. Thence, our work supports the idea that EGCs might play a role

in expansion of inflammation, perhaps by attracting immune cells and facilitating the shaping of adaptive immunity, but do not seem to participate in its initiation. In addition, these results underscore that even low levels of contamination of a cell type in a culture (often seen in primary cultures) may result in important misinterpretation of results and biased conclusions.

TLRs, enteric neurons, resident macrophages and the microenvironment of embryonic ENS culture

As already stated, the role of TLRs in enteric neurons was not addressed in our studies, although these were the main TLR-expressing cells of the ENS. Interestingly, TLR2 expression was essentially located in neurons and neuronal fibres (chapter 3), and intracolonic administration of 500 µg zymosan caused an increase in nNOS expression in the colon of naïve mice (Annex 2). Indeed, zymosan intracolonic administration has been shown to increase the number of nNOS-positive neurons in the spinal cord [90]. These observations might settle a basis to formulate new hypotheses linking the role of TLRs in enteric neurons and the frequent alterations in their neurochemical coding during inflammation [73, 91]. Other enteric neuron functions that might be regulated by TLRs are production of chemokines, which has been described elsewhere [92], as well as apoptosis and survival. In this regard, recent work by Anitha and colleagues has demonstrated that TLR4 is associated to nitrergic neuron survival, thereby regulating intestinal motility [93]. Our results after combined TLR stimulation in ENS cultures showed that there was no neuronal cell death despite pro-inflammatory cytokine release, and may therefore agree with the existence of TLR-mediated mechanisms promoting cell survival (chapter 4).

Although our experiments in ENS cultures did not aim to focus on the functions of TLRs in resident macrophages, most of the responses we observed might be attributed to the activation of these cells. Interestingly, all TLR ligands used to stimulate ENS cultures activated the NF-κB pathway, but only LPS induced production of inflammatory mediators. This may indicate that these cells respond to Gram-negative bacteria but are permissive to Gram-positives - among them probiotic bacteria -, displaying some sort of hyporesponsiveness to the latter. Indeed, cytokine production and chemoattraction were enhanced after costimulation with different MAMPs that mimicked the Gram-

negative cell wall and unmethylated microbial DNA (LPS and ODN 1826, respectively), as reported elsewhere [13, 94]. These findings demonstrate that specific responses are triggered in resident macrophages depending on the quality and diversity of the stimuli provided by invading microorganisms.

Previous observations have shown that intestinal resident macrophages display important phagocytic activity, but have limited secretion of soluble mediators in response to LPS [95, 96]. This state of hyporesponsiveness, in terms of cytokine secretion, is achieved through production and release of TGF-β by surrounding stromal cells [96]. Interestingly, TGF-β is an intermediate-expressed gene ($\Delta Ct_{TGF\text{-}\beta/S6}$= 9; data not shown) [28] in ENS cultures in resting conditions. Expression of this and other molecules might help to create a regulatory microenvironment in which immunocytes, such as macrophages, display low-activation phenotypes, even after exposure to pro-inflammatory stimuli. Indeed, some of these soluble molecules are possibly produced by such resident macrophages and over-expressed during inflammation, since hyporesponsiveness in RAW 264.7 cells appeared after conditioning with LPS sENS supernatants, and were enhanced by LPS and ODN 1826 sENS supernatants (chapter 4). Other evidences supporting this rationale come from the fact that enteric neurons and glial cells have been associated to pro-inflammatory events [85, 97], and from the absence of significant tolerance induction when incubating macrophages with fibroblast conditioned media (chapter 4). However, taking into account that these ENS cultures have been established from embryonic tissue, the presence of mesenchymal stem cells producing anti-inflammatory factors cannot be dismissed [98]. Although the physiological meaning of this regulatory mechanism is still unknown, it might be possibly related to limiting uncontrolled inflammation through modulating of soluble mediator release and enhancing phagocytic and bactericidal activities [96]. Characterisation of the cell types and soluble mediators that elicit such regulatory microenvironment in these cultures might be of great value to design new therapeutic approaches to IBD, as well as to disclose potential roles involving TLR activation in immunomodulation.

TLRs in the lower GI tract: lessons learned

When this project was initially conceived, most of the data concerning TLRs expression and functions had been obtained in immunocytes. Such PRRs were involved

in microbe recognition, bridged innate and adaptive immunity, shaped the immune response [1, 4] and were associated to inflammatory conditions such as IBD [20, 21]. As expression and signalling of these receptors showed cross-responsiveness [12, 51], hyporesponsiveness [24] and tolerance phenomena [15], controlled administration of their ligands represented a potentially valid mechanism to modulate their deregulated functions during GI inflammation.

Knowledge of the roles that TLRs play in the GI tract during homeostasis and inflammation has rapidly evolved, and so did our work. Currently, we have come to the conclusion that TLRs are expressed in all tissues [99], and probably in most of the cell types found in humans and rodents. Depending on the cell type and on the expressed TLR, the specific functions they play might vary, but they are generally associated to antimicrobial and immune mediator production [4, 30] and/or promotion of cell survival and maturation [31, 100].

In the GI tract, in physiological conditions massive contact between both epithelial and immune cells with luminal microbiota is hampered by the mucus layers [53], but still low signalling through TLRs is probably allowed. Signals provided by microbiota elicit barrier strengthening and controlled epithelial proliferation and maturation [23, 30, 101], as well as tolerance induction by professional APCs [102]. Upon disruption of the mucus layers, bacteria reach the mucosa and submucosa, contacting different cell populations (chapter 1) [54]. Among these cell populations, resident immune cells initiate the pro-inflammatory response, chemoattracting peripheral leukocytes that orchestrate such response [103]. In addition, other cell types such as EGCs might recognise MAMPs and inflammation-generated DAMPs, acquiring an activated phenotype and contributing to inflammation by recruiting more immune cells (chapter 3) [84, 85].

During inflammation, TLRs expressed in IECs participate in repair responses, as described elsewhere [42-44]. In these stages, pharmacologic management of TLR functions through administration of their ligands is facilitated by the loss of the mucus layers. Activation of TLR2, which is involved in antiapoptotic and barrier strengthening responses, has proven useful to improve mucosal repair [44]. Furthermore, stimulation of TLR4 during the acute inflammatory phase might improve epithelial barrier recovery

through secretion of epithelial protective factors [43, 58] and enhancement of IEC proliferation (chapter 2) [47, 63].

Enhancing mucosal healing during inflammatory flares has emerged as an interesting therapeutic strategy in IBD, and has already entered clinical trials [104]. Hence, it is conceivable that TLR ligands might also be used in the future with these same purposes, since promising results have been reported in IBD experimental models [44, 58]. On the other hand, research in new biologics to address treatment of inflammation is still wide open, awaiting for characterisation of molecules with potent immunomodulatory effects, such as those we observed in chapter 4. Indeed, attenuation of the symptoms suffered by IBD patients during the active phases is still the best strategy to manage these processes until identification of the etiologic agent and a more thorough understanding of this process be achieved.

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Conclusions

1) In the lower GI tract and in physiological conditions, TLR2 is an intermediate-expression gene preferentially located in colonocytes of the crypt bottom, in discrete cells in the *lamina propria*, in muscle fibres and in the intrinsic plexuses of the ENS. During inflammation, expression of this receptor is increased in colonocytes from both the upper and lower parts of the crypt, infiltrating immune cells within the submucosal layer, ENS plexuses and EGCs. Up-regulation of this receptor is associated to colitis severity, since treatments attenuating colitis signs are accompanied by significant reductions in TLR2 expression. Therefore, targeting dysregulation of this receptor might be considered a valid therapeutic approach in studies intended to reduce the severity of experimentally-induced colitis.

2) Intracolonic administration of zymosan in naïve mice causes transient alterations in IL-1β and IL-10 expression when co-administered with 30% ethanol. After repeated instillations on days 0, 3 and 6, combination of ethanol and 100 µg zymosan provokes a tendency towards colon shortening, IL-1β and IL-10 production and more severe histologic lesions when compared to other combinations at day 7 post-administration. Intracolonic administration of zymosan whenever the mucus layer is intact does not elicit overt inflammation, although it might have sub-inflammatory effects when regularly given in combination with other substances causing epithelial mucus layer disruption.

3) In physiological conditions, intracolonic instillation of 500 µg zymosan or LPS on two alternate days does not produce alterations in the parameters classically used to evaluate colitis severity, at least when evaluated 3 days after the last administration. These results highlight the effectiveness of the epithelial barrier in separating the host internal milieu from the large amounts of antigenic molecules present in the gut lumen.

4) Whereas administrations of 200 and 500 µg zymosan on two alternate days provide no benefit in improving inflammation severity, same doses of LPS can be used to attenuate experimental colitis phenotype. Intracolonic LPS given on days 2 and 4 after starting the DSS regime decreases colon shortening, down-regulates TLR2 and TLR4 aberrant expression and ameliorates the DAI and histologic findings. The LPS-

improving effects seem to rely on increased epithelial preservation through induction of a proliferative drive, probably on day 4. Even though the proliferative pathway mediating such effect must still be determined, modulation of epithelial TLR4 activation through occasional administration of LPS raises as a possible therapeutic approach to promote epithelial healing during active phases of IBD. Given its potential harmful and tumorigenic effects, extensive work must still be performed in this field to improve targeting of the cell types of interest and to verify safety issues.

5) EGCs participate in spreading and/or amplifying inflammation through expression of functional TLR4. Upon LPS challenge, the NF-κB signalling pathway becomes activated in these cells, inducing IL-6 and MCP-1 release to the external milieu, and increasing chemoattraction of immunocytes. Special care must be taken when preparing primary cultures of these cells, since they are easily contaminated by resident macrophages that might contribute to the secretion of cytokines and provoke over-estimation mistakes, as it has been reported for CNS astrocyte cultures and microglia.

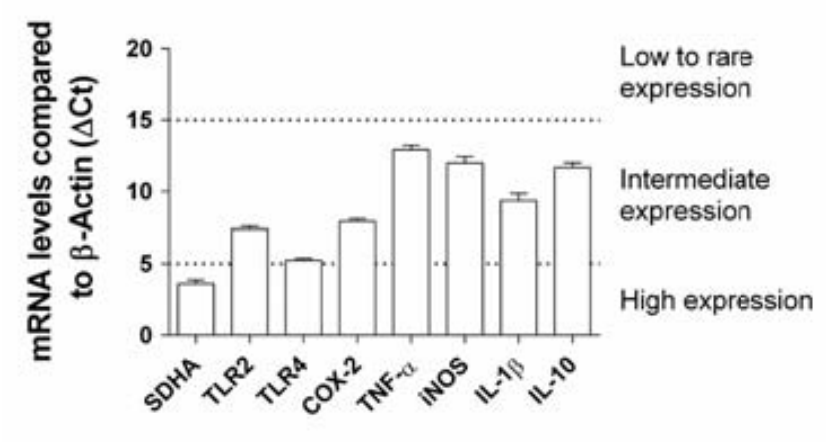
6) ENS cultures express functional TLR2, TLR4 and TLR9. Upon stimulation with their different MAMPs, cultures become activated, but production of pro-inflammatory mediators is only elicited after TLR4 challenge. A mechanism of tolerance may underlie these observations, since costimulation with TLR2 ligands had no remarkable effects in TLR4-induced mediator profile, whereas addition of a bacterial DNA-mimicking motif synergistically enhanced such pro-inflammatory profile. Most of the functions described in these cultures probably depend on resident macrophage activity.

7) In response to TLR4 challenge, ENS cultures do not only potentiate a pro-inflammatory phenotype to fight possible invading microbes, but also increase their release of immunomodulatory substances that attenuate TNF-α production by RAW 264.7 macrophages. Such mediators are increased in ENS cultures after TLR4+TLR9 costimulation, since increased inhibition responses were observed in macrophages upon combined challenge. Although the nature of these substances remains elusive their identification could be of enormous value to develop new biologic therapies in the

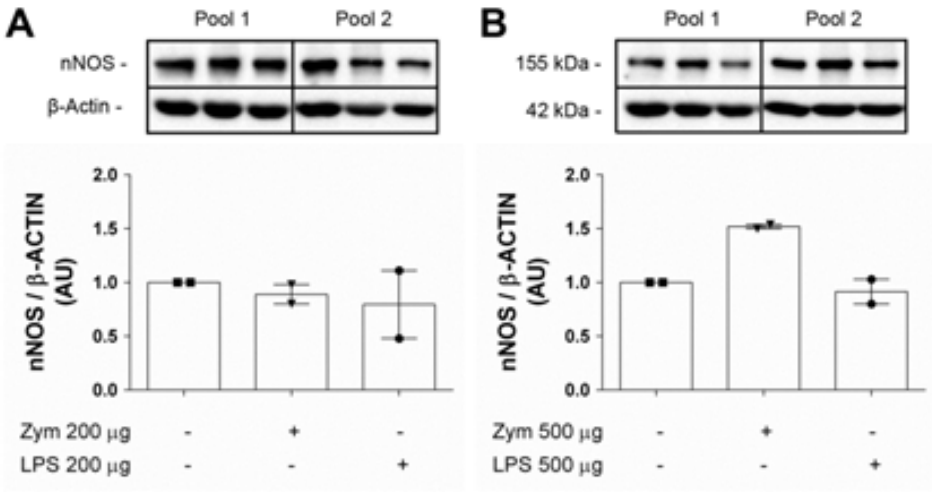
context of IBD, as well as to understand the mechanisms through which intestinal resident cells regulate the responses of inflammation-recruited immune cells.

The findings summarised in this manuscript have aimed to improve the understanding of the functions that TLRs develop in the lower GI tract during homeostasis and inflammation. Overall, TLR roles may vary depending on the challenged cell type and its environmental situation. Some of the responses driven by TLRs can be used to modulate inflammation, such as those observed in IECs, whereas some others must be avoided to prevent potentiation of these processes (those in EGCs, for instance). Selectivity is the key, and might be achieved through accurate dosage and precise administration regimes.

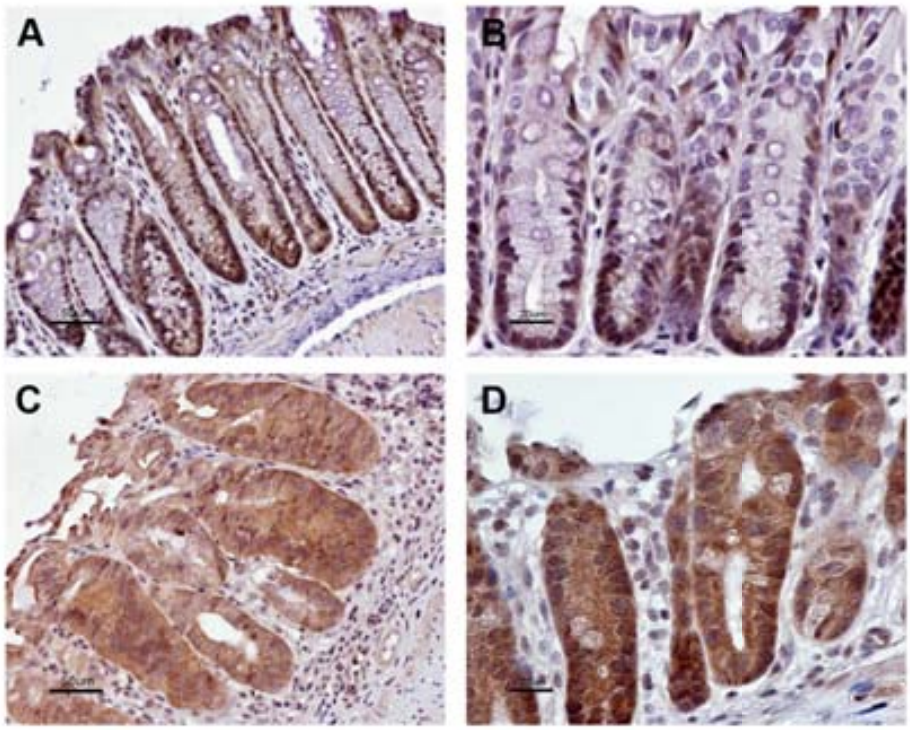
Annexes



Annex 1 – Colonic relative expression of the transcripts evaluated in our studies in physiologic conditions. Relative expression was calculated by performing the ΔCt between the indicated transcripts and β-actin. N=5 mice from control groups were used in each case.



Annex 2 – nNOS protein levels at the end of intracolonic administration experiments. The nNOS protein of two pools of naïve mice administrated with intracolonic zymosan or LPS was evaluated by WB following the protocols detailed in chapter 2. Rabbit polyclonal anti-nNOS antibody (1:10,000) was from Santa Cruz, whereas secondary HRP-linked goat anti-rabbit antibody (1:10,000) was purchased from Cell Signaling. B-actin was labeled as described in chapter 2. **A)** "Low-dose" experiments. **B)** "High dose experiments.



Annex 3 – TLR4 immunoreactivity in colon epithelial crypts from control and DSS-treated mice. IHC was performed as described in chapter 3. **(A)** and **(B)** represent pictures in different magnifications of control mice immunoreactivity. **(C)** and **(D)** represent different pictures of DSS-treated mice tissue. Dysplastic crypts in the mucosa of DSS-treated mice show increased staining of colonocytes. Scale bars correspond to 50 µm in **(A)** and **(C)**, and to 20 µm in **(B)** and **(D)**.