UAB

Universitat Autònoma de Barcelona

Influencia de la Deficiencia de Il-10 sobre el Fenotipo Mastocitario, la Microbiota y la Respuesta Inmune Intestinal en un Modelo Murino

Presentada por

Roberto Andrés Riquelme Neira

Memoria para optar al grado de Doctor

Programa de Doctorado en Inmunología Avanzada
Departamento de Biología Celular, Fisiología e Inmunología
Facultad de Veterinaria
Universidad Autónoma de Barcelona

Directores:
Patrocinio Vergara Esteras
Joan Antoni Fernández Blanco
Bellaterra, junio 2018

Universitat Autònoma de Barcelona

Patrocinio Vergara Esteras, Catedrática de Fisiología del Departamento de Biología Celular, Fisiología e Inmunología de la Universidad Autónoma de Barcelona y **Joan Antoni Fernández Blanco**, Profesor Asociado del Departamento de Biología Celular, Fisiología e Inmunología de la Universidad Autónoma de Barcelona

HACEN COSTAR:

Que la memoria titulada "**Influencia de la Deficiencia de Il-10 Sobre el Fenotipo Mastocitario, la Microbiota y la Respuesta Inmune Intestinal en un Modelo Murino**" presentada por ROBERTO ANDRÉS RIQUELME NEIRA para optar al grado de Doctor se ha realizado bajo su dirección y, al considerarla concluida, autorizan su presentación para ser evaluada por el Tribunal correspondiente.

Y, para que conste a los efectos oportunos, firman el presente documento.

Bellaterra, 21 junio de 2018

Firma de los directores:

Patrocinio Vergara Esteras   **Joan Antoni Fernández Blanco**

Imagen Portada: mastocito de tipo mucosa (MLMC). Microscopía electrónica de transmisión obtenida por Jackub Rychter.

AGRADECIMIENTOS

Una mención especial para Martín, primero por ayudarme con mis ratoncitos y experimentos, y segundo, por haberme presentado a esa compañera tuya tan risueña, simpática y bonita, Sophie, gracias por todo y gracias por ser tú.

A mis amigos incondicionales, Eduardo y Luis, gracias por siempre preocuparse y por esas largas conversaciones por skype que me mantenían desvelado por el desfase horario.

Finalmente, a mi familia, hermanos, cuñadas, sobrinos, gracias por su apoyo y por siempre darme ánimo para continuar el camino. Especialmente a mis padres, tatitas como siempre les he dicho, todo lo que soy se los debo a ustedes "A Son Never Forgets".

Gracias Totales.

<div align="right">

Roberto Andrés Riquelme Neira

Bellaterra, junio 2018

</div>

Tabla de contenidos

ABREVIACIONES

16S rRNA: ARN ribosomal 16S.

AMPs: péptidos antimicrobianos.

BMMC: mastocitos derivados de médula ósea.

CD: enfermedad de Crohn.

CD14: proteína de unión a lípido A.

CRLs: receptores tipo lectina C.

CTMC: mastocitos de tejido conectivo.

DAMPs: patrones moleculares asociados a daño.

DC: célula dendrítica.

DSS: dextrano sulfato de sodio.

FcεRI: receptor de alta afinidad para la fracción constante de IgE.

HSC: célula madre hematopoyética.

IBD: enfermedad inflamatoria intestinal.

IFNγ: interferón gamma.

IgA: inmunoglobulina A.

IgE: inmunoglobulina E.

KO: deficiente.

IL: interleucina.

LBP: proteína de unión a lípido.

LPS: lipopolisacárido.

LTA: ácido lipoteicoico.

LTs: leucotrienos.

MC: mastocito.

MC$_T$: mastocito que expresa triptasa.

MC$_{TC}$: mastocito que expresa triptasa y quimasa.

MD-2: proteína de diferenciación mieloide 2.

MDP: muramíl dipéptido.

MLMC: mastocitos de tipo mucosa.

MMC: mastocitos de mucosa.

mMCP: proteasa mastocitaria de ratón.

NLRs: receptores tipo NOD.

NOD2: dominio de oligomerización de nucleótidos 2.

OTUs: unidades taxonómicas operacionales.

PAMPs: patrones moleculares asociados a patógeno.

PCMC: mastocitos cultivados derivados de peritoneo.

PGD2: prostaglandina D2.

PGN: peptidoglicano.

PMC: mastocitos peritoneales.

PRRs: receptores de reconocimiento de patrones.

RLRs: receptores tipo lectina C.

SCF: factor de células madre.

SMC: mastocitos derivados de piel.

TLRs: receptores tipo toll.

TNFα: factor de necrosis tumoral alfa.

Treg: linfocito T regulador.

UC: colitis ulcerativa.

SUMMARY

Mast cells (MC) can participate in the response to microorganisms by various pattern recognition receptors (PRRs). After their activation through these receptors, MC can orchestrate a response by secreting immunological mediators such as cytokines. Among these, interleukin 10 (IL-10) is an important cytokine due to its immunomodulatory characteristics, as well as its ability to regulate the expression of MC proteases. Additionally, thanks to the existence of genetically modified murine models, such as IL-10 deficient (IL-10$^{-/-}$) mice that develop colitis spontaneously, it is possible to investigate the potential role of IL-10 in MC response to activation with antigens of different microorganisms. On the other hand, the use of this animal model allows investigating the influence of this cytokine on the composition of the intestinal microbiota.

This work has explored the functional role of IL-10 in differentiated MC *in vitro*, as well as the effects of IL-10 deficiency on the composition of the microbiota and the expression of factors related to the immune response, before (6 weeks) and at the onset (20 weeks) of colitis. For this purpose, the effect of IL-10 deficiency has been characterized on MC of different phenotype and after its activation via PRRs. Additionally, the effect produced by the lack of IL-10 on the microbiota composition, the expression of TLRs and proinflammatory cytokines, as well as the production of luminal IgA, in the same stages and after antibiotics treatment was evaluated.

The results obtained indicated that the IL-10 deficiency produced different effects depending on the MC phenotype, age and type of PRR ligand. Thus, in the absence of IL-10, mucosal-like MC (MLMC) showed lower expression of TLR4 and NOD2 at

week 6 and TLR7 at week 20. In addition, both MC phenotypes (mucosa and connective), showed a lower secretion of IL-6 and TNFα after TLR2 activation. The TLR4 and TLR7 activation in MLMC generated a lower secretion of IL-6 at week 6, while MLMC secreted less TNFα at week 20. Finally, after NOD2 stimulation, no cytokine secretion was observed in any of the MC phenotypes.

On the other hand, it was observed that in IL-10$^{-/-}$ animals there are factors that potentially favor the development of colitis. Thus, IL-10$^{-/-}$ mice at week 6 showed representatives of Verrucomicrobia phylum and a lower relative abundance of Rikenellaceae and Lachnospiraceae taxa. Meanwhile at week 20 in IL-10$^{-/-}$ mice, microorganisms of the phylum TM7 were observed, as well as, a lower expression of IL-1β, IL-6, TLR6, -7 and -8, and an increase of TNFα and IgA. Additionally, the use of antibiotics before the development of colitis induced a decrease in diversity and a restructuring of the microbiota, together with a decrease in TLRs and cytokines expression, and a lower production of luminal IgA.

In summary, these findings provide new insights on the role of MC and IL-10 in the host-microorganism interaction. They show how the IL-10 deficiency can affect the microbiota composition and the expression of factors associated with the immune response. And they suggest that early modification of the microbiota through the use of antibiotics in genetically susceptible individuals could alter the colitis progression.

RESUMEN

Los mastocitos (MC) pueden participar en la respuesta a microorganismos mediante diversos receptores de reconocimiento de patrones (PRRs). Luego de su activación a través de estos receptores, los MC pueden orquestar una respuesta mediante la secreción de mediadores inmunológicos como las citocinas. Entre éstas, la interleucina 10 (IL-10) es una citocina importante por sus características inmunomoduladoras, así como también, por su capacidad de regular la expresión de proteasas en MC. Adicionalmente, gracias a la existencia de modelos murinos modificados genéticamente, como los ratones IL-10 deficientes (IL-10$^{-/-}$), que desarrollan colitis de forma espontánea, es posible investigar el rol potencial de la IL-10 en la respuesta de MC frente a la activación con antígenos de diversos microorganismos. Por otra parte, el uso de este modelo animal permite investigar la influencia de esta citocina sobre la composición de la microbiota intestinal.

Este trabajo ha explorado el rol funcional de la IL-10 en mastocitos diferenciados in vitro, así como los efectos de la deficiencia de IL-10 sobre la composición de la microbiota y la expresión de factores relacionados con la respuesta inmune, antes (6 semanas) y al inicio (20 semanas) de la colitis. Para este propósito, se ha caracterizado el efecto de la deficiencia de IL-10 sobre el fenotipo mastocitario y tras su activación vía PRRs. Adicionalmente, se evaluó el efecto que produce la carencia de IL-10 sobre la composición de la microbiota, la expresión de TLRs y citocinas proinflamatorias, así como la producción de IgA luminal, en las mismas etapas y tras el tratamiento con antibióticos.

Los resultados obtenidos indicaron que la deficiencia de IL-10 produjo distintos efectos dependiendo del fenotipo mastocitario, de la edad y del tipo de ligando PRR. Así, en ausencia de IL-10, MC de tipo mucosa (MLMC) mostraron una menor expresión de TLR4 y NOD2 a las 6 semanas y TLR7 a las 20 semanas. Además, ambos fenotipos de MC (mucosa y conectivo), mostraron una menor secreción de IL-6 y TNFα tras la activación de TLR2 La activación de TLR4 y TLR7 en MLMC generó una menor secreción de IL-6 a las 6 semanas, mientras MLMC secretaron menos TNFα a las 20 semanas. Finalmente, tras la estimulación de NOD2 no se observó secreción de citocinas en ninguno de los fenotipos mastocitarios.

Por otra parte, se observó que en animales IL-10$^{-/-}$ existen factores que potencialmente favorecerían el desarrollo de colitis. Así, los ratones IL-10$^{-/-}$ a las 6 semanas mostraron representantes del filo Verrucomicrobia y una menor abundancia relativa de los taxa Rikenellaceae y Lachnospiraceae. Mientras que a las 20 semanas en los ratones IL-10$^{-/-}$ se observaron microorganismos del filo TM7, una menor expresión de IL-1β, IL-6, TLR6, -7 y -8, y un incremento de TNFα e IgA. Adicionalmente, el uso de antibióticos antes del inicio de la colitis indujo una disminución en la diversidad y una reestructuración de la microbiota, junto con una disminución en la expresión de TLRs, citocinas y menor producción de IgA luminal.

En resumen, estos hallazgos proveen nuevas perspectivas sobre la función de los MC y la IL-10 en la interacción microorganismo-huésped. Muestran cómo la ausencia de IL-10 puede afectar la composición de la microbiota y la expresión de factores asociados a la respuesta inmune. Y sugieren que la modificación temprana de la microbiota mediante la utilización de antibióticos en individuos genéticamente susceptibles podría alterar la progresión de la colitis.

INTRODUCCIÓN

LOS MASTOCITOS (MC)

ASPECTOS GENERALES

Reportados por primera vez por Paul Ehrlich, los mastocitos (MC) se distinguen microscópicamente por presentar gránulos citoplasmáticos que se tiñen de color púrpura con tinciones como el azul de toluidina, fenómeno conocido como metacromasia (Beaven 2009). Estas células provienen de la médula ósea y migran a través del torrente sanguíneo en forma de precursores inmaduros. Éstos, una vez alcanzado el tejido diana, completan el proceso de maduración al verse expuestos a factores locales específicos como las interleucinas (IL)-3, IL-4, IL-9, IL-10 y el factor de células madre (SCF) (Figura 1) (Tsai et al. 1991; Robbie-Ryan & Brown 2002; Galli et al. 2008; Galli et al. 2011).
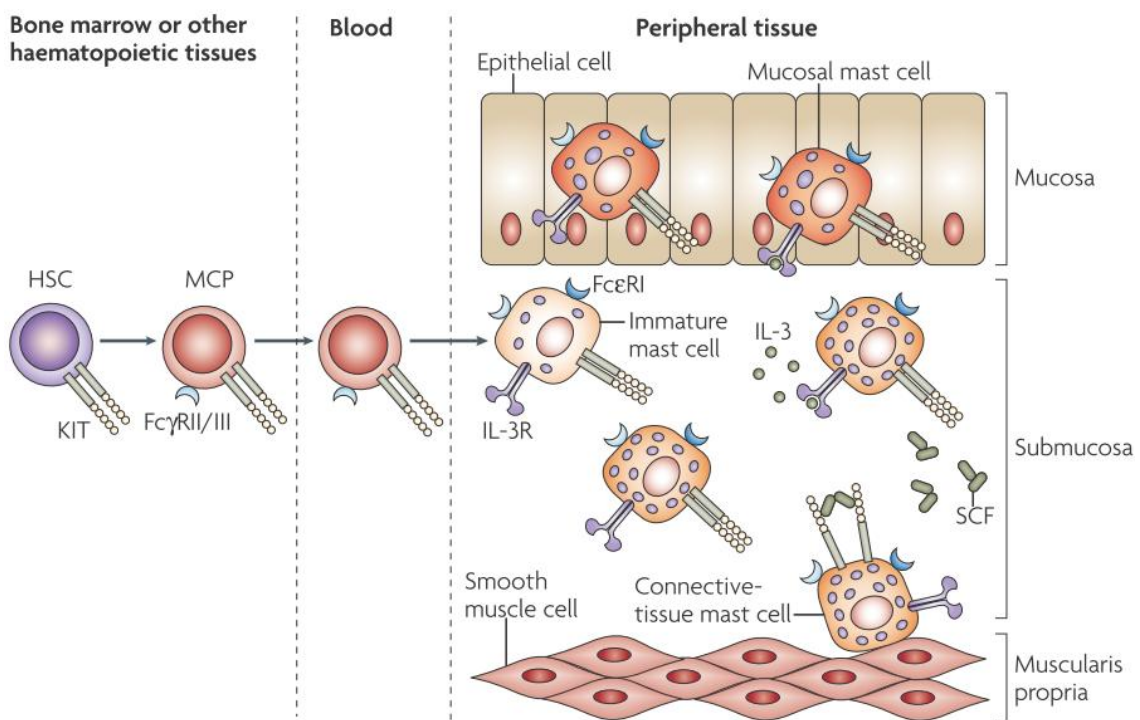
Figura 1. Desarrollo y distribución tisular de los mastocitos. HSC, células madre hematopoyéticas; MCP, progenitor de mastocitos; SCF, factor de células madre (también conocido como ligando KIT); FcεRI, receptor de alta afinidad para la fracción constante de IgE; FcγRII/III, receptores Fc de activación de baja afinidad (FcγRIII) e inhibidor (FcγRIIβ) para IgG; IL-3R, receptor de interleucina-3 (IL-3). Extraído de: Galli, S., Grimbaldeston, M., & Tsai, M. (2008). Immunomodulatory mast cells: negative, as well as positive, regulators of immunity. *Nature Reviews Immunology*, *8*(Box 1).

Los MC son células inflamatorias ampliamente distribuidas en el organismo realizando funciones de vigilancia y protección. Se encuentran localizados predominantemente en tejidos expuestos al medio externo, como el tracto genitourinario, el epitelio respiratorio, la piel y el tracto gastrointestinal (Echtenacher et al. 1996; Metcalfe et al. 1997; Galli et al. 2005; Feng et al. 2007; Abraham & St John 2010; Bąbolewska et al. 2012; Urb & Sheppard 2012). En dichas localizaciones, actúan como primera línea de defensa contra estímulos nocivos orquestando el desarrollo de la respuesta inmune innata y adaptativa (Abraham & St John 2010; Urb & Sheppard 2012).

A causa de su amplia distribución en el organismo, estas células se han visto implicadas en el desarrollo de afecciones inmuno-mediadas como el asma, la artritis, la dermatitis y los procesos inflamatorios intestinales, incluyendo la enfermedad inflamatoria intestinal (IBD) (Nigrovic & Lee 2005; Bradding et al. 2006; Liu et al. 2011; De Winter et al. 2012).

TIPOS DE MASTOCITOS/MODELOS DE ESTUDIO

Los MC pueden tener diferentes funciones y fenotipos, y su diferenciación se ve influida por el microambiente circundante. En función de su localización y la expresión diferencial de proteasas, los MC se clasifican en dos subtipos (Tabla 1) (Williams & Galli 2000; Miller & Pemberton 2002; Pejler et al. 2007; Pejler et al. 2010; Westerberg et al. 2012; Wernersson & Pejler 2014). En humanos, los MC que expresan triptasa y quimasa (MC_{TC}) residen principalmente en tejido conectivo, mientras que los MC que expresan solo triptasa (MC_T) se encuentran localizados predominantemente en la mucosa. En el caso de los ratones, los MC que se localizan en el tejido conectivo se denominan "mastocitos de tejido conectivo" (CTMC) y los que se ubican en la mucosa "mastocitos de mucosa" (MMC), los cuales se pueden diferenciar por la expresión de las proteasas mastocitarias mMCP6 y mMCP1, respectivamente (Figura 1) (Miller & Pemberton 2002; Pejler et al. 2007; Malbec et al. 2007; Pejler et al. 2010; Wernersson & Pejler 2014).

Tabla 1. Subtipos de mastocitos y expresión de proteasas mastocitarias en humano y ratón.

Subtipo de MC	Quimasas	Triptasas	Carboxipeptidasa A3
Humano			
MC$_{TC}$	CMA1 *(CMA1)**	α/βI *(TPSAB1)* βII/βIII *(TPSB2)*	CPA3 *(CPA3)*
MC$_T$	-	α/βI *(TPSAB1)* βII/βIII *(TPSB2)*	-
Ratón			
CTMC	mMCP4 *(Mcpt4)* mMCP5 *(Mcpt5)*	mMCP7 *(Tpsab1)* mMCP6 *(Mcpt6)*	CPA3 *(Cpa3)*
MMC	mMCP1 *(Mcpt1)* mMCP2 *(Mcpt2)*	-	-
* Entre paréntesis la anotación genética			

Adaptado de: Wernersson, S., & Pejler, G. (2014). Mast cell secretory granules: armed for battle. *Nature Reviews. Immunology*, *14*(7), 478–94. CMA1, quimasa mastocitaria 1; mMCP, proteasa mastocitaria de ratón, CPA3, carboxipeptidasa 3.

Ambos subtipos mastocitarios pueden responder de modo diferente frente a los mismos estímulos. Por tanto, generalizar en base a los hallazgos encontrados en una población mastocitaria específica podría conducir a errores (Gurish & Boyce 2002; Malbec et al. 2007; Westerberg et al. 2012). En consecuencia y con el fin de dilucidar la respuesta producida tras la activación de diferentes poblaciones de MC, se han realizado estudios *in vitro* con modelos que expresan características de cada fenotipo mastocitario. Entre estos modelos destacan los basados en el empleo de MC derivados de la piel (SMC) (Matsushima et al. 2004), MC peritoneales (PMC) (Hochdörfer et al. 2011), MC derivados de la médula ósea (BMMC) (Supajatura et al. 2001), MC de tipo mucosa (MLMC) (Miller & Wright 1999; Wright et al. 2002; Ekoff et al. 2007) y MC cultivados derivados de peritoneo (PCMC) (Malbec et al. 2004; Malbec et al. 2007; Mrabet-Dahbi et al. 2009; Vukman et al. 2012). Un ejemplo de este tipo de estudio es el realizado por Mrabet-Dahbi y colaboradores (Mrabet-Dahbi et al. 2009). En el mismo, se demuestra que los BMMC, en comparación con los PCMC, tienen una menor expresión de los receptores tipo toll (TLRs) 2 y 4, y una menor secreción de citocinas en respuesta a su estimulación.

ACTIVACIÓN DE LOS MASTOCITOS

Los MC pueden ser activados en respuesta a una gran variedad de estímulos. Entre ellos, se ha descrito en detalle como la unión del complejo antígeno-IgE al receptor de alta afinidad para la fracción constante de la IgE (FcεRI) conduce a las reacciones de hipersensibilidad asociadas a las respuestas alérgicas (Bischoff 2007; Rijnierse et al. 2007; Galli et al. 2011; Galli & Tsai 2012). A su vez, los MC pueden ser activados a través de los receptores de reconocimiento de patrones (PRRs), como los receptores de tipo NOD (NLRs) o los TLRs. Estos, pueden reconocer y responder frente a distintos patrones moleculares asociados a patógenos (PAMPs), tales como el lipopolisacárido (LPS) o peptidoglicano (PGN) (Figura 2A) (Bischoff 2007; Rijnierse et al. 2007; Hofmann & Abraham 2009; Hochdörfer et al. 2011; St John & Abraham 2013).

En respuesta a su activación, los MC pueden liberar y producir una serie de mediadores. En función de los mediadores liberados, la activación mastocitaria se asociará a un aumento de la permeabilidad vascular, una remodelación tisular o la infiltración de células inflamatorias (Miller & Pemberton 2002; McDermott et al. 2003; Malbec et al. 2007; Galli et al. 2011; Kunder et al. 2013). Algunos mediadores, tales como la histamina, el factor de necrosis tumoral alfa (TNFα) y algunas proteasas, se encuentran preformados y almacenados en gránulos. En otras ocasiones, los mediadores deben ser sintetizados *de novo* tras la activación celular, como es el caso de algunas interleucinas como IL-6, IL-10, interferón gamma (IFNγ) y TNFα, quimiocinas, leucotrienos (LTs) y la prostaglandina D2 (PGD2) (Figura 2B) (Marshall 2004; Krishnaswamy & Chi 2005; Rijnierse et al. 2007).
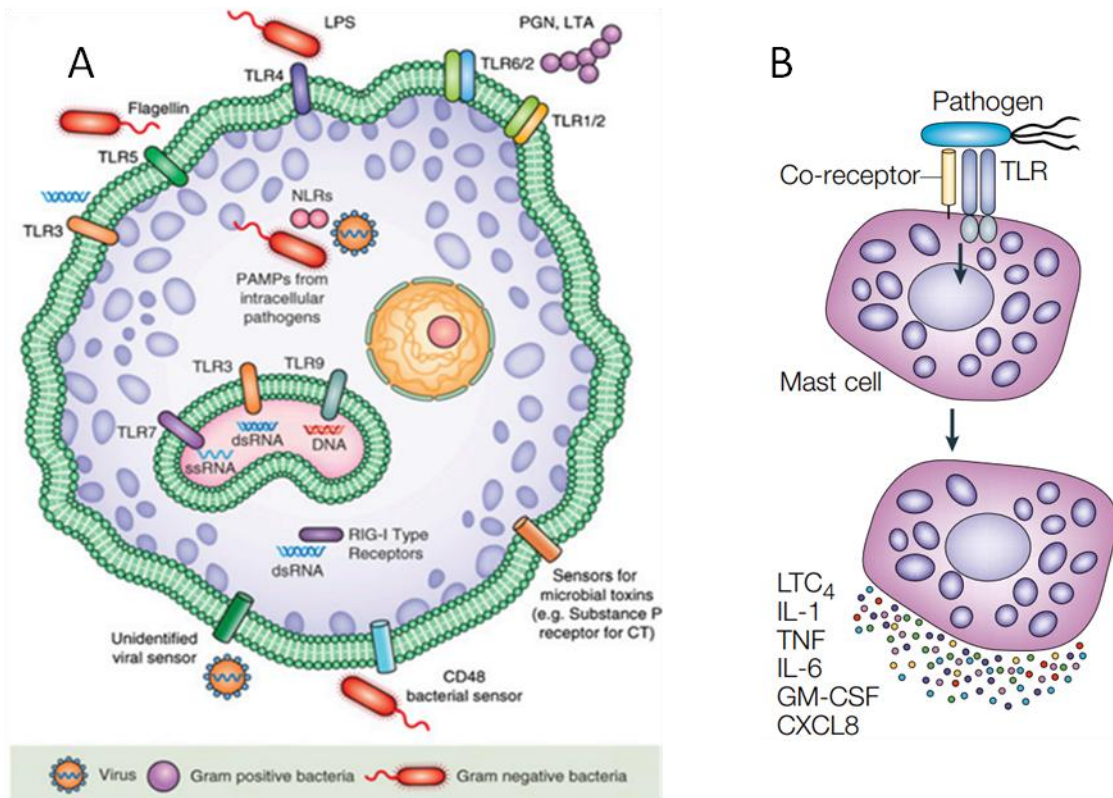
Figura 2. Receptores de reconocimiento de patrones y activación mediada por TLR en mastocitos. **(A)** Los mastocitos responden a diversos subtipos de patógenos y motivos asociados a patógenos debido a su expresión de receptores de reconocimiento de patrones (PRRs) y receptores celulares adicionales. Resumen de los receptores utilizados por MC, la ubicación celular del receptor (superficie celular versus endosomal versus citosólica) y la clase de patógeno o patrones moleculares asociado a patógenos (PAMPs) que el receptor reconoce. **(B)** La activación mastocitaria directa incluye eventos mediados por receptores tipo toll (TLRs). Con frecuencia, la activación mediada por TLR no conduce a la degranulación, aunque se ha informado sobre la producción de citocinas, quimiocinas y mediadores lipídicos. LPS, lipopolisacárido; LTA, ácido lipoteicoico; PGN, peptidoglicano; CD48, grupo de diferenciación 48; LTC_4, leucotrieno C4; IL, interleucina; TNF, factor de necrosis tumoral; GM-CSF, factor estimulante de colonias granulocitos/macrófagos; CXCL8, CXC-quimiocina ligando 8. Extraído de: (A) St John, A. L., & Abraham, S. N. (2013). Innate immunity and its regulation by mast cells. Journal of Immunology (Baltimore, Md.: 1950), 190(9), 4458–63. (B) Marshall, J. S. (2004). Mast-cell responses to pathogens. Nature Reviews. Immunology, 4(10), 787–799.

En ocasiones, los niveles de activación de los MC se ven alterados. La consiguiente liberación excesiva y sostenida de mediadores mastocitarios contribuye al inicio y perpetuación de diversas condiciones patológicas, incluyendo la IBD.

MASTOCITOS Y ENFERMEDAD INFLAMATORIA INTESTINAL

Numerosos estudios han demostrado el papel potencial de los MC en la patogenia de la IBD (Fox et al. 1990; Nolte et al. 1990; Raithel et al. 1995; Rijnierse et al. 2007; Chen et al. 2008; Okumura et al. 2009; Chichlowski et al. 2010). En concreto, los MC contribuirían al desarrollo de la enfermedad al propiciar un aumento en la permeabilidad intestinal acompañado de una mayor exposición a los antígenos luminales, por ejemplo los derivados de la microbiota (Keita & Söderholm 2010; Hering et al. 2012). Ello, en conjunto con factores de predisposición tanto genéticos como ambientales favorecería al desarrollo de la enfermedad (Podolsky 2002; Bouma & Strober 2003; Girardin, Hugot, et al. 2003; Thompson-Chagoyán et al. 2005; Chichlowski et al. 2010).

Por esto, junto con analizar la función mastocitaria, debemos tener en cuenta el papel fundamental que cumple la microbiota en el desarrollo de los procesos inflamatorios intestinales.

MICROBIOTA

Las microbiotas son comunidades microbianas complejas que contienen cientos de especies y que podemos encontrar en todas partes, desde los humanos hasta el ambiente (Shin et al. 2016). Asociadas a un huésped, estas comunidades son diversas y difieren de las comunidades microbianas en el ambiente externo, sin embargo, los procesos que determinan su composición son actualmente materia de diversos estudios (Adair & Douglas 2017). Hace un par de décadas, nuestra apreciación de la riqueza y la diversidad de la microbiota estaban restringidas por los métodos basados en cultivo, no obstante, estos no permiten la identificación de microorganismos no cultivables. En la última década, con el desarrollo de metodologías de secuenciación de alto rendimiento y análisis bioinformáticos, la complejidad y las diversas funciones de la microbiota se han ido paso a paso descubriendo (Chang & Lin 2016). En este contexto, existen algunos conceptos que es necesario conocer cuando se llevan a cabo análisis de diversidad microbiana. En primer lugar, la alfa diversidad describe la riqueza (o abundancia) de especies dentro de una muestra. Por otro lado, la beta diversidad señala las diferencias

en la composición de especies entre diferentes muestras (Goodrich et al. 2014; Levy et al. 2017). Por último, las unidades taxonómicas operacionales (OTUs), corresponden a un grupo de microorganismos definidos por su similitud de secuencia. Por ejemplo, generalmente se considera que OTUs a 97 % de similitud definen una especie, por lo que para una especie todas las secuencias del gen 16S rRNA son al menos un 97 % idénticas (Ley et al. 2008).

Por definición, la microbiota comprende el conjunto de microorganismos (incluyendo bacterias, virus, hongos y eucariotas) que reside en un huésped o en un lugar específico de éste, como por ejemplo el tracto gastrointestinal (Sommer & Bäckhed 2013; Donaldson et al. 2015). Mediante la aplicación de las técnicas de secuenciación masiva para el estudios del gen que codifica para el ARN ribosomal 16S (16S rRNA), es posible realizar estudios que permiten llevar a cabo clasificaciones filogenéticas y estudiar la diversidad dentro de una comunidad microbiana (Ashelford et al. 2005; Antonopoulos et al. 2009). En cuanto a su composición, la microbiota intestinal está estructurada por diversos microorganismos, entre ellos, levaduras, hongos, protozoos, virus y bacterias. Las bacterias están representadas por aproximadamente 100 trillones de microorganismos comensales, clasificados principalmente en 7 filos distintos: Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Fusobacteria, Proteobacteria y Verrucomicrobia. Entre éstos, Bacteroidetes y Firmicutes son los más abundantes tanto en humanos como en ratones (Qin et al. 2010; Kamdar et al. 2013; Sommer & Bäckhed 2013).

Dicho esto, es importante tener en consideración que a lo largo del tracto gastrointestinal la microbiota no posee una composición homogénea. Desde el nacimiento, la colonización y el establecimiento de la microbiota se ve modulado por factores tales como el pH, la concentración de oxígeno, los cambios en la dieta, el desarrollo de infecciones y la administración de antimicrobianos (Wang et al. 2005; Ley et al. 2006; Ley et al. 2008; Garner et al. 2009). Ello conlleva a que se establezcan diferencias dependientes del segmento anatómico del tracto digestivo estudiado. De este modo, en el intestino delgado encontramos de forma dominante las familias Enterobacteria y Lactobacillales, mientras que el colon es principalmente colonizado por Bacteroidetes y Clostridia (Figura 3A) (Swidsinski et al. 2005; Dinoto et al. 2006; Sekirov et al. 2010; Kamada et al. 2013).

Al mismo tiempo, es posible clasificar la microbiota intestinal como transitoria o permanente de acuerdo a su habilidad de colonización. La primera corresponde a bacterias que, debido a su falta de adaptación para competir con la microbiota residente, no pueden perpetuarse en el tracto digestivo, como son los microorganismos patógenos o los probióticos. Por el contrario, la microbiota permanente está formada por comunidades bacterianas que han desarrollado adaptaciones evolutivas para establecer una relación permanente con el hospedador. Ejemplo de esta última son los comensales verdaderos (Ivanov & Honda 2012).

El análisis de la microbiota intestinal ha permitido establecer algunas funciones de los microorganismos que son vitales para el funcionamiento del huésped. Entre ellas encontramos la producción de ácidos grasos y vitaminas, la modulación de la sensibilidad visceral, el control de la renovación epitelial, la protección frente a la colonización por patógenos y la modulación de la respuesta inmune local (Bouskra et al. 2008; Soret et al. 2010; Chung et al. 2012; Aguilera et al. 2013; Buffie & Pamer 2013; Ramakrishna 2013; Pagliari et al. 2015). Sin embargo, esta relación de mutualismo sólo se mantiene cuando la comunidad microbiana posee una estructura, composición, riqueza y uniformidad particulares. Si este equilibrio se altera, la salud puede verse comprometida produciéndose una disbiosis (Figura 3B) (Lepage et al. 2005; Dethlefsen et al. 2007; Chang et al. 2008; Chang & Lin 2016).

Definida como los cambios en la composición normal de la microbiota. La disbiosis puede ser dividida en tres categorías, producto de: (i) la pérdida de la diversidad microbiana general, (ii) la expansión de patógenos o microorganismos potencialmente dañinos (patobiontes), y (iii) la pérdida de microorganismos beneficiosos (Petersen & Round 2014). La disbiosis ha sido asociada con diversas patologías como el autismo, la esclerosis múltiple, la diabetes, la obesidad y desordenes gastrointestinales como la IBD (Kalliomäki et al. 2008; Sartor 2008; Camilleri et al. 2012; Cryan & Dinan 2012; Kamdar et al. 2013; Simreń et al. 2013; Mayer et al. 2014). Hasta la fecha, algunos estudios en individuos que padecen de IBD han caracterizado una reducción en la diversidad microbiana junto con una reducción de algunas especies comensales, particularmente en los filos Firmicutes y Bacteroidetes (Manichanh et al. 2006; Manichanh et al. 2012; Gevers et al. 2014; Kostic et al. 2014).

Todo ello sugiere que la microbiota y su interacción con el huésped forman parte de la fisiopatología de las alteraciones que ocurren en el desarrollo de la IBD. Sin embargo, se desconoce si la disbiosis se produce como causa o consecuencia de la enfermedad (Salzman & Bevins 2013).
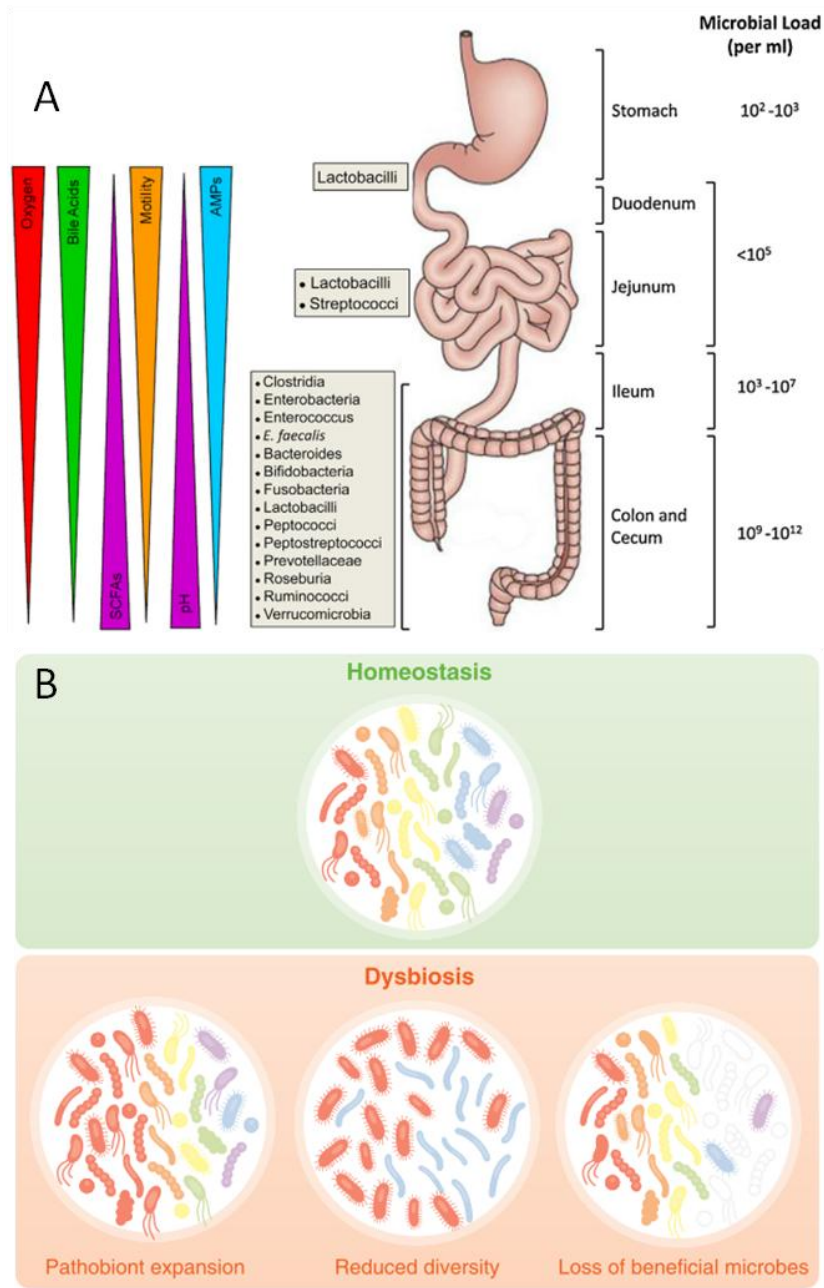
Figura 3. Organización y equilibrio de las comunidades microbianas en el tracto digestivo. (A) Organización espacial de comunidades microbianas y gradientes fisiológicos a lo largo del tracto gastrointestinal animal. **(B)** Homeostasis y disbiosis. La pérdida de microbios beneficiosos, la expansión de los patobiontes y la pérdida de diversidad son eventos que abarcan la disbiosis. En condiciones homeostáticas, la microbiota está compuesta por una diversidad de microorganismos conocidos por beneficiar el desarrollo y la salud del huésped. AMPs, péptidos antimicrobianos; SCFAs, ácidos grasos de cadena corta. Extraído de: (A) Reinoso Webb, C., Koboziev, I., Furr, K. L., & Grisham, M. B. (2016). Protective and pro-inflammatory roles of intestinal bacteria. *Pathophysiology*, *23*(2), 67–80. (B) Petersen, C., & Round, J. L. (2014). Defining dysbiosis and its influence on host immunity and disease. *Cellular Microbiology*, *16*(7), 1024–1033.

INTERACCIONES MICROORGANISMO-HUESPED

El sistema inmune es un elemento de vital importancia tanto en el mantenimiento de la salud como en la resolución de una enfermedad. Por ello, es importante comprender los fenómenos que suceden en una de las mayores interfaces que tiene el huésped con el ambiente, el tracto gastrointestinal. En éste interactúan los componentes internos del sistema inmune con los elementos provenientes del ambiente, incluyendo los microorganismos (Round & Mazmanian 2009). En el intestino, el alto riesgo de encontrarse con elementos externos nocivos resulta en una actividad constante del sistema inmunológico, responsable de mantener el equilibrio homeostático. Este equilibrio es crucial para prevenir reacciones innecesarias, que podrían ser perjudiciales, y asegurar que la respuesta de los mecanismos de defensa ocurra en el momento oportuno. En este sentido, la tolerancia oral es crucial para mantener la homeostasis. Esto significa que el sistema inmune necesita tolerar la exposición a los epítopes derivados de los alimentos y componentes del medio externo no perjudiciales sin iniciar una respuesta hostil frente a ellos (Pabst & Bernhardt 2010; Chistiakov et al. 2014; McLean et al. 2015).

En el mantenimiento de este estado de equilibrio, están involucrados tanto los mecanismos de defensa innata como adaptativa. Éstos determinan un adecuado reconocimiento de la microbiota, dando como resultado el mantenimiento de la homeostasis intestinal. Por lo tanto, alteraciones en las interacciones microorganismos-huésped pueden generar respuestas intestinales anómalas, llevando al desarrollo de respuestas inflamatorias e incluso a un estado de disbiosis. Entre los componentes de la inmunidad innata presentes en el intestino encontramos el moco, las células epiteliales, las células dendríticas, los macrófagos y los mastocitos. La respuesta inmune innata ejercida por dichos componentes, si bien es rápida, es altamente inespecífica. Por otra parte, la respuesta adaptativa, que a diferencia de la respuesta innata es más lenta pero más específica, involucra la activación selectiva de los linfocitos B y T (Figura 4) (Mayer 2010; Khor et al. 2011; Rescigno 2011; Geremia et al. 2014; Zhang et al. 2017).

INMUNOGLOBULINA A (IgA)

La inmunoglobulina A es una clase de anticuerpo producido por las células plasmáticas residentes en la lámina propia. Ésta es secretada a través del epitelio al lumen intestinal donde funciona como un mecanismo de protección frente a patógenos a través de su unión a moléculas de superficie expresadas por estos (Perez-Lopez et al. 2016).

Frente a antígenos bacterianos, la IgA cumple una serie de funciones promoviendo la homeostasis intestinal, mediante la neutralización de microorganismos potencialmente peligrosos, ayudando a dar forma a la microbiota comensal y previniendo una respuesta inflamatoria inapropiada contra antígenos microbianos y de los alimentos (Macpherson et al. 2012; Rogier et al. 2014; Macpherson et al. 2015). Este isotipo de inmunoglobulina puede ser producido de forma independiente (innata) o dependiente (adaptativa) de los linfocitos T. Si bien la IgA innata puede excluir microorganismos del intestino, ésta no es tan potente como la IgA adaptativa a la hora de determinar la relación mutualista huésped-microbiota (Kawamoto et al. 2014; Sutherland et al. 2016; Belkaid & Harrison 2017). En cuanto a su función bajo condiciones patológicas, se ha descrito que en los pacientes de afecciones que involucran la inflamación intestinal, como la IBD, existe un aumento en la secreción de IgA (MacDermott et al. 1986; Macpherson et al. 2015).
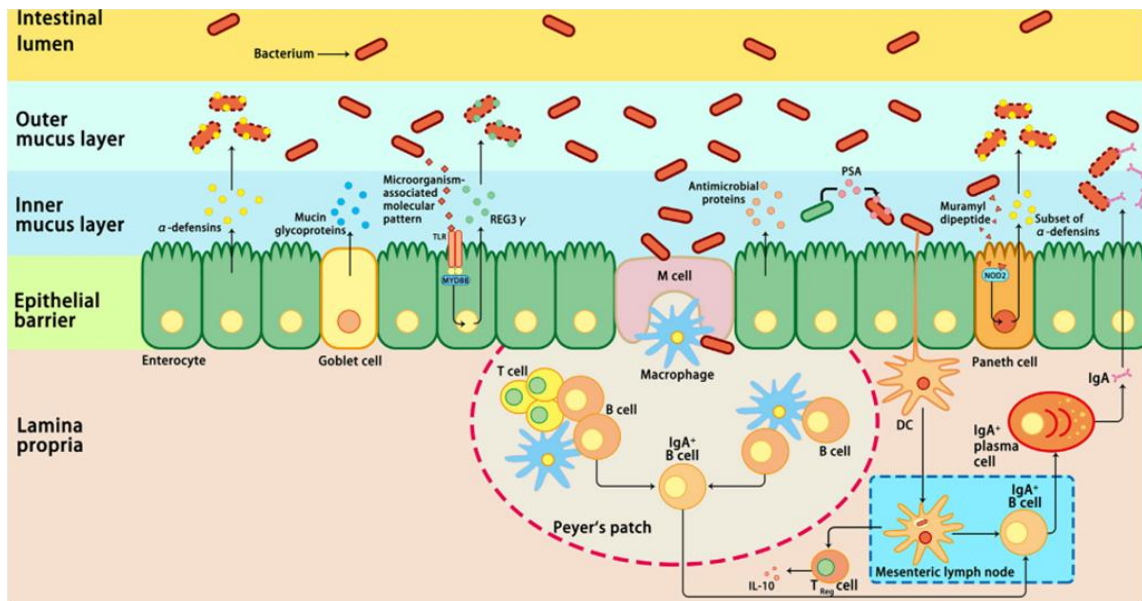
Figura 4. Respuesta inmune del huésped frente a la microbiota intestinal. TLR, receptor tipo toll; IgA, inmunoglobulina A; DC, célula dendrítica; NOD2, dominio de oligomerización de nucleótidos 2; PSA, polisacárido A; Treg, linfocito T regulador; IL-10, interleucina 10; MYD88, proteína de respuesta primaria de diferenciación mieloide 88; REG3γ, receptor de tipo lectina C. Extraído de: Zhang, M., Sun, K., Wu, Y., Yang, Y., Tso, P., & Wu, Z. (2017). Interactions between Intestinal microbiota and host immune response in inflammatory bowel disease. *Frontiers in Immunology*, *8*(AUG), 1–13.

CITOCINAS

Las citocinas, son polipéptidos cuyos efectos incluyen la modulación de las respuestas inmune e inflamatoria. Caracterizadas por su pleiotropismo (pueden tener más de un receptor) y redundancia (sus receptores comparten subunidades) estas moléculas que participan tanto de la respuesta inmune innata como adaptativa (O'shea et al. 2008). Son producidas por diversos tipos celulares y a menudo muestran actividades superpuestas que regulan la proliferación o diferenciación celular, dependiendo del tipo y estado de desarrollo de las células diana involucradas (Scheller et al. 2011). Entre estas proteínas, la IL-6 y el TNFα son importantes en el desarrollo de la IBD por sus características pro-inflamatorias. La IL-10 por su parte, es importante en el mantenimiento de la homeostasis intestinal, por sus características principalmente antiinflamatorias.

En el caso concreto de la IL-6, ésta es una citocina involucrada en diferentes aspectos de la inmunidad innata y adaptativa. Es secretada por diversos tipos celulares, entre ellos los monocitos, macrófagos, fibroblastos, células endoteliales y linfocitos (Kishimoto et

al. 1992; Naugler & Karin 2008). Se ha descrito su participación en la inducción de la secreción de proteínas de la fase aguda de la inflamación, así como en la regulación de la diferenciación de células del sistema inmune incluyendo los macrófagos y linfocitos T (Kishimoto et al. 1992; Diehl & Rincón 2002; Waldner & Neurath 2014). En el contexto de la IBD, se ha detectado que la producción de IL-6 por parte de macrófagos y linfocitos T aislados de lámina propia se ve incrementada tanto en humanos como en animales (Atreya & Neurath 2010; Kai et al. 2005; Neurath 2014).

Por lo que se refiere al TNFα, es una citocina que puede ser producida por distintos tipos celulares como macrófagos, linfocitos T, fibroblastos y mastocitos (Baert & Rutgeerts 1999; McLachlan et al. 2003; Rijnierse et al. 2006; Billiet et al. 2014). Cumple también con una amplia gama de funciones en el desarrollo de la inflamación, entre las que destacan la remodelación tisular, la alteración de la permeabilidad epitelial y vascular, la activación de macrófagos y el reclutamiento de células inflamatorias (Baert & Rutgeerts 1999; Bischoff et al. 1999; Suryaprasad & Prindiville 2003; Rijnierse et al. 2006). Como mediador pro-inflamatorio, se ha establecido que juega un rol clave en enfermedades inflamatorias crónicas como la artritis reumatoide, la psoriasis y la IBD (Tracey et al. 2008; Billiet et al. 2014). En el contexto de esta última, se han encontrado una elevada concentración de TNFα en sangre, mucosa intestinal y materia fecal de los pacientes (MacDonald et al. 1990; Murch et al. 1991; Braegger et al. 1992; Nicholls et al. 1993; Breese et al. 1994; Komatsu et al. 2001; Billiet et al. 2014; Pedersen et al. 2014).

IL-10, como se ha señalado previamente, es una citocina con características principalmente antiinflamatorias. Puede ser producida tanto por células del sistema inmune innato (incluyendo células dendríticas, eosinófilos, macrófagos, mastocitos, monocitos y neutrófilos) como adaptativo (linfocitos B y T) (Saraiva & O'Garra 2010; Gabryšová & Howes 2014). Las células responsables de la presencia de IL-10 en situaciones específicas depende del tipo de estímulo, del tipo de tejido afectado y del momento en el que se evalúa la respuesta inmune (Couper et al. 2008; Sabat et al. 2010). La acción de IL-10 en el mantenimiento de la homeostasis intestinal viene dada por su interferencia en los procesos de maduración de las células dendríticas y en la activación y proliferación de los macrófagos. De este modo, la IL-10 favorece el mantenimiento del fenotipo anérgico de ambos tipos celulares, característico del estado

de homeostasis intestinal (Paul et al. 2012; Kole & Maloy 2014; Keubler et al. 2015). Por otra parte, la IL-10 ejerce un efecto directo sobre los linfocitos T, promoviendo el mantenimiento, expansión y función de los linfocitos T reguladores (Treg), los cuales contribuyen a preservar la homeostasis en la interface del intestino con el ambiente (Paul et al. 2012; Kole & Maloy 2014; Keubler et al. 2015). Adicionalmente, en los MC se ha demostrado que la IL-10 influye en el proceso de maduración y expresión de proteasas mastocitarias, así como también, la producción de IL-10 por los MC interviene en la inmunomodulación descrita previamente (Galli et al. 2008).

En lo referente al rol de la IL-10 en el desarrollo de la colitis, se ha demostrado que una disminución en su secreción por parte de las células epiteliales puede llevar al desarrollo de IBD (Bijjiga & Martino 2011; Shah et al. 2012). Al mismo tiempo, se ha descrito que en los ratones deficientes en IL-10 se produce una respuesta exacerbada de linfocitos T (Th1) con una secreción excesiva de citocinas pro-inflamatorias (Davidson et al. 1996; Paul et al. 2012; Keubler et al. 2015).

RECEPTORES DE RECONOCIMIENTO DE PATRONES (PRRs)

Los PRRs son expresados en el intestino principalmente por las células epiteliales y las células del sistema inmune. Su función es la detección de microorganismos a través del reconocimiento de PAMPs y la detección de potenciales señales de peligro a través de patrones moleculares asociados a daño (DAMPs) (Abreu 2010; Frosali et al. 2015).

Estos receptores se han clasificado mayoritariamente en cuatro familias: receptores de tipo lectina C (CRLs), receptores de tipo RIG-I (RLRs), y los ya mencionados NLRs y TLRs (Tabla 2) (Creagh & O'Neill 2006; Takeuchi & Akira 2010; De Nardo 2015; Plato et al. 2015; Cao 2016; Liu & Ding 2016). Entre estos, los NLRs y TLRs son los que mejor se han caracterizado hasta el día de hoy. Los NLRs, entre los que destacan NOD1 y NOD2, son proteínas citoplasmáticas que reconocen moléculas derivadas del metabolismo del peptidoglicano (Chamaillard et al. 2003; Girardin, Boneca, et al. 2003; Hasegawa et al. 2006; Takeuchi & Akira 2010; Elia et al. 2015). Por su parte, han sido identificados 13 TLRs, habiéndose descrito los TLR del 1 al 9 tanto en humanos como en ratones, el TLR10 sólo en humanos y los TLR del 11 al 13 sólo en ratones (De Nardo

2015; Liu & Ding 2016). De acuerdo a su localización a nivel celular, los TLRs pueden ser clasificados en 2 grupos. El grupo comprendido por TLR 1, 2, 4, 5, 6 y 11, que residen sobre la membrana plasmática y reconocen PAMPs como lípidos, proteínas y lipoproteínas. El otro grupo lo componen los TLR 3, 7, 8, 9, 11, 12 y 13, los cuales se pueden localizar en las membranas de vesículas intracelulares como el endosoma, lisosoma y endolisosoma, y reconocen principalmente ácidos nucleicos (TLRs 3-9) o algunos componentes microbianos (TLR 11 y 12) (Takeuchi & Akira 2010; Di Gioia & Zanoni 2015; Liu & Ding 2016).

Tanto los NLRs como los TLRs son importantes en el mantenimiento de la homeostasis intestinal, así como en la mediación de la respuesta inmune frente a agentes patógenos. Su activación resulta en una respuesta que involucra la producción de mediadores como citocinas y quimiocinas, así como la activación de otros genes importantes en el control de las respuestas inflamatorias (Takeda & Akira 2005; Cario et al. 2007; Leulier & Lemaitre 2008; Abreu 2010).

Tabla 1. PRRs localización y ligandos

PRRs	Localización	Ligando	Origen del ligando
TLRs			
TLR1	Membrana plasmática	Lipopéptidos triacilados	Bacteria
TLR2	Membrana plasmática	Lipoproteínas/lipopéptidos peptidoglicano, ácido lipoteicoico	Bacterias, virus, parásitos, endógenos
TLR3	Endolisosoma	dsRNA	Virus
TLR4	Membrana plasmática	Lipopolisacárido (LPS)	Bacterias, virus, endógenos
TLR5	Membrana plasmática	Flagelina	Bacteria
TLR6	Membrana plasmática	Lipopéptidos diacilados	Bacterias, virus
TLR7	Endolisosoma	ssRNA	Virus, bacterias, endógenos
TLR8	Endolisosoma	ssRNA	Virus, bacterias, endógenos
TLR9	Endolisosoma	CpG-DNA	Virus, bacterias, protozoos, endógenos
TLR10	Membrana plasmática	Desconocido	Desconocido
TLR11	Membrana plasmática	Profilina/flagelina	Protozoos
TLR12	Endolisosoma	Profilina	Desconocido
TLR13	Endolisosoma	23S rRNA	Desconocido
NLRs			
NOD1	Citoplasma	iE-DAP	Bacteria
NOD2	Citoplasma	MDP	Bacteria
RLRs			
RIG-1	Citoplasma	dsRNA corto	ARN y ADN viral
MDA5	Citoplasma	dsRNA largo	ARN viral
LGP2	Citoplasma	Desconocido	ARN viral
CLRs			
Dectin-1	Membrana plasmática	β-Glucano	Hongos
Dectin-2	Membrana plasmática	β-Glucano	Hongos
MINCLE	Membrana plasmática	SAP130	Hongos, endógenos

Adaptado de: De Nardo, D. (2015). Toll-like receptors: Activation, signalling and transcriptional modulation. *Cytokine*, *74*(2), 181–189, y Takeuchi, O., & Akira, S. (2010). Pattern Recognition Receptors and Inflammation. *Cell*, *140*(6), 805–820. MDA5, gen asociado a la diferenciación de melanoma 5; MINCLE, lectina tipo C de macrófago; dsRNA, ARN bicatenario; ssRNA, ARN monocatenario; CpG-DNA, motivos ADN ricos en guanina y citocina; 23S rRNA, ARN ribosomal 23S; iE-DAP, ácido g-D-glutamil-meso-diaminopimélico; MDP, muramíl dipéptido; SAP130, proteína asociada al espliceosoma.

En este trabajo nos hemos centrado en los fenómenos que ocurren tras la activación mastocitaria de dos PRRs que ejercen su función principalmente en la superficie celular, los TLR 2 y 4, y dos receptores que realizan su función intracelularmente, como son el TLR7 y el NOD2.

TLR2, TLR4, TLR7 Y NOD2

El TLR2 reconoce una amplia variedad de PAMPs asociados con bacterias tanto Gram positivas como negativas. Entre ellos se incluyen lipopéptidos, ácido lipoteicoico (LTA) y componentes derivados del PGN. Además, se ha descrito que el TLR2 puede realizar interacciones heterodiméricas con TLR 1 y 6, con los cuales puede reconocer lipopéptidos triacilados o diacilados respectivamente (Akira et al. 2006; Cario 2008; Kawai & Akira 2011; McClure & Massari 2014).

El TLR4 es esencial en el reconocimiento de LPS, uno de los componentes principales de la membrana externa de bacterias Gram negativas. La activación mediada por TLR4 se produce mediante la formación de un complejo entre LPS y algunas moléculas accesorias, como la proteína de unión a lípidos (LBP) y la proteína de unión a lípido A (CD14). Este complejo se presenta luego a TLR4 en presencia de la proteína de diferenciación mieloide 2 (MD-2), activando la cascada de señalización intracelular (Kawai & Akira 2009; Park & Lee 2013; McClure & Massari 2014).

Por su parte, el TLR7 reconoce ARN monocatenario derivado de virus (ssRNA), compuestos antivirales sintéticos de imidazoquinolina (ej. Imiquimod) y ARN bacteriano derivado de estreptococos del grupo B. Una de las principales características de este receptor de ácidos nucleicos es que su activación promueve la producción de IFN de tipo I, además de otras citocinas inflamatorias (Schiller et al. 2006; Mancuso et al. 2009; Takeuchi & Akira 2010; Kawai & Akira 2011).

Con respecto al NOD2, éste es un receptor citosólico que confiere resistencia a una amplia variedad de bacterias debido a que reconoce muramíl dipéptido (MDP), el cual es un componente del PGN presente en la pared bacteriana tanto de bacterias Gram positivas como negativas (Girardin, Boneca, et al. 2003; Inohara et al. 2003; Kawai & Akira 2009; Takeuchi & Akira 2010; Biswas et al. 2012; Elia et al. 2015). Este receptor

se ha identificado también como un factor crítico en la regulación de la expresión de péptidos antimicrobianos en las superficies mucosas (Kobayashi et al. 2005; Elia et al. 2015).

Tanto los TLRs como los NLRs están involucrados en la regulación de la proliferación y regeneración de células epiteliales, la producción de IgA, la integridad de la barrera intestinal, y la expresión y liberación de péptidos antimicrobianos (Abreu 2010; Cario 2010). Debido a su participación en el desarrollo de las respuestas inmunes frente a los microorganismos, es probable que la generación de una respuesta anómala por parte de estos receptores hacia la microbiota pueda conducir a una inflamación intestinal crónica. En este sentido, estudios recientes han mostrado que existen asociaciones entre algunos polimorfismos o mutaciones en TLR2, TLR4, TLR5 y NOD2 con el desarrollo de la IBD (Cho 2008; Cario 2010; Takeuchi & Akira 2010; Frosali et al. 2015; Meena et al. 2015).

ENFERMEDAD INFLAMATORIA INTESTINAL (IBD)

La IBD es una enfermedad crónica, recidivante, caracterizada por una respuesta inmunomediada descontrolada en el tracto digestivo. Esta enfermedad se presenta en individuos genéticamente susceptibles expuestos a determinados factores de riesgo ambientales. Comúnmente la IBD se ha agrupado en dos subtipos, colitis ulcerativa (UC) y enfermedad de Crohn (CD). La UC se caracteriza por la presencia de lesiones superficiales normalmente restringidas a colon y recto. En el caso de la CD, las lesiones pueden estar presentes a lo largo del tracto gastrointestinal (Podolsky 2002; Thoreson & Cullen 2007; Molodecky et al. 2012; Bendtsen et al. 2015; Malik 2015).

Desde el punto de vista epidemiológico, es una enfermedad de gran importancia. Ambas formas de presentación (UC y CD) han mostrado una tasa de incidencia creciente alrededor del mundo (Molodecky et al. 2012), observándose en países occidentalizados una tasa de entre 10-30 casos/100.000 personas por año e incrementando su incidencia en países en vías de desarrollo a medida que estos se han industrializado (Molodecky & Kaplan 2010). Además, cabe destacar que hoy en día las opciones terapéuticas son solo paliativas. Todo ello conlleva que la IBD tenga un considerable impacto económico

derivado de los costes médicos (hospitalización, cirugía, medicamentos, etc.) y la disminución de la productividad en los individuos afectados por la enfermedad (Molodecky et al. 2012). Sólo en Europa se estima un gasto aproximado entre 2.1-16.7 billones de euros asociado a la enfermedad de Crohn (Peng Yu et al. 2008).

El aumento en la incidencia de la IBD coincide con mejoras en las condiciones de vida de las personas en algunas regiones alrededor del mundo. Ello ha llevado a proponer que la exposición a ciertos factores ambientales y su interacción con el sistema inmune podrían resultar en una respuesta inflamatoria anormal a la microbiota intestinal. Hoy en día, la teoría más aceptada en este sentido es la "hipótesis de la higiene", la cual propone que la creciente frecuencia de trastornos inmunológicos puede atribuirse a la falta de exposición en etapa infantil a patógenos (Bernstein & Shanahan 2008; Shanahan & Bernstein 2009; Molodecky & Kaplan 2010). Las mejoras en las condiciones de higiene en conjunto con una menor exposición a microorganismos entéricos en la etapa infantil, podrían conducir al desarrollo de una respuesta inmune inapropiada al exponerse a nuevos antígenos (por ejemplo, infecciones gastrointestinales) en la etapa adulta (Gent et al. 1994; Molodecky & Kaplan 2010).

Dado que la IBD es producida por la convergencia de múltiples factores, se han realizado diversos estudios para esclarecer cuales son las causas del desarrollo de la enfermedad. Entre éstas, se han identificado numerosos factores de riesgo tales como, el uso de píldoras anticonceptivas, los antiinflamatorios no esteroidales, el tratamiento con antibióticos, la dieta, fumar, la condición socioeconómica o el estrés. Sin embargo, aunque afectan de distintas formas la función de barrera intestinal, la respuesta inmune o la composición de la microbiota, ninguno de ellos por si solo puede explicar de manera determinante el desarrollo de la enfermedad (Figura 5) (Li et al. 2015; Molodecky & Kaplan 2010)
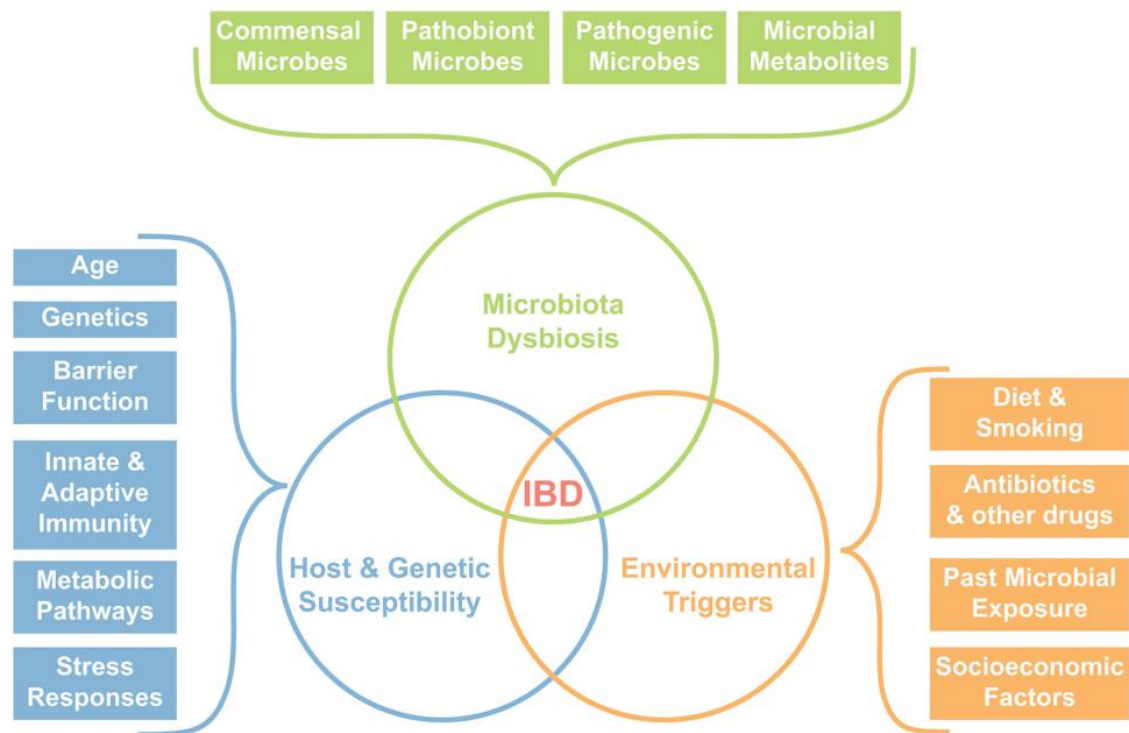
Figura 5. La interacción entre disbiosis de la microbiota del huésped, el estado genético y los factores ambientales contribuyen a la patogenia de la enfermedad inflamatoria intestinal (IBD). Extraído de: Li, J., Butcher, J., Mack, D., & Stintzi, A. (2015). Functional Impacts of the Intestinal Microbiome in the Pathogenesis of Inflammatory Bowel Disease. *Inflammatory Bowel Diseases*, *21*(1), 139–153.

MODELOS ANIMALES Y ENFERMEDAD INFLAMATORIA INTESTINAL

La elección de un modelo animal de enfermedad se basa en su capacidad de imitar o reproducir las características de la patología descritas en los seres humanos. Un modelo puede ser utilizado para responder preguntas específicas relativas a diferentes aspectos y fases del desarrollo de la enfermedad. Sin embargo, por lo general, se hace necesaria la utilización de diversos modelos animales con el fin de entender las bases fisiopatológicas de una enfermedad (Wirtz et al. 2007; Strober 2008).

Los modelos animales han contribuido de manera relevante al mayor entendimiento de los mecanismos subyacentes al desarrollo de la IBD (Jurjus et al. 2004). Un modelo óptimo debería mostrar características similares en cuanto a la etiología, los síntomas y los hallazgos histopatológicos de la IBD. Además, los animales utilizados deben tener

un fondo genético definido, un sistema inmune bien caracterizado, así como unos criterios establecidos relativos a su manejo y manipulación (Jurjus et al. 2004).

En la actualidad, existen diversos modelos murinos orientados a la comprensión de los diferentes aspectos de la IBD. Estos permiten abordar cuestiones que son difíciles de investigar en humanos, como los cambios que suceden en etapas tempranas de la enfermedad o los efectos de nuevas aproximaciones terapéuticas (Jurjus et al. 2004). De manera general, los modelos murinos de IBD pueden ser clasificados en cuatro categorías: (i) colitis producida por defectos en la barrera epitelial, (ii) colitis producida por defectos en la respuesta inmune innata, (iii) colitis producida de forma espontánea, y (iv) colitis caracterizada por respuestas adaptativas anómalas de linfocitos T. Esta última se puede separar en dos sub-categorías, la colitis producida por respuestas excesivas de células efectoras o la colitis producida por un desequilibrio entre linfocitos T efectores y reguladores (Tabla 3) (Strober et al. 2002; Pizarro et al. 2003; Rijnierse et al. 2007; Valatas et al. 2015).

La elección de los modelos pertenecientes a cada una de estas categorías será llevada a cabo por los investigadores en función de los objetivos a abordar. Por ejemplo, los ratones deficientes en TLRs, IL-2 o IL-10 han sido utilizados para investigar la contribución de las correspondientes alteraciones genéticas y del sistema inmune en el desarrollo de la enfermedad (Dieleman et al. 2000; Madsen et al. 2000; Pizarro et al. 2003). A la hora de estudiar el rol potencial de la microbiota en la IBD, se han empleado mayoritariamente animales libres de gérmenes o tratamientos con antibióticos con distintos espectros de acción (Dieleman et al. 2000; Madsen et al. 2000). En relación al empleo de agentes antimicrobianos, se ha demostrado que pueden alterar la composición de las comunidades microbianas intestinales en modelos murinos (Croswell et al. 2009; Garner et al. 2009). Del mismo modo, se ha visto como la utilización de antibióticos puede prevenir el desarrollo de la inflamación intestinal en los modelos murinos de IBD inducidos por dextrano sulfato de sodio (DSS) o por la deficiencia en IL-10 (Hoentjen et al. 2003; Ward et al. 2016).

Tabla 3. Modelos experimentales de enfermedad inflamatoria intestinal

Defectos en barrera epitelial	Respuesta adaptativa anómala de células T Respuesta excesiva de células efectoras
- DSS	- TNBS
- N-cadherina dominante negativa	- Oxazolona
- Muc1/2 KO	- TNF$^{\Delta ARE}$
- Mdr1a KO	- STAT4 tg
Defectos en inmunidad innata	- TCRα KO
- A20 KO	**Desequilibrio de células T efectoras y reguladoras**
- STAT3 KO	- CD45RB^{high} transferencia adoptiva
- CD40 mAb ->RAG KO	- IL-2 KO
- *Helicobacter hepaticus*->SCID/RAG KO	- TGFβ1 KO
- IEC/IKK-γ KO	- IL-10 KO
- TRUC	- Gαi2 KO
Colitis espontánea	- Tgε26
- C3H/HeJBir	- TGFβRII DN
- SAMP1/Yit(Fc)	

DSS, dextrano sulfato de sodio; Muc1/2; mucina 1 y 2; KO, deficiente; Mdr1a, resistencia a múltiples drogas murino 1a; A20, proteína citoplasmática; STAT3, transductor de señales y activador de transcripción 3; RAG, gen de activación de la recombinación; SCID, inmunodeficiencia combinada severa; IEC, células epiteliales intestinales; IKK-γ, IκB quinasa-γ; TRUC, modelo murino de colitis ulcerativa; C3H/HeJBir y SAMP1/Yit(Fc), sub-cepas de ratones que desarrollan colitis espontánea; TNBS, ácido dinitrobencenosulfónico; TNF, factor de necrosis tumoral; ΔARE, deleción en elementos ricos en adenina y uracilo; STAT4 tg, transductor de señales y activador de transcripción 4 transgénico; TCRα, cadena alfa del receptor de linfocitos T; CD45RB^{high}, grupo de diferenciación 45RB de cadena pesada; IL, interleucina; TGFβ1, factor de crecimiento transformante β1; Gαi2, sub-unidad αi2 de receptor acoplado a proteína G; Tgε26, ratones transgénicos para el grupo de diferenciación 3ε humano; TGFβRII DN, Receptor de TGFβ dominante-negativo tipo II. Adaptado de: Valatas, V., Bamias, G., & Kolios, G. (2015). Experimental colitis models: Insights into the pathogenesis of inflammatory bowel disease and translational issues. *European Journal of Pharmacology*, *759*, 253–264.

MODELO IL-10 DEFICIENTE (IL-10$^{-/-}$)

La IL-10 como se ha señalado previamente, es una citocina que desempeña un papel principalmente antiinflamatorio. Su ausencia en los ratones deficientes de IL-10 (IL-10$^{-/-}$) da lugar al desarrollo de una inflamación intestinal crónica al verse impedida la función reguladora que lleva a cabo esta citocina sobre el sistema inmune en condiciones fisiológicas (Sellon et al. 1998).

Los animales IL-10$^{-/-}$ desarrollan colitis bajo condiciones de estabulación convencionales. Por el contrario, bajo condiciones de barrera microbiológica o en caso de ser criados bajo condiciones libres de gérmenes, no se observa inflamación intestinal.

Ello ha llevado a sugerir que la microbiota intestinal residente es necesaria para el establecimiento de la colitis en los ratones que poseen dicha alteración en el sistema inmune (Sellon et al. 1998).

Todas estas observaciones, junto con las evidencias existentes de disbiosis, respaldan la teoría de que las interacciones anómalas entre huésped-microorganismos mediadas por PRRs contribuyen al desarrollo de la IBD.

En este contexto, se hace interesante investigar la función que cumplen los distintos PRRs presentes en los MC y la respuesta que generan tras su activación. A día de hoy, se ha demostrado evidencia de expresión de diversos PRRs, tales como TLR2, TLR3, TLR4, TLR6, TLR7, TLR9 y NOD2 en poblaciones mastocitarias murinas (Inohara et al. 2003; Jean S. Marshall et al. 2003; Jean S Marshall et al. 2003; Marshall 2004; Galli et al. 2008; Mrabet-Dahbi et al. 2009; Haidl et al. 2011; Vukman et al. 2012; Xie et al. 2012).

Por esta razón, es importante observar la respuesta producida por los MC en un modelo de IBD como el que se expresa en los ratones IL-10 deficientes, para así caracterizar su respuesta y el rol que cumple la carencia de IL-10 en el desarrollo de la respuesta. Y por otra parte, observar cómo la deficiencia de IL-10 afecta la composición de la microbiota en este modelo.

HIPÓTESIS Y OBJETIVOS

De lo anteriormente expuesto se puede señalar que la microbiota intestinal es un componente activo en el mantenimiento de la homeostasis intestinal. Para ello son clave las interacciones entre los microorganismos presentes en el tracto gastrointestinal y las células del huésped. Éstas son posibles debido a la expresión de mecanismos de identificación, como los receptores de reconocimiento de patrones (PRRs), por parte de diversos tipos celulares del hospedador, incluyendo los mastocitos. Las alteraciones en la microbiota intestinal, así como en la interacción de ésta con el huésped, pueden llevar a estados patológicos como la enfermedad inflamatoria intestinal. Al mismo tiempo, se ha descrito ampliamente que la IL-10 es una citocina importante tanto en el mantenimiento del equilibrio homeostático intestinal, como en la maduración de los mastocitos. Así como también, la participación de los mastocitos en procesos inflamatorios gastrointestinales.

Basado en esto, nos planteamos la **HIPÓTESIS** de que la ausencia de IL-10 causa alteraciones en el fenotipo y la activación mastocitarios asociados a cambios en la expresión de factores asociados a la respuesta inmune. Ello contribuye a la afectación de la composición de la microbiota intestinal y al desarrollo de procesos inflamatorios intestinales descritos en los ratones IL-10$^{-/-}$.

Haciendo uso de un modelo murino deficiente de IL-10 y a fin de demostrar la citada hipótesis, los **OBJETIVOS** del presente trabajo fueron caracterizar:

- La influencia de la IL-10 en la expresión de receptores de reconocimiento de patrones (PRRs) y su respuesta frente a ligandos específicos en poblaciones mastocitarias de tipo mucosa (MLMC) y conectivo (PCMC).

- La regulación por parte de la IL-10 de la composición de la microbiota intestinal en estadios previos (6 semanas de edad) y posteriores (20 semanas de edad) al desarrollo de la inflamación intestinal.

- La influencia de la IL-10 sobre los cambios que tienen lugar tras la administración de antibióticos vía oral a nivel de la microbiota y de las respuestas inmunes intestinales asociadas a receptores de tipo toll (TLR) y a la secreción de inmunoglobulina A (IgA).

CAPÍTULO 1

IL-10 Modulates the Expression and Activation of Pattern Recognition Receptors in Mast Cells

Roberto Riquelme-Neira,[1] Patrocinio Vergara,[1] and Joan Antoni Fernández-Blanco[1]

[1]Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, 08193, Spain.

Abstract

Mast cells (MC) identify microorganisms by pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NODs). Consequently, a defensive response is activated, inducing the secretion of immune mediators. At the same time, interleukin (IL)-10 could be an important modulator of MC responses through PRRs. However, the functional role of IL-10 in PRR-mediated activation of MC is not fully understood. Therefore, we decided to analyze the expression and activation of TLR2, TLR4, TLR7 and NOD2 in mucosal-like MC (MLMC) and peritoneum-derived cultured MC (PCMC) from IL-10$^{-/-}$ and wild type mice. Cells were isolated from 6 and 20-week-old animals, before and after the progression of colitis in IL-10$^{-/-}$. IL-10$^{-/-}$ mice showed a reduced expression of TLR4 and NOD2 at week 6 and TLR7 at week 20 in MLMC. In both MLMC and PCMC, TLR2 activation induced IL-6 and TNFα secretion, which was reduced in the case of IL-10$^{-/-}$ MC. TLR4- and TLR7-mediated secretion of IL-6 and TNFα was detected in MLMC but not in PCMC. MLMC isolated from 6-week-old IL-10$^{-/-}$ mice secreted lower levels of IL-6 in response to TLR4 and TLR7 activation. However, IL-10$^{-/-}$ MLMC released less TNFα when derived from animals at 20 weeks of age. Finally, no cytokine release was induced by the NOD2 ligand Muramyl dipeptide in any MC subtype. Concerning age-related changes, responses to TLR2 and TLR4 activation were lower in cells isolated at 20 weeks than at 6 weeks. Taken together, our results indicate that PRR activation in MC depends on the MC phenotype, the PRR ligand, age and IL-10 presence. These findings provide new insights into MC functions and should help in the understanding of inflammatory processes in which IL-10 and the interaction microorganism-host play a key role, such as inflammatory bowel disease.

Introduction

Mast cells (MC) are known for their role in allergic disorders, such as anaphylaxis and asthma, and their participation in defense responses against pathogens [1–3]. These cells are distributed in proximity to host-environment interfaces and have different functions and phenotypes depending on their tissue location [4–6]. Accordingly, they are classified as mucosal MC (MMC), found predominantly in the mucosa of the airways and the gastrointestinal tract, and connective tissue MC (CTMC), and mainly located in the skin and peritoneal cavity [4–8]. In these locations, MC act as sentinel cells, initiating immune responses to exogenous stimuli [6,9,10]. MC subtypes show differences in the production and secretion of mediators in response to external stimuli [6–8].

The detection of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) plays a crucial role in the innate immune response. PRRs include Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NODs). Regarding MC, many studies have detected the expression of PRRs, including TLR2, TLR3, TLR4, TLR6, TLR7, TLR9 and NOD2 [1,11–15]. In this respect, TLR2 participates in the recognition of peptidoglycans, lipoteichoic acid and lipopeptides present in bacterial cell wall [14,16]. TLR4, for its part, is involved in the recognition of lipopolysaccharides (LPS) found in the outer membrane of Gram negative bacteria [17,18]. TLR7 mediates responses to synthetic imidazoquinoline compounds and virus-derived single-stranded RNA [19]. In the case of NOD2, it detects intracellularly the bacterial-derived muramyl dipeptide (MDP) [11,20].

Therefore, via PRRs, MC can recognize and respond to bacteria and viruses located both intracellularly and intracellularly [2,21]. Once activated after PAMP-PRR interactions, MC release immune mediators [4,10,14,16,22–25]. These mediators generate a diversity of biological effects, including the recruitment and activation of inflammatory cells, smooth muscle contractions and the increase of vascular permeability [8,21,26]. In this context, interleukin 6 (IL)-6 and tumor necrosis factor alpha (TNFα) are critical cytokines involved in inflammatory responses related to inflammatory diseases such as rheumatoid arthritis, asthma, and inflammatory bowel disease (IBD) [13,21,22]. In the case of IBD, it has been suggested that IL-10 modulates

the interaction of the host with the microbiota [27,28]. In agreement, IL-10 knockout (IL-10$^{-/-}$) mice develop enterocolitis under conventional housing conditions which mimics IBD signs [29]. In addition, deficient mice in both mast cells and IL-10 exhibit exacerbated colitis compared to single IL-10 knockout mice, which clearly reinforces the relationship between mast cells and IL-10 [21,30].

In addition, responses to PRR activation are conditioned by the level of expression of PRRs in different MC subtypes. For instance, previous reports suggest that, in comparison to peritoneum-derived cultured MC, bone marrow-derived MC show a lower expression of TLR4 and TLR2 together with a lower secretion of cytokines when stimulated with LPS and lipoteichoic acid [14]. Therefore, it is necessary to analyze each specific MC subpopulation individually to properly understand their modulation by PRRs.

The aim of this study was to compare the role of IL-10 in PRR-mediated responses in MMC and CTMC. To this end, experiments were performed in peritoneum-derived cultured mast cells (PCMC) and mucosal-like mast cells (MLMC) from IL-10$^{-/-}$ and wild-type mice. We then assessed the expression of TLR2, TLR4, TLR7 and NOD2 and the release of inflammatory cytokines after their activation by specific ligands at different time points and concentrations.

Materials and Methods

Animals

B6.129P2-$Il10^{tm1Cgn}$/J (IL-10$^{-/-}$) and C57BL/6J (WT) female mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) (5-week-old, weight 15-17 g). Upon arrival, animals were randomly divided into 4 groups [6-week-old WT (n=4), 6-week-old IL-10$^{-/-}$ (n=4), 20-week-old WT (n=6) and 20-week-old IL-10$^{-/-}$ (n=5)]. Mice were housed in individually ventilated cages in groups of four to six per cage under conventional conditions in an environmentally controlled room (temperature: 20-22°C; humidity: 40-70%; photoperiod: 12h/12h light-dark cycle). Mice had access to tap water and Teklad Global 14% protein rodent maintenance diet (2014; ENVIGO CRS S.A., Cambridgeshire, UK) *ad libitum*. All the experimental procedures were approved by the

Ethics Committee of the Universitat Autònoma de Barcelona and the Generalitat de Catalunya (protocols 2773 and 8814, respectively).

Collection and culture of peritoneum-derived cultured mast cells (PCMC)

At the time of the experiments, mice were deeply anesthetized with isoflurane (Isoflo; Esteve, Barcelona, Spain) and euthanatized by exsanguination through intracardiac puncture followed by cervical dislocation. Immediately after, a peritoneal lavage was performed with 10 ml sterile 1X PBS to collect peritoneal mast cells. These cells were used to obtain peritoneum-derived cultured mast cells (PCMC), as previously described [8] with some modifications. Briefly, the lavage fluid was centrifuged (250 g, 7 min, 4°C). Thereafter, cells were resuspended in fresh Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 1% penicillin/streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine, all of them purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA), 10 ng/ml recombinant mouse IL-3 (rmIL-3; R&D Systems, Inc., Minneapolis, MN, USA) and 30 ng/ml recombinant mouse stem cell factor (rmSCF; Peprotech Inc., Rocky Hill, NJ, USA). Cells were cultured in the same medium for 48 h in 75-cm^2 flat-bottomed flasks at 37 °C under 10% CO_2. Next, non-adherent cells were removed and the medium replaced and changed twice a week to achieve expansion and purification of residual peritoneal cells. After 4 weeks, cells were harvested, their viability was monitored using Trypan blue staining and their surface expression of c-kit and FcεRI was measured by flow cytometry.

Mucosal-like Mast Cells (MLMC) culture

After euthanasia, intact femurs and tibias were removed from the mice. The bone marrow was washed using a 23-gauge needle and a 5 ml syringe filled with DMEM containing 10% FCS, 1% penicillin/streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine (DMEM/FCS). Then, mucosal-like mast cells (MLMC) were cultured and differentiated as previously described [31]. In short, the cell suspension was passed three times through a 19-gauge needle, and centrifuged (250 g, 7 min, room temperature). Cells were resuspended in 10 µl DMEM/FCS, stained with Trypan blue and counted in a hemocytometer to determine cell density and viability. Subsequently, 24 ml of cell suspension (5x10^5 cells/ml) were cultured in 75 cm^2 flat-bottomed flasks in a humidified 10% CO_2 incubator at 37°C in DMEM/FCS containing 50 ng/ml rmSCF, 1

ng/ml rmIL-3, 5 ng/ml recombinant mouse IL-9 (rmIL-9; R&D Systems, Inc., Minneapolis, MN, USA), and 1 ng/ml recombinant human transforming growth factor (rhTGF-β1; Sigma-Aldrich CO., St. Louis, MO, USA). Cultures were maintained for 9 days and were fed every 2-3 days by centrifuging (250 g, 7 min, room temperature) and resuspending them in half-volume of the original culture medium and half-volume of fresh medium.

Cytospin and staining of mast cell

Cellular suspensions obtained from MLMC and PCMC cultures were cytocentrifuged using a Cytocentrifuge Cytospin^{TM} 4 (Thermo Fisher Scientific Inc., Waltham, MA, USA) onto a slide (600 rpm, 6 min, room temperature), air-dried for 15 min, fixed in Carnoy's fixative solution (60% pure ethanol, 30% chloroform, 10% acetic acid) for 15 min and stained with a 1% Toluidine Blue O solution (Sigma-Aldrich CO., St. Louis, MO, USA) at pH = 7 for 5 min. Samples were dehydrated and mounted in DPX medium.

In vitro cell stimulation

MLMC and PCMC were seeded in 12-well flat-bottom plates (Nunclon® of Thermo Fisher Scientific Inc., Waltham, MA, USA) in triplicate at a density of $5x10^5$ cells/ml in complete culture medium in a humidified 10% CO_2 incubator at 37°C. To study responses related to FcεRI activation, cells were sensitized with 1 μg/ml anti-dinitrophenyl IgE (clone: SPE-7; ref: D8406, Sigma-Aldrich CO., St. Louis, MO, USA) overnight, and then challenged for 6 h with 50 ng/ml dinitrophenyl-human serum albumin (DNP-HAS; Sigma-Aldrich CO.). To evaluate TLR2, TLR4, TLR7 and NOD2 mediated responses, cells were stimulated with Pam_2CSK4 (InvivoGen, San Diego, CA, USA), Lipopolysaccharide (LPS) from *Escherichia coli* serotype O55:B5 (Sigma-Aldrich CO.), Imiquimod (InvivoGen) and Muramyl dipeptide (MDP; InvivoGen), respectively. Before and after stimulation, cells were collected by centrifugation (250 g, 7 min, room temperature). Cell fractions were used to perform morphology and flow cytometry analysis. Cells and supernatants were frozen and stored at -80°C until analysis.

RNA extraction and reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA from MLMC and PCMC was extracted using RNeasy® Mini Kit (Qiagen, Hilden, Germany). RNA was quantified by Nanodrop (NanoDrop Technologies, Rockland, DE, USA) and 1 μg of RNA was reverse-transcribed in a 20 μl reaction volume for cDNA synthesis using iScript™ cDNA Synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The temperature profile for reverse transcription was 25 °C for 5 min, 42 °C for 30 min and 85 °C for 5 min.

Validated TaqMan® gene expression assays with hydrolysis probes for mouse mast cell proteases (mMCPs), cytokines, TLRs and reference genes were used (Applied Biosystems, Foster City, CA, USA; Table 1). PCR reaction mixtures were transferred to clear 384-well reaction plates (Bio-Rad Laboratories, Inc., Hercules, CA, USA); sealed by adhesives and C1000 Touch™ Thermal Cycler platform (Bio-Rad Laboratories, Inc.) for 40 cycles (95 °C for 15 s, 60 °C for 1 min). Fluorescence signals measured during amplification were processed after amplification. Bio-Rad CFX Manager 2.1 software was used to obtain the cycle threshold (CT) for each sample. Each sample was run in triplicate and data were analyzed by the comparative CT method [$2^{-\Delta\Delta CT}$], as previously described [32]. Actb and β-2-microglobulin were tested as reference genes. β-2-microglobulin expression levels were used for normalizing the mRNA levels of the target genes because of their constancy across the different experimental groups. Controls of analytical specificity included omission of reverse transcriptase, to exclude contamination with genomic DNA, and no-template controls, omitting the cDNA.

Table 1. TaqMan® gene expression assays.

Protein	Gene symbol	Assay reference
Beta-2-microglobulin	*B2m*	Mm00437762_m1
Beta-actin	*Actb*	Mm00607939_s1
Interleukin 1 beta (IL-1β)	*Il1b*	Mm00434228_m1
Interleukin 6 (IL-6)	*Il6*	Mm00446190_m1
Tumor necrosis factor-alfa (TNFα)	*Tnf*	Mm00443258_m1
Mast cell protease 1 (mMCP-1)	*Mcpt1*	Mm00656886_g1
Mast cell protease 6 (mMCP-6)	*Tspb2*	Mm01301240_g1
Nucleotide-binding oligomerization domain-containing protein 2	*Nod2*	Mm00467543_m1
Toll-like receptor 7	*Tlr7*	Mm00446590_m1

Flow cytometry

$5x10^5$ PCMC and MLMC were harvested, washed twice in FACS buffer (2% FCS and 2mM EDTA in 1X PBS) and pre-incubated for 15 min with 200 µl of FACS buffer with 1 µg of purified anti-mouse CD16/CD32 (clone: 93; ref: 14-0161) to block non-specific binding to Fc Receptor (FcR). Without washing, 50 µl ($1,25x10^5$) of pre-incubated cells were simultaneously stained for cell surface markers for 30 min at 4 °C by adding 50 µl of a mix of monoclonal antibodies (eBioscience, San Diego, CA, USA) anti-mouse CD117 (c-Kit) conjugated with allophycocyanin (clone: 2B8; ref: 17-1171), anti-mouse Fc epsilon Receptor I alpha (FcεR1α) conjugated with fluorescein isothiocyanate (clone: MAR-1; ref: 11-5898) and anti-mouse CD282 (TLR2) (clone: 6C2; ref: 12-9021) or anti-mouse CD284 (TLR4) conjugated with phycoerythrin (clone: UT41; ref: 12-9041). All the antibodies were previously titrated. After two washes, cells were resuspended in 0.2 ml of FACS buffer and the analysis of 20.000 events was performed using a six-colour BD FACSCanto flow cytometer and BD FACSDiva Software v7.0 (BD Biosciences, San Jose, CA). The strategy of gating used was as follows: we first set a gate of live cells based on forward and side scatter properties and then a second gate on the CD117 and FcεR1 double positive population to select MC. To set the negative controls for TLRs on MC, we used unstained cells and fluorescence minus one control (only CD117 and FcεR1 antibodies).

Quantification of cytokines concentration

Supernatants obtained from PCMC and MLMC cultures, as described before, were analyzed to determine the concentration of the cytokines IL-1β, IL-6, IL-10, IL-12p70, IFN-γ, MCP-1 and TNFα, using a cytometric bead analysis Mouse Inflammation Kit (CBA) (BD Biosciences, San Jose, CA, USA). Data were obtained using FACScan and analyzed with the BD™ Cytometric Bead Array (CBA) Analysis Software (BD Biosciences).

Data Analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 6.0 software (Graph Prism Software Inc., La Jolla, CA, USA). Comparisons between multiple groups were performed using one-way ANOVA or two-way ANOVA, followed, when necessary, by a Bonferroni multiple-comparison test. P-values <0.05 were considered statistically significant.

Results

Morphological analysis of MLMC and PCMC

MC stained with toluidine blue were identified by their red-purple cytoplasmic metachromatic granular content and blue nuclei. Light microscopy analysis revealed that both subsets of MC had a similar size, with each subset comprising a homogeneous cell population. MLMC and PCMC displayed typical MC phenotypes, as shown by intense metachromatic staining, with PCMC heavily granulated compared to MLMC. There was no obvious morphologic difference between WT and IL-10$^{-/-}$ MC (Fig 1).
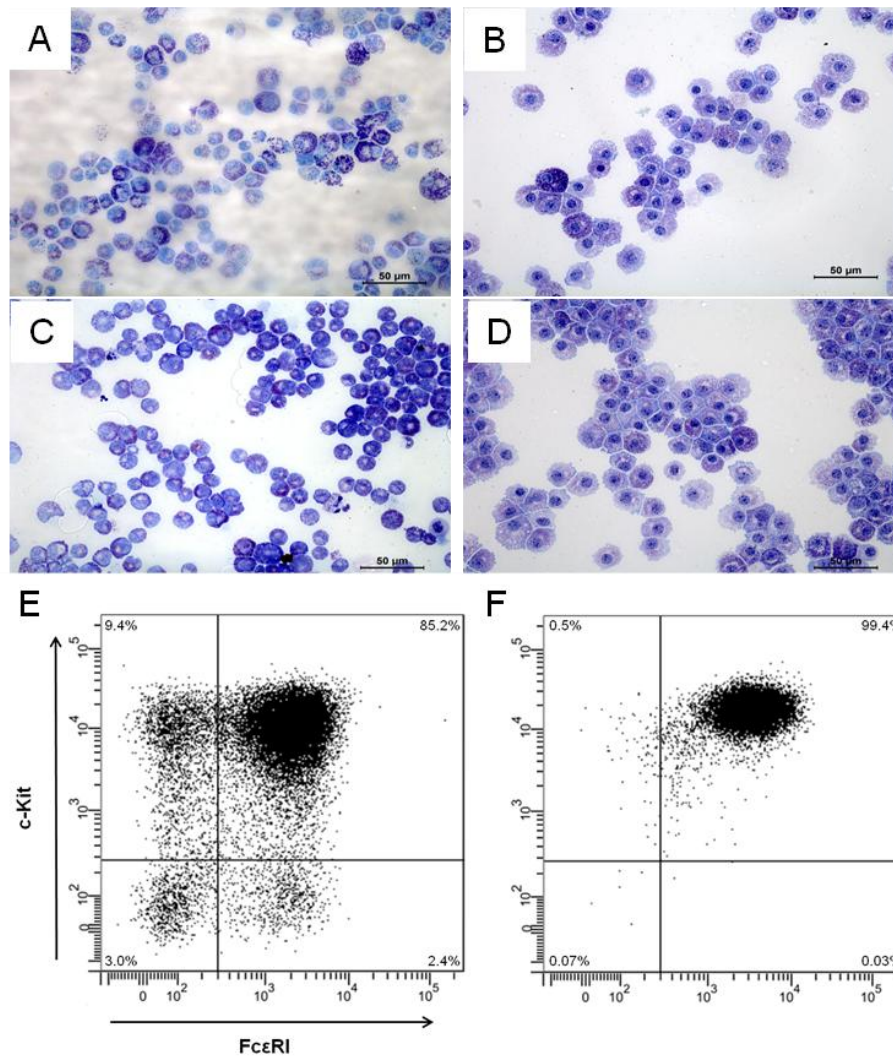
Figure 1. Characterization of the morphology and purity of MLMC and PCMC isolated from wild type and IL-10$^{-/-}$ mice. The images correspond to Toluidine blue-stained cytospin preparations of mucosal-like mast cells (MLMC; A, C) and peritoneum-derived cultured mast cells (PCMC; B, D) from wild type (A, B) or IL-10$^{-/-}$ (C, D) mice. Toluidine blue-positive cells contain dark blue cytoplasmic granules. Original magnification: 40X. Scale bars: 50 μm. (E, F) Two-color FACS® analysis of high-affinity IgE receptor (y-axis) and c-kit (x-axis) expression of MLMC (E) or PCMC (F).

mMCP-1 and mMCP-6 characterization in MLMC and PCMC

In MLMC, similar levels of mMCP-1 were observed in both genotypes at week 6. In WT MLMC, we detected a higher expression of mMCP-1 when cells were isolated at week 20. Conversely, no variations in the expression of mMCP-1 were observed between weeks 6 and 20 for knockout MLMC. A similar pattern was observed when mMCP-6 expression was analyzed in MLMC (Fig 2 A, B).

In PCMC, no expression of mMCP-1 was detected, whereas expression of mMCP-6 was similar in both genotypes when PCMC were isolated from 6-week-old mice. However, in PCMC derived from IL-10$^{-/-}$ mice at 20 weeks of age, we detected a lower expression of mMCP-6 compared to age-matched WT (Fig 2 C, D).
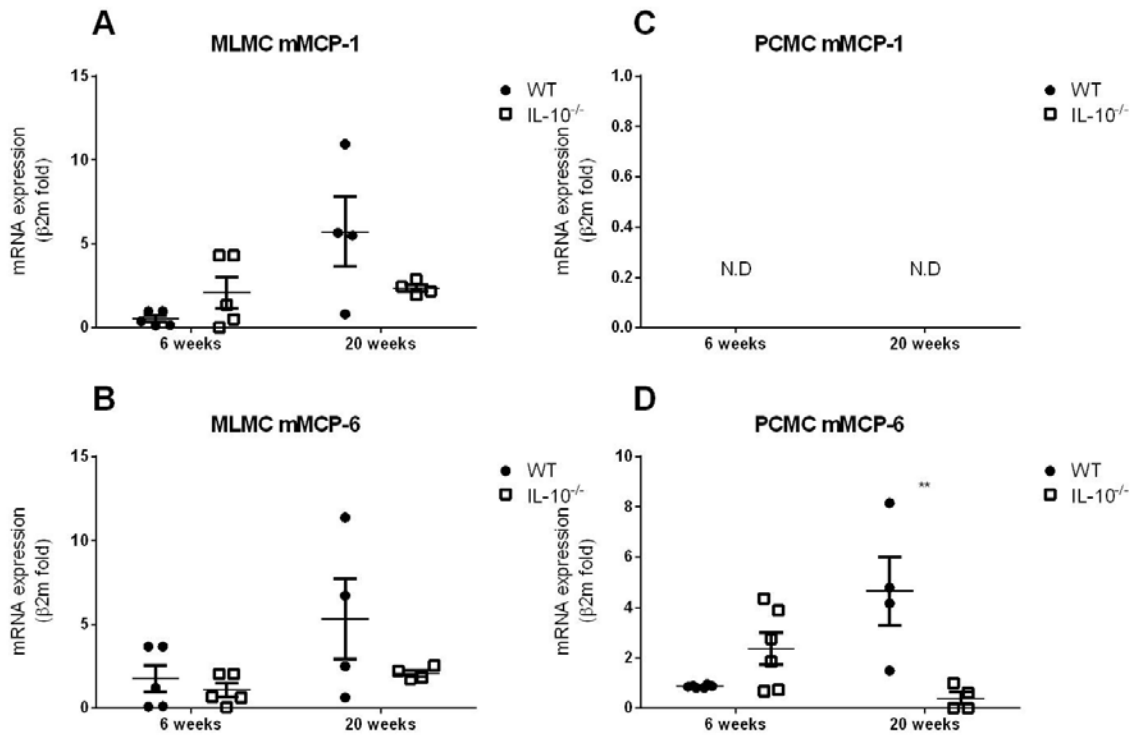
Figure 2. Gene expression of mMCP-1 and mMCP-6 by MLMC and PCMC derived from wild type and IL-10$^{-/-}$ mice. Gene expression of mouse mast cell protease (mMCP)-1 (A, C) and mMCP-6 (B, D) was assesses in mucosal-like mast cells (MLMC; A, B) and peritoneum-derived cultured mast cells (PCMC; C, D). Relative expression of mMCPs mRNA was analyzed by quantitative reverse transcription-PCR and normalized to transcript levels of β-2-microglobulin. Expression levels were measured in wild type (WT; filled circles) and IL-10$^{-/-}$ (empty squares). All the analyses were performed using cells isolated from 6 (left panels) and 20 (right panels) week old mice. Data are mean ± standard error of 4-6 animals per group. **P<0.01 vs the wild type, 2-way ANOVA with Bonferroni post test. N.D.: not detected.

Effects of IL-10 deletion on TLR2 expression and release of inflammatory mediators related to its activation by Pam$_2$CSK4 in MLMC and PCMC

The percentage of MLMC and PCMC expressing TLR2 on their surface in basal conditions (time zero, unstimulated) was not significantly different when derived from

WT or IL-10$^{-/-}$ mice. In contrast, a decrease in the number of TLR2^{+} MC was observed in 20-week-old mice of both genotypes compared to 6-week-old mice (Fig 3 A, D).

However, differences in TLR2 expression between both genotypes were unmasked when cells were stimulated with the TLR2 agonist Pam$_2$CSK4. Firstly, when analyzed together before and after stimulation, we detected that the general expression of TLR2 was lower in MC obtained from IL-10$^{-/-}$ mice. More specifically, at 20 weeks, MLMC stimulation for 24 h with 100 µg/mL Pam$_2$CSK4 increased the percentage of TLR2^{+} cells in WT but not in IL-10$^{-/-}$ cells. Similarly, the percentage of TLR2^{+} PCMC detected after stimulation was lower in the IL-10$^{-/-}$ compared with the WT cells.

The stimulation of both MLMC and PCMC with Pam$_2$CSK4 resulted in the secretion of the proinflammatory cytokines IL-6 and TNFα (Fig 3). Interestingly, TLR2-mediated responses were conditioned by IL-10 expression. In the case of MLMC, IL-10 deletion reduced the secretion of IL-6 at 6 weeks and secretion of IL-6 and TNFα at 20 weeks. Regarding PCMC, the secretion of both cytokines was lower in the absence of IL-10 by week 6, when it was maximal for WT cells. It was also observed that the magnitude of the response regarding the secretion of TNFα by the PCMC (~500 pg/mL) was higher than that observed in MLMC (~250 pg/mL) (Fig 3). It is noteworthy that, when MLMC and PCMC were subjected to any of the stimuli used in the study, it was not possible to detect IL-10, IL-1β, IL-12p70 nor IFN-γ (data not shown).
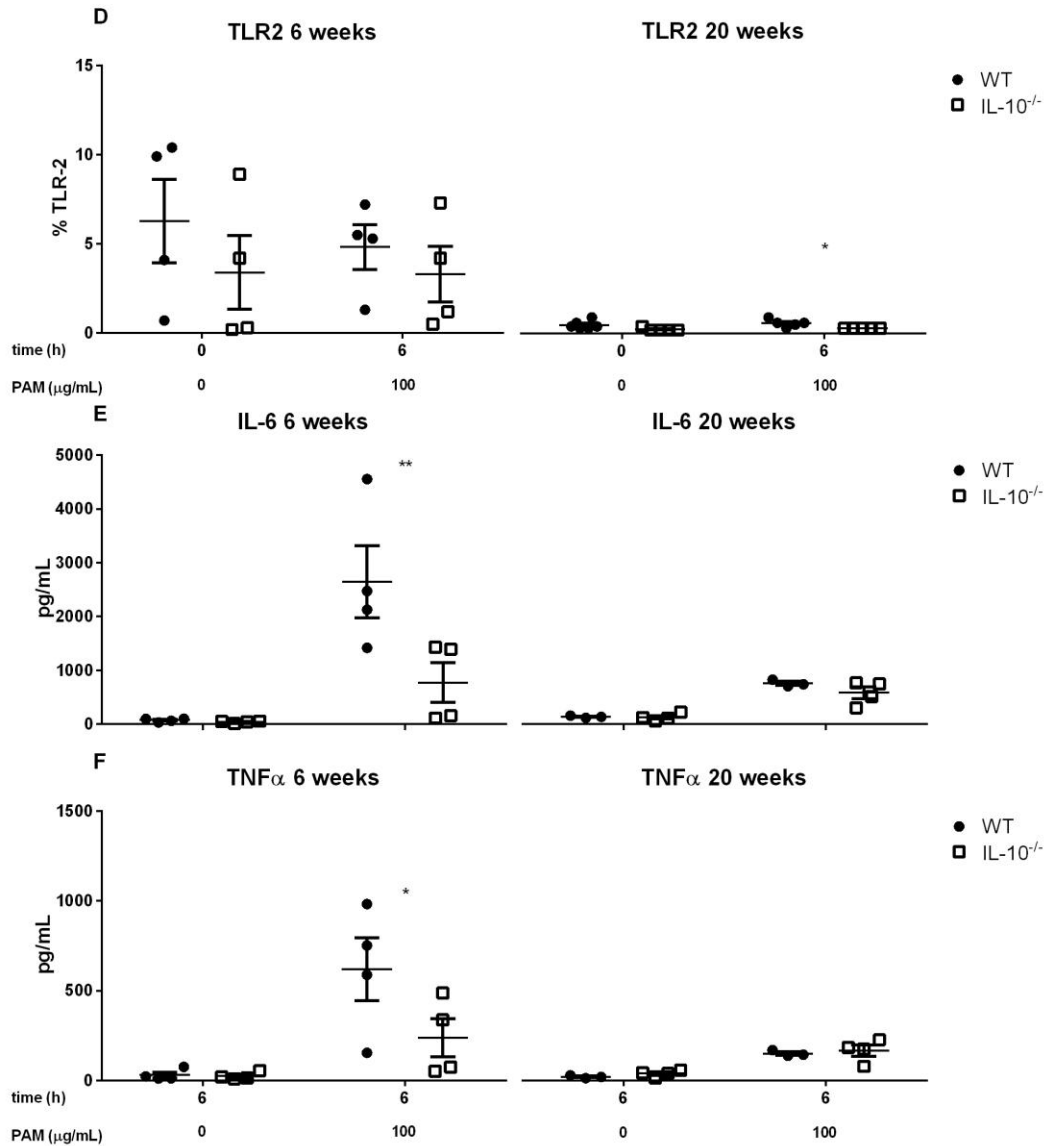
Figure 3. Effects of IL-10 deletion on TLR2 expression and release of inflammatory mediators related to its activation by Pam₂CSK4 in MLMC and PCMC. The expression of toll-like receptor 2 (TLR2) was assesses in mucosal-like mast cells (MLMC; A) and peritoneum-derived cultured mast cells (PCMC; D). The percentage of cells positive for TLR2 was characterized in basal conditions (unstimulated; time 0) and after the stimulation with synthetic diacylated lipoprotein Pam₂CSK4 (PAM). IL-6 (B, E) and TNFα (C, F) levels were measured in wild type (WT; filled circles) and IL-10$^{-/-}$ (empty squares) MLMC (B, C) and PCMC (E, F) left unstimulated or stimulated with PAM. All the analyses were performed using cells isolated from 6 (left panels) and 20 (right panels) week old mice. Data are mean ± standard error of 4-6 animals per group. *P<0.05, **P<0.01 and ***P<0.001 vs wild type, 2-way ANOVA with Bonferroni post test.
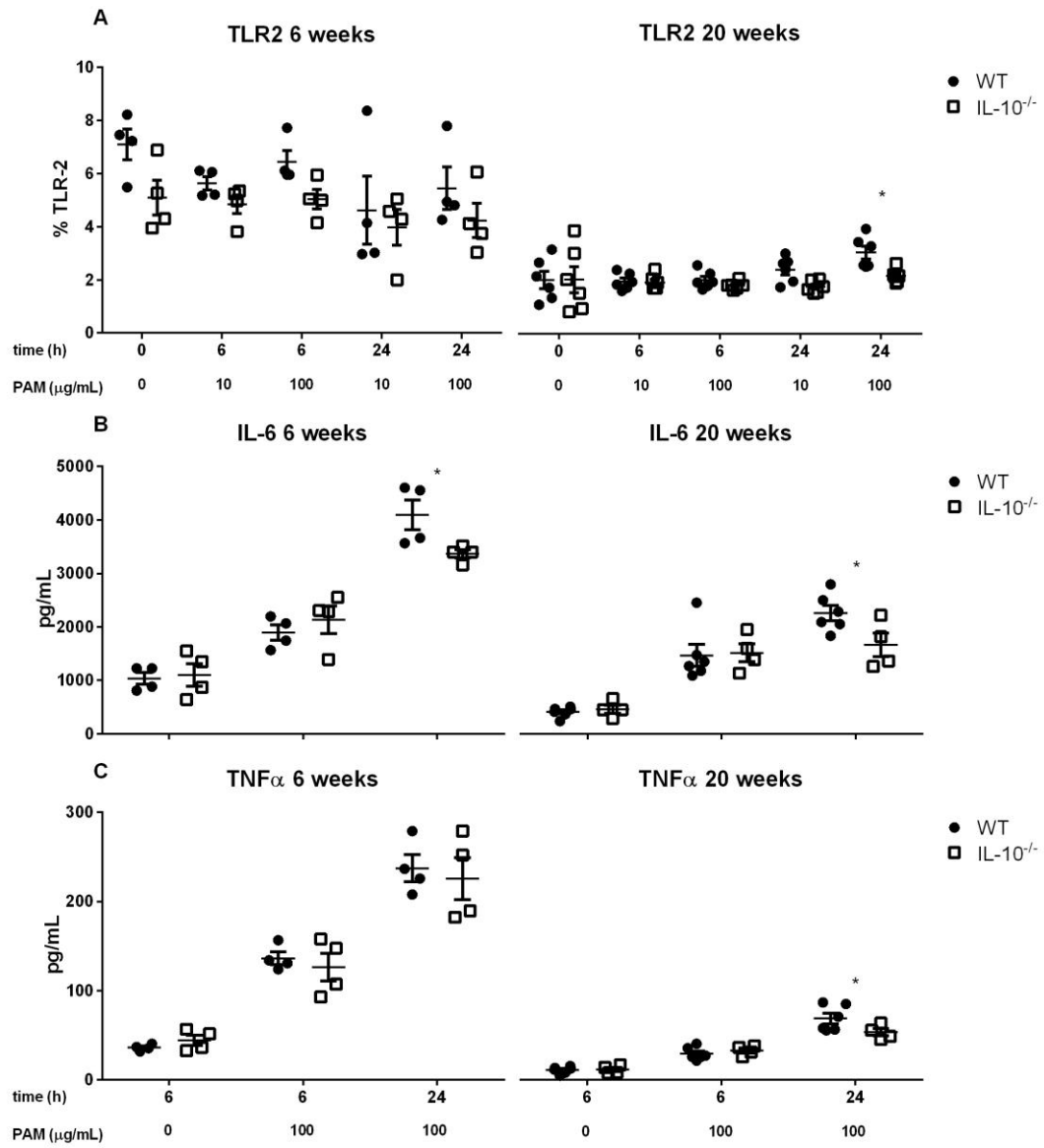
Effects of IL-10 deletion on TLR4 expression and release of inflammatory mediators related to its activation by LPS in MLMC and PCMC

The basal proportion of MLMC expressing TLR4 on their surface at week 6 was lower in MLMC derived from IL-10$^{-/-}$ mice than in those derived from WT animals. This difference was reverted by week 20, when MLMC expression of TLR4 was equivalent in both genotypes. In the case of unstimulated PCMC, no differences were detected in the basal expression of TLR4 at week 6 and the cell surface expression of the receptor was no longer detected by week 20 (Fig 4 A, D).

After the stimulation of TLR4 with LPS, the expression of TLR4 in MLMC trended to decrease and the differences detected between both genotypes at week 6 were preserved. Regarding PCMC, LPS did not affect the surface expression of TLR4 neither in knockout nor in WT cells.

Concerning the secretion of proinflammatory mediators, TLR4 activation induced the release of IL-6 and TNFα by MLMC (Fig 4). Levels of IL-6 increased progressively in time while TNFα levels peaked at 6 h after LPS addition. When both phenotypes were compared, MLMC derived from 6-week-old IL-10$^{-/-}$ mice secreted more IL-6 after TLR4 stimulation than age-matched WT animals. On the other hand, at week 20, in agreement with what we described for TLR2 expression, differences between both genotypes in IL-6 secretion were no longer observed. Secretion of TNFα by MLMC was not influenced by IL-10. In addition, we observed a reduction in the proinflammatory response of MLMC to LPS at 20 weeks compared with 6 weeks. As opposed to what we detected in MLMC, LPS did not induce the secretion of IL-6 or TNFα by PCMC (Fig 4). That said, the levels of these cytokines detected in the medium of PCMC were dependent on the genotype. While IL-6 levels were higher at week 6 in WT cells, TNFα basal secretion was higher at week 20 in the case of IL-10$^{-/-}$ PCMC.

A

TLR4 6 weeks   TLR4 20 weeks

● WT
□ IL-10$^{-/-}$

| time (h) | 0 | 6 | 6 | 24 | 24 |
| LPS (ng/mL) | 0 | 10 | 100 | 10 | 100 |

B

IL-6 6 weeks   IL-6 20 weeks

● WT
□ IL-10$^{-/-}$

C

TNFα 6 weeks   TNFα 20 weeks

● WT
□ IL-10$^{-/-}$

| time (h) | 6 | 6 | 24 | 6 | 6 | 24 |
| LPS (ng/mL) | 0 | 100 | 100 | 0 | 100 | 100 |

Figure 4. Effects of IL-10 deletion on TLR4 expression and release of inflammatory mediators related to its activation by LPS in MLMC and PCMC. The expression of toll-like receptor 4 (TLR4) was assesses in mucosal-like mast cells (MLMC; A) and peritoneum-derived cultured mast cells (PCMC; D). The percentage of cells positive for TLR4 was characterized in basal conditions (unstimulated; time 0) and after the stimulation with lipopolysaccharide (LPS) from *Escherichia coli* O55:B5. IL-6 (B, E) and TNFα (C, F) levels were measured in wild type (WT; filled circles) and IL-10^{-/-} (empty squares) MLMC (B, C) and PCMC (E, F) left unstimulated or stimulated with LPS. All the analyses were performed using cells isolated from 6 (left panels) and 20 (right panels) week old mice. Data are mean ± standard error of 4-6 animals per group. *P<0.05, **P<0.01 and ***P<0.001 vs wild type, 2-way ANOVA with Bonferroni post test. N.D.: not detected.
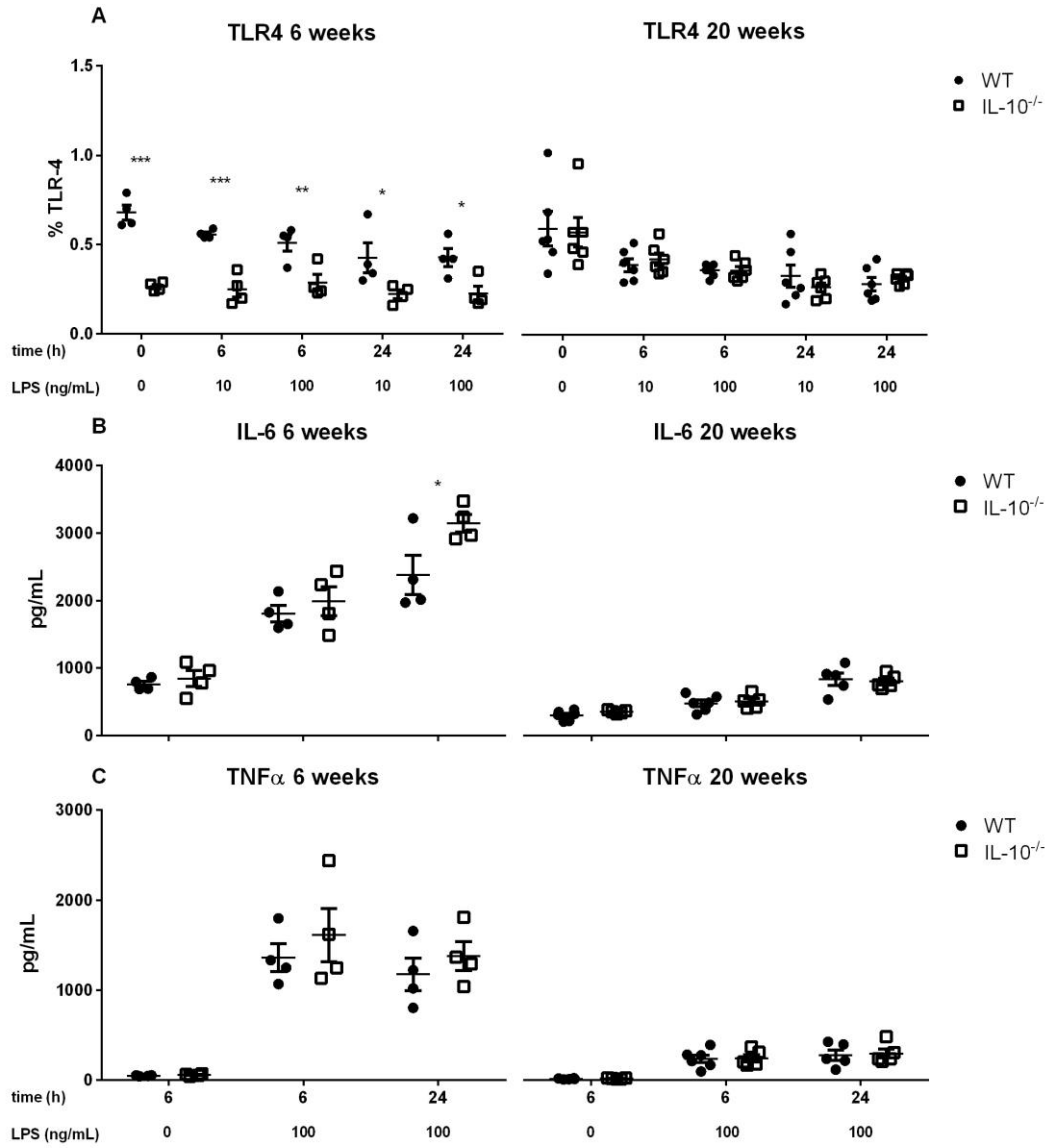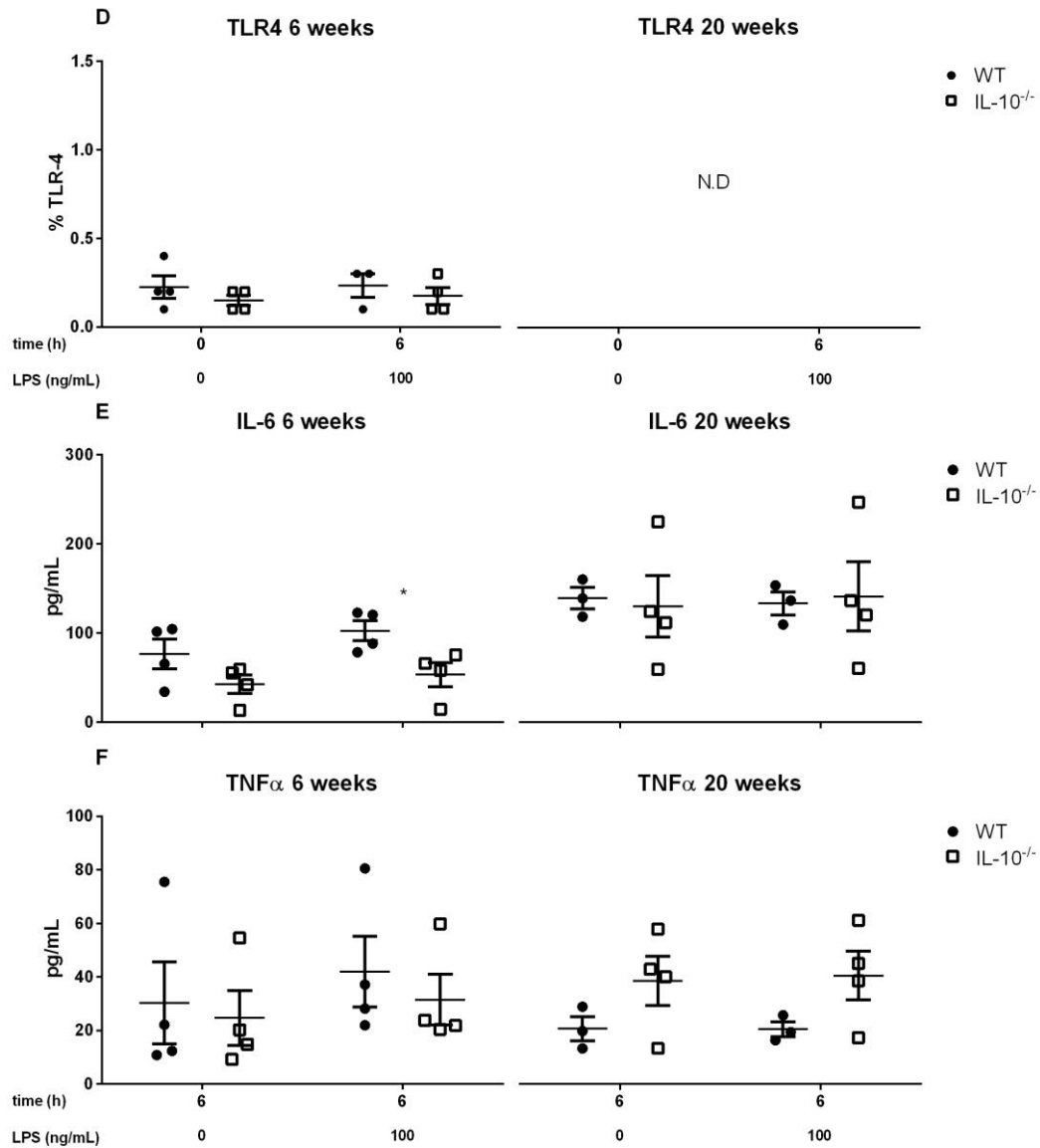
Effects of IL-10 deletion on TLR7 expression and release of inflammatory mediators related to its activation by Imiquimod in MLMC and PCMC

In basal conditions, MLMC derived from 6-week-old mice of both genotypes showed similar levels of TLR7 expression. On the other hand, at week 20, the expression of TLR7 was lower in MLMC when IL-10 was not present. In contrast to MLMC, TLR7 was not detected in PCMC isolated from any of the genotypes at any time point (Fig 5).

The exposure of MLMC to the agonist Imiquimod at week 6 was associated with a drop in time of the gene expression of TLR7 in both genotypes (P <0.001; two-way ANOVA). At 20 weeks, after 6 h of stimulation, we detected a lower expression of TLR7 in IL-10$^{-/-}$ cells, similar to what we observed in basal conditions.

The activation of TLR7 by Imiquimod induced the secretion of both IL-6 and TNFα by MLMC (Fig 5). The proinflammatory pattern depended on the genotype of the cells and the age of the animals from which were isolated. At week 6 and after 24 h of stimulation, IL-6 levels detected were higher in cells obtained from IL-10$^{-/-}$ mice. By contrast, at 20 weeks, TNFα secretion in response to Imiquimod was larger in WT MLMC. With respect to unstimulated PCMC, those isolated from animals at week 6 secreted more IL-6 and similar levels of TNFα than those obtained at week 20 (Fig 5). Finally, in agreement with the lack of detection of TLR7 in this cell type, we did not observe any effect of Imiquimod on the secretion of proinflammatory cytokines.

A

TLR7 6 weeks

TLR7 20 weeks

mRNA expression (β2m fold)

WT
IL-10⁻/⁻

B

IL-6 6 weeks

IL-6 20 weeks

pg/mL

WT
IL-10⁻/⁻

C

TNFα 6 weeks

TNFα 20 weeks

pg/mL

WT
IL-10⁻/⁻

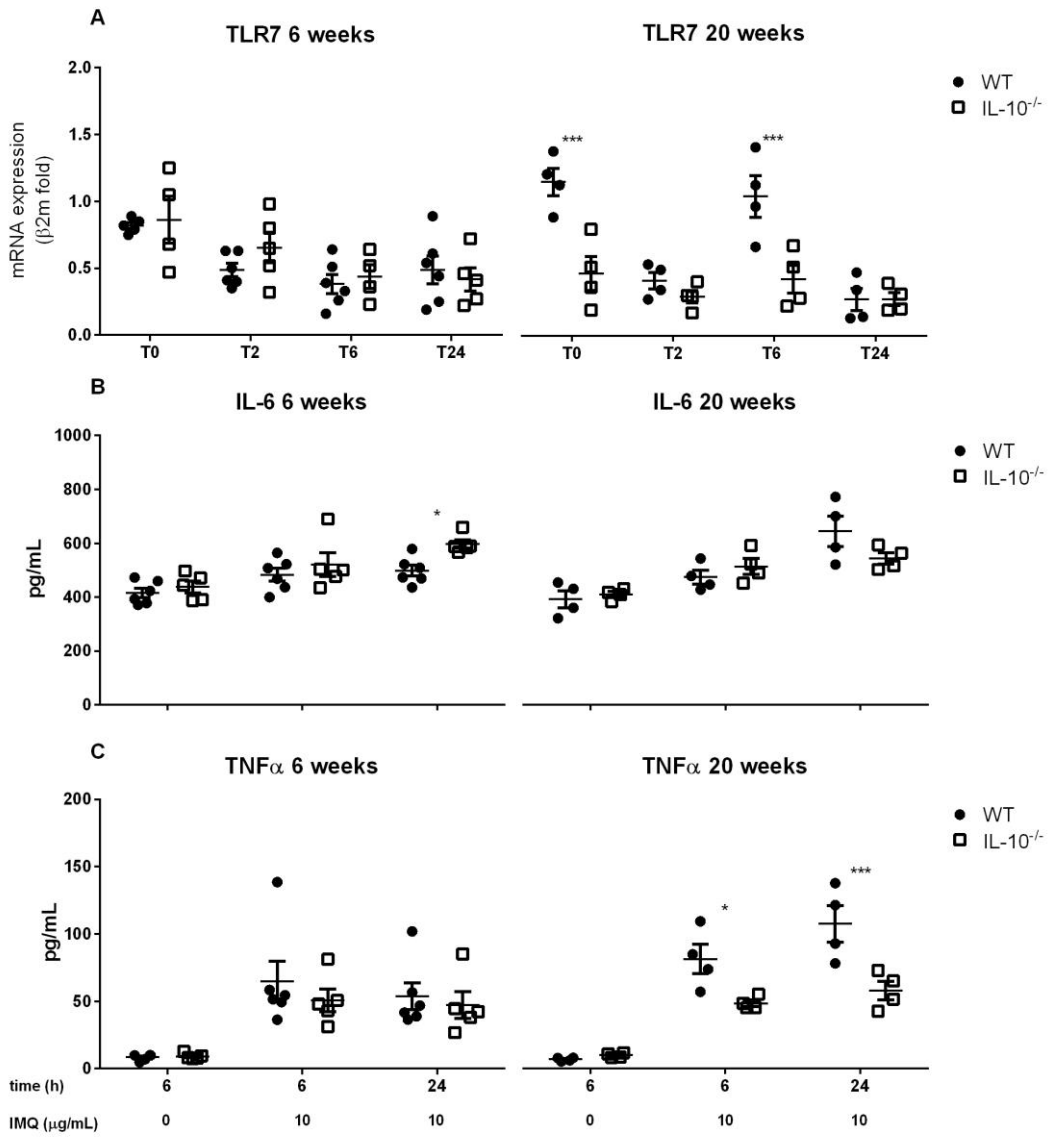| time (h) | 6 | 6 | 24 | 6 | 6 | 24 |
| IMQ (µg/mL) | 0 | 10 | 10 | 0 | 10 | 10 |

Figure 5. Effects of IL-10 deletion on TLR7 expression and release of inflammatory mediators related to its activation by Imiquimod in MLMC and PCMC. Gene expression of toll-like receptor 7 (TLR7) was assesses in mucosal-like mast cells (MLMC; A) and peritoneum-derived cultured mast cells (PCMC; D). Relative expression of TLR7 mRNA was analyzed by quantitative reverse transcription-PCR and normalized to transcript levels of β-2-microglobulin in basal conditions (unstimulated; time 0) and after the stimulation with imiquimod (IMQ). IL-6 (B, E) and TNFα (C, F) levels were measured in wild type (WT; filled circles) and IL-10$^{-/-}$ (empty squares) MLMC (B, C) and PCMC (E, F) left unstimulated or stimulated with IMQ. All the analyses were performed using cells isolated from 6 (left panels) and 20 (right panels) week old mice. Data are mean ± standard error of 4-6 animals per group. *P<0.05, **P<0.01 and ***P<0.001 vs wild type, 2-way ANOVA with Bonferroni post test. N.D.: not detected.
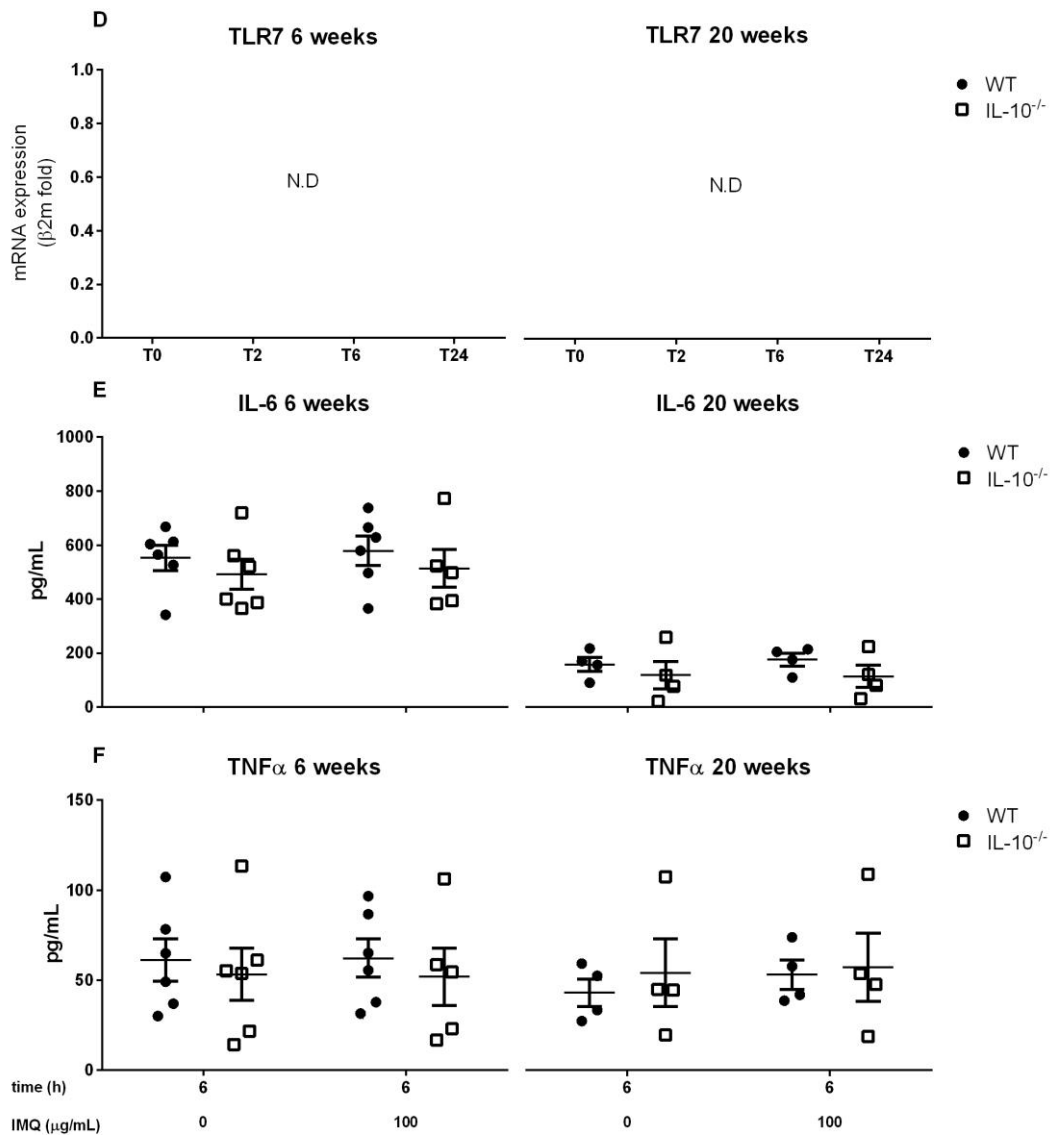
Effects of IL-10 deletion on NOD2 expression and release of inflammatory mediators related to its activation by MDP in MLMC and PCMC

The gene expression of the NOD2 receptor was only detected in MLMC isolated from 6- week-old mice. In this case, we observed a downregulation of gene expression in IL-$10^{-/-}$ animals compared with WT mice. However, these differences were no longer detected after cell culture and stimulation with MDP, which induced a reduction in NOD2 expression (Fig 6).

In relation to cytokine release, no changes were observed in IL-6 or TNFα levels associated with the stimulation with MDP or with the expression of IL-10 in any MC subtype (Fig 6).
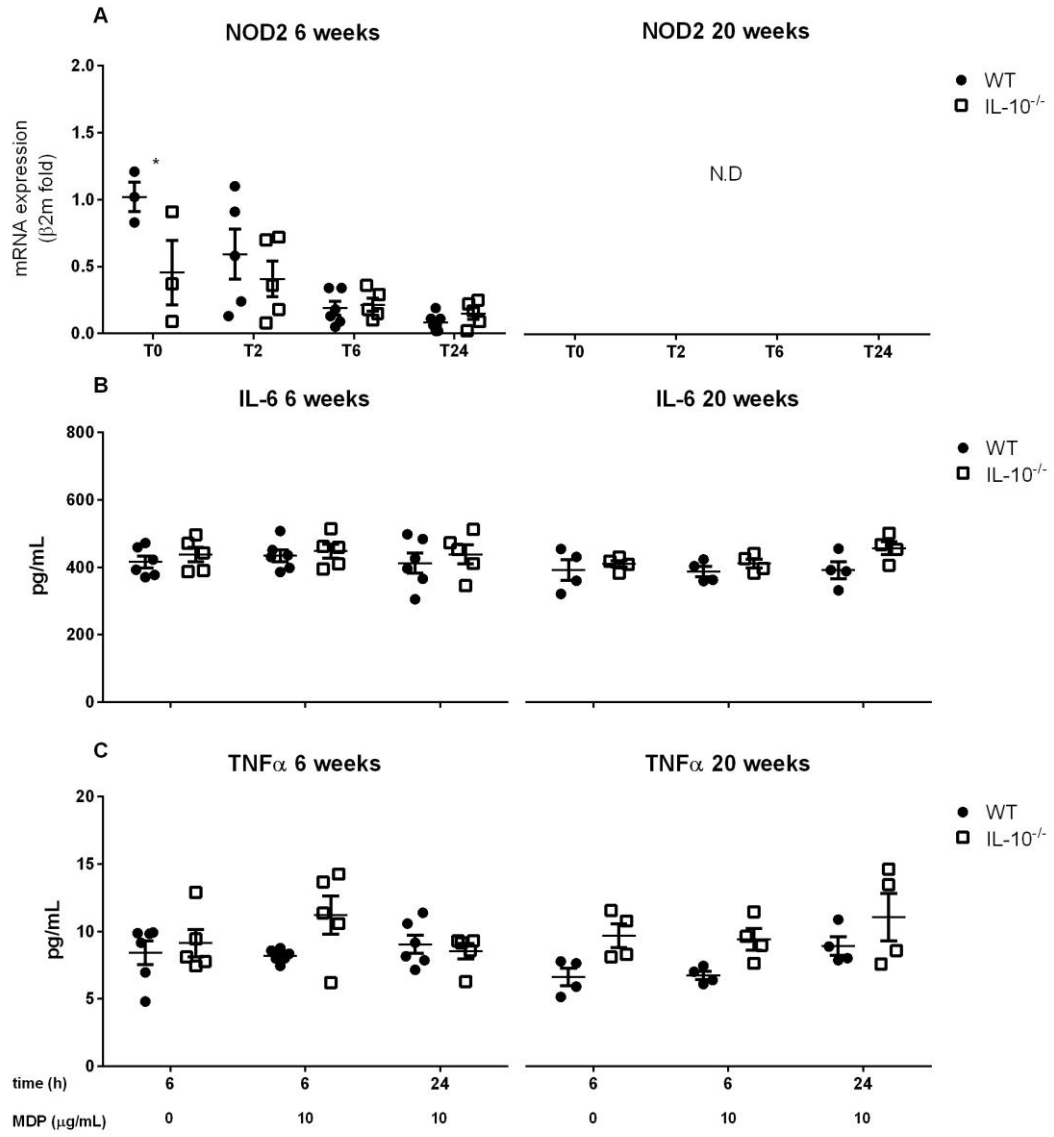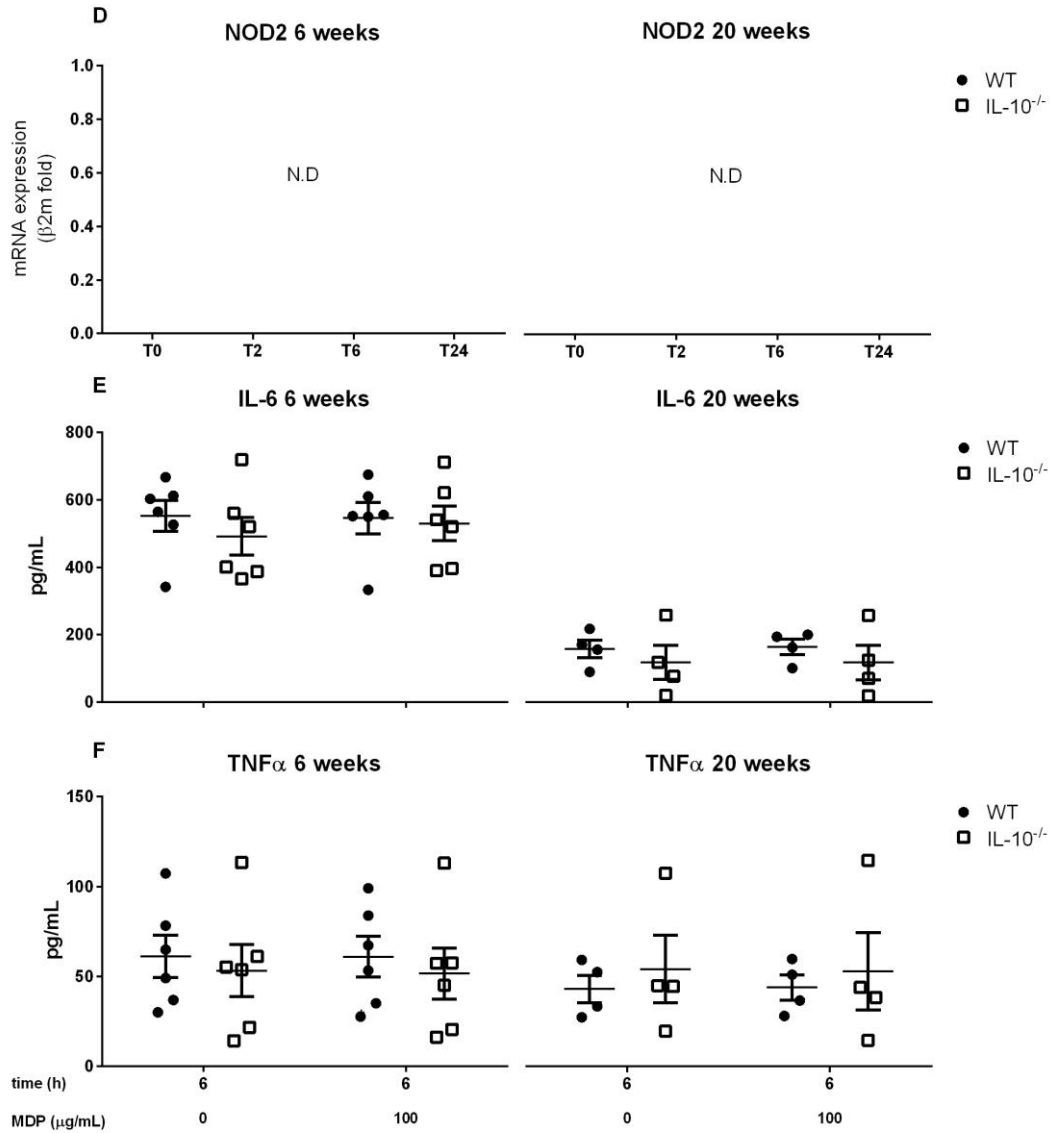
Figure 6. Effects of IL-10 deletion on NOD2 expression and release of inflammatory mediators related to its activation by MDP in MLMC and PCMC. Gene expression of nucleotide-binding oligomerization domain-containing protein 2 (NOD2) was assesses in mucosal-like mast cells (MLMC; A) and peritoneum-derived cultured mast cells (PCMC; D). Relative expression of NOD2 mRNA was analyzed by quantitative reverse transcription-PCR and normalized to transcript levels of β-2-microglobulin in basal conditions (unstimulated; time 0) and after the stimulation with muramyldipeptide (MDP). IL-6 (B, E) and TNFα (C, F) levels were measured in wild type (WT; filled circles) and IL-10^{-/-} (empty squares) MLMC (B, C) and PCMC (E, F) left unstimulated or stimulated with MDP. All the analyses were performed using cells isolated from 6 (left panels) and 20 (right panels) week old mice. Data are mean ± standard error of 4-6 animals per group. *P<0.05, **P<0.01 and ***P<0.001 vs the wild type, 2-way ANOVA with Bonferroni post test. N.D.: not detected.

Effects of the lack IL-10 on FcεRI-mediated responses in MLMC and PCMC

The activation of FcεRI in MLMC and PCMC induced a significant secretion of the cytokines IL-6 and TNFα cytokines (Fig 7).

In MLMC derived from 6-week-old mice, we observed a similar response in both genotypes. On the other hand, when MLMC were obtained from 20 week-old-mice, the FcεRI-mediated secretion of IL-6 and TNFα was lower in IL-10$^{-/-}$ cells (Fig 7).

In the case of PCMC, IL-10 deletion reduced cytokines levels detected after FcεRI activation in MC isolated at week 6. In contrast, when obtained at week 20, IL-10$^{-/-}$ MC produced similar levels of IL-6 and higher levels of TNFα compared to WT cells (Fig 7).
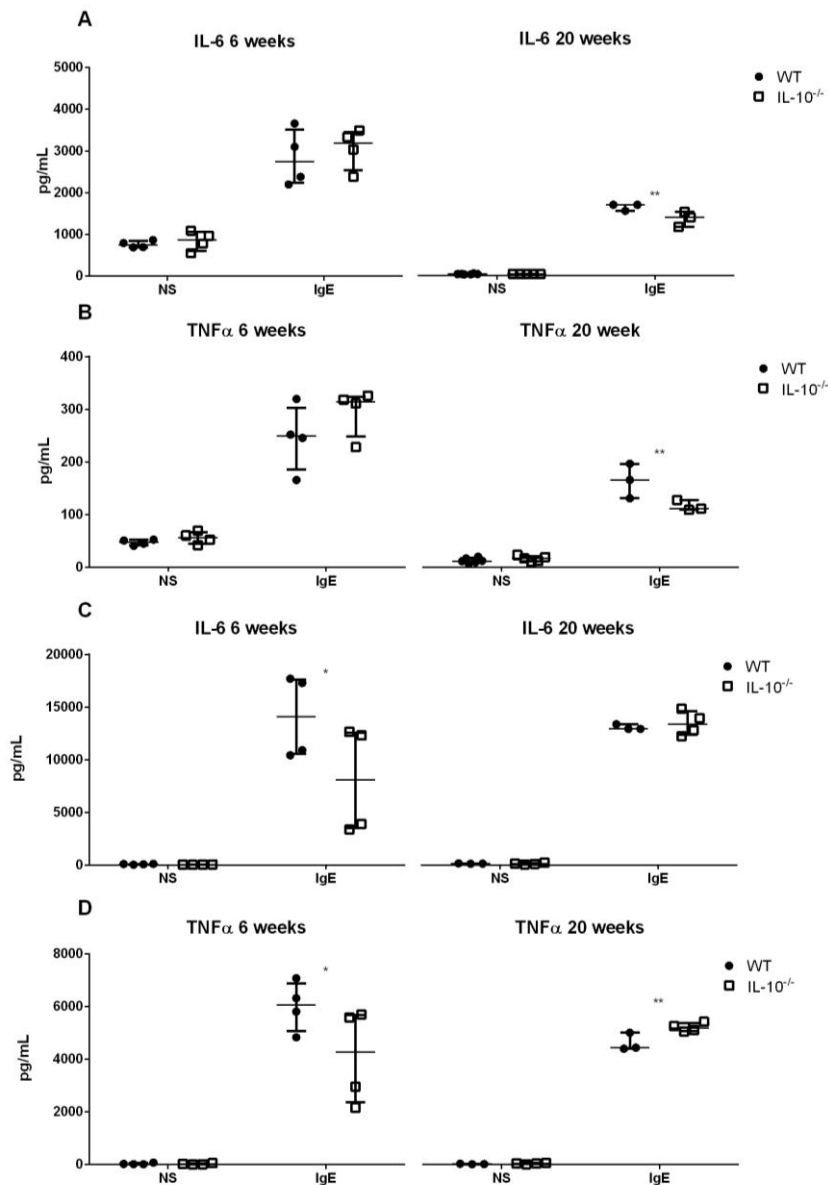
Figure 7. Effects of the lack IL-10 on FcεRI-mediated release of proinflammatory mediators in MLMC and PCMC. IL-6 (A, C) and TNFα (B, D) levels were measured in wild type (WT; filled circles) and IL-10$^{-/-}$ (empty squares) MLMC (A, B) and PCMC (C, D) left unstimulated (NS) or stimulated with dinitrophenyl-human serum albumin. All the analyses were performed using cells isolated from 6 (left panels) and 20 (right panels) week old mice. Data are mean ± standard error of the mean of 3-4 animals per group. *P<0.05 and **P<0.01 vs the wild type, 2-way ANOVA with Bonferroni post test.

Discussion

Interleukin (IL)-10 has been suggested to modulate mast cell (MC) functions and inflammatory responses related to host-microorganisms interactions in an age-related way [21,29,33,34]. In the present study, we describe for the first time the role of IL-10

in pattern recognition receptor (PRR) expression and MC activation. With that purpose, we assessed cytokines production induced by PRRs ligands in mucosal-like MC (MLMC) and peritoneum-derived cultured MC (PCMC) from IL-10$^{-/-}$ and wild type (WT) mice. Interestingly, we found that both the expression and the activation of PRRs depend on the MC subtype and animals' age and are conditioned by IL-10 deficiency.

To perform these studies, we first assessed the phenotype of both MC subtypes after their *in vitro* differentiation. We were able to confirm that MLMC and PCMC were positively stained for both FcεRI and c-kit (CD117), as observed specifically in MC [35,36]. Cell differentiation was further corroborated by the study of their morphology and the expression of mouse mast cell proteases (mMCPs) [37]. As previously described by others, MLMC were smaller and contained fewer toluidine blue-stained granules than PCMC [38]. In addition, mMCP-1, a protease typically related to mucosal MC, was detected in MLMC but not in PCMC. This demonstrates that the protocol used to derive MLMC from bone marrow cells induces a differentiation to a mucosal MC phenotype [31]. It also confirms that mMCP-1 is a useful marker for distinguishing mucosal from connective tissue MC in mice. On the other hand, mMCP-6, usually considered a marker for connective tissue MC [3], was detected in both MLMC and PCMC cultures. This could be explained by the expression of proteases generally associated with CTMC, including mMCP-6, by mucosal and transitional populations of MC [3,39,40]. Regarding the influence of IL-10 in MC differentiation, while no obvious differences in morphology were detected between both genotypes, we show that IL-10 modulates mMCPs expression. As IL-10 can induce the expression of mMCPs, the lower expression of mMCP-6 detected in knockout MC could be related to the absence of IL-10 and the lack of its autocrine activity [39,41].

We then studied PRR expression in IL-10$^{-/-}$ and WT MLMC and PCMC and found that TLR2, TLR4, TLR7 and NOD2 were expressed by MLMC. In comparison, TLR2 and TLR4, but neither TLR7 nor NOD2, could be detected in PCMC. This supports the constitutive expression of TLR2 and TLR4 in MMC and CTMC and the heterogeneity in TLR7 and NOD2 expression in different MC populations [14,25,42–44]. However, the exact proportion of MC of each subtype expressing PRRs and the factors behind the modulation of their expression is more difficult to specify. For instance, in our study, the proportion of MLMC and PCMC expressing TLR2 was similar. In contrast, Mrabet-

Dahbi et al. (2009) found that the percentage of TLR2$^+$ cells was higher in PCMC than in bone marrow cultured MC, used as a model of MMC. In the same study, they observed a higher percentage of PCMC expressing TLR2 and TLR4 than we did. The difference detected between their results and ours could be related to changes in the *in vivo* model and the *in vitro* maturation of the cells. For instance, they cultured PCMC for 9 days in comparison to our 28 day culture protocol. In addition, we show for the first time how PRR expression evolves with aging in MC, similar to what others have described for macrophages [45]. In particular, we observed that the proportion of TLR2$^+$ and TLR4$^+$ PCMC was higher when derived from younger mice. So, the results obtained by Mrabet-Dahbi et al. could be conditioned by the age of the animals used to isolate cells. Unfortunately, that information was not specified in their manuscript. That said, it seems clear that both *in vivo* and *in vitro* variables modulate the expression of PRRs in MC and that this should be considered in future experiments.

When we characterized the role of IL-10 in PRR expression, we observed that it depended on the receptor and the age of the animals when cells were isolated. In the case of MLMC, IL-10 deletion reduced the expression of TLR4 and NOD2 at week 6 and TLR7 at week 20. Regarding CTMC, IL-10 could have contributed to the observed TLR2 expression by week 20, a time point when the other PRRs evaluated were not detected in this cell type. To date, the mechanisms by which IL-10 regulates PRRs have not been characterized. However, the modulation by IL-10 of the repertoire of PRRs expressed by MC could be involved in the ability of this cytokine to induce MC expansion, survival and activation [46,47].

After studying PRR expression in MC, we characterized their activation and the consequent release of immune mediators. Firstly, Pam$_2$CSK4-mediated activation of TLR2 induced the secretion of IL-6 and TNFα in both genotypes and MC subtypes. These results complement other studies in which a TLR2-dependent production of cytokines was observed in bone marrow-derived MC and PCMC after their stimulation with the TLR2 agonists peptidoglycan and lipoteichoic acid [10,14,48]. However, we cannot exclude Pam$_2$CSK4 recognition by a heterodimeric interaction of TLR2 with other TLRs, such as TLR1 or TLR6 [10,49]. Therefore, although out of the scope of the present study, the interaction among different TLRs in MC should be addressed in future experiments.

TLR4 and TLR7 activation, as discussed above for TLR2, also induced the secretion of pro-inflammatory cytokines in WT and IL-10^{-/-} MLMC. In contrast, TLR4 and TLR7-mediated secretion of IL-6 and TNFα was not observed in PCMC. While the lack of response of PCMC to the TLR7 ligand Imiquimod matches with previous reports, the finding that TLR4 activation did not induce cytokine secretion was unexpected [14,42]. This could be related to a lower expression of TLR4 or the absence of membrane CD14 in the PCMC we used for our studies, in comparison with those used by groups that observed a positive response [14,48].

It is noteworthy that our study clearly shows how the release of cytokines by MC in response to TLR2 and TLR4 ligands is age-dependent. In particular, we observed a reduction in IL-6 and TNFα secretion in cells isolated at week 20 compared to those obtained from 6-week-old mice. These changes could be attributed to differences in TLRs expression discussed above. At the same time, this could be associated with a general reduction in the production and secretion of proinflammatory mediators by MC with age. This hypothesis is supported, at least in the case of MLMC, by the results obtained with IgE stimulation. As PRR activation and their regulation of immune responses changes with age, immune response regulated by PRRs would be affected. This would condition the interaction of the host with its microbiota. As a result, the secretion of cytokines would be reduced, facilitating the overgrowth of bacteria and their interaction with the epithelium. These mechanisms could be involved in the age-dependent activation and perpetuation of colitis observed in IL-10^{-/-} mice [21,29,45].

Finally, NOD2 ligands, according to our results and those obtained by others, do not induce the release of cytokines by either WT or IL-10^{-/-} MC [25,43]. In general, this would be justified by the lack of expression of the receptor. In this respect, the expression of NOD2 depends on IFNγ, which we did not detect on our cell cultures [25]. Therefore, further assays to study NOD2 in MC exposed to IFNγ would help to better understand PRR-mediated responses in inflammatory diseases.

Regarding the modulation of cytokine secretion by IL-10, it must be stressed that the results obtained are different depending on the PRR evaluated. On the one hand, in the case of TLR2, the secretion of IL-6 and TNFα by MC was reduced in absence of IL-10. This would explain, at least partially, the role of IL-10 in LPS-mediated responses [50].

Interestingly, we observed a similar pattern for the production of TNFα after TLR7 stimulation in MLMC. In contrast, TLR4 ligands induced larger immune responses in cells derived from IL-10$^{-/-}$ mice. This could be due to the potential role of IL-10 in the modulation of the expression of inflammatory mediators, as shown for other cell types including macrophages and monocytes [22,51–53]. Therefore, a complex and poorly understood scenario exists in which immune responses are a result of the counter-regulation of different PRRs by IL-10. In addition, the pro-inflammatory or anti-inflammatory roles of MC are conditioned not only by the kind of stimulus and the MC subtype, but also by their tissue location and interactions with other components of the immune system [34,54].

In summary, this study shows that both mucosal and connective tissue MC have the potential to respond to a variety of microorganisms via the activation of a diverse repertoire of PRRs. We describe in detail how the secretion of cytokines depends on the PRRs stimulated, the MC subtype and the age of the individuals from which MC or their precursors are isolated. Finally, we demonstrate that IL-10 deficiency affects the expression of PRRs and cytokine production in differentiated MC. Although the clinical relevance of this study remains to be determined, these observations provide new insights into MC functions in conditions other than allergy. As a result, this may help in the understanding of inflammatory processes in which the interaction microorganism-host seems to be key, such as inflammatory bowel disease.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

References

[1]   J.S. Marshall, "Mast-cell responses to pathogens.," *Nature Reviews. Immunology*, vol. 4, no. 10, pp. 787–799, 2004.

[2]   T. Hochdörfer, M. Kuhny, and C. Zorn, "Activation of the PI3K pathway increases TLR-induced TNF-α and IL-6 but reduces IL-1β production in mast cells," *Cellular Signalling*, vol. 23, pp. 866–875, 2011.

[3]   E. Rönnberg, G. Calounova, B. Guss, A. Lundequist, and G. Pejler, "Granzyme D is a novel murine mast cell protease that is highly induced by multiple pathways of mast cell activation.," *Infection and Immunity*, vol. 81, no. 6, pp. 2085–94, 2013.

[4]   D.D. Metcalfe, D. Baram, and Y.A. Mekori, "Mast cells," *Physiol Rev*, vol. 77, no. 4, pp. 1033–1079, 1997.

[5]   C.M. Williams, and S.J. Galli, "Mast cells can amplify airway reactivity and features of chronic inflammation in an asthma model in mice.," *The Journal of Experimental Medicine*, vol. 192, no. 3, pp. 455–462, 2000.

[6]   C.M. Westerberg, E. Ullerås, and G. Nilsson, "Differentiation of mast cell subpopulations from mouse embryonic stem cells.," *Journal of Immunological Methods*, vol. 382, no. 1–2, pp. 160–6, 2012.

[7]   M.F. Gurish, and J.A. Boyce, "Mast cell growth, differentiation, and death.," *Clinical Reviews in Allergy & Immunology*, vol. 22, no. 2, pp. 107–18, 2002.

[8]   O. Malbec, K. Roget, C. Schiffer, et al., "Peritoneal Cell-Derived Mast Cells: An In Vitro Model of Mature Serosal-Type Mouse Mast Cells," *The Journal of Immunology*, vol. 178, no. 10, pp. 6465–6475, 2007.

[9]   S.J. Galli, and B.K. Wershil, "The two faces of the mast cell," *Nature*, vol. 381, no. 6577, pp. 21–22, 1996.

[10]   V. Supajatura, H. Ushio, A. Nakao, et al., "Differential responses of mast cell Toll-like receptors 2 and 4 in allergy and innate immunity.," *The Journal of Clinical Investigation*, vol. 109, no. 10, pp. 1351–1359, 2002.

[11]   N. Inohara, Y. Ogura, A. Fontalba, et al., "Host recognition of bacterial muramyl dipeptide mediated through NOD2: Implications for Crohn's disease," *Journal of Biological Chemistry*, vol. 278, no. 8, pp. 5509–5512, 2003.

[12]   J.S. Marshall, J.D. McCurdy, and T. Olynych, "Toll-like receptor-mediated activation of mast cells: Implications for allergic disease?," *International Archives of Allergy and Immunology*, vol. 132, no. 2, pp. 87–97, 2003.

[13]   S. Galli, M. Grimbaldeston, and M. Tsai, "Immunomodulatory mast cells: negative, as well as positive, regulators of immunity," *Nature Reviews Immunology*, vol. 8, no. Box 1, 2008.

[14]   S. Mrabet-Dahbi, M. Metz, A. Dudeck, T. Zuberbier, and M. Maurer, "Murine

mast cells secrete a unique profile of cytokines and prostaglandins in response to distinct TLR2 ligands," *Experimental Dermatology*, vol. 18, no. 5, pp. 437–444, 2009.

[15]   X. Xie, L. Wang, F. Gong, et al., "Intracellular Staphylococcus Aureus-induced NF-κB Activation and Proinflammatory Responses of P815 Cells Are Mediated by NOD2," vol. 32, no. 3, pp. 317–323, 2012.

[16]   T. Ikeda, and M. Funaba, "Altered function of murine mast cells in response to lipopolysaccharide and peptidoglycan," *Immunology Letters*, vol. 88, no. 1, pp. 21–26, 2003.

[17]   B. Beutler, and A. Poltorak, "Sepsis and evolution of the innate immune response," *Critical Care Medicine*, vol. 29, no. 7 Suppl, pp. S2-6; discussion S6-7, 2001.

[18]   S. Varadaradjalou, F. Féger, N. Thieblemont, et al., "Toll-like receptor 2 (TLR2) and TLR4 differentially activate human mast cells," *European Journal of Immunology*, vol. 33, no. 4, pp. 899–906, 2003.

[19]   H. Matsushima, N. Yamada, H. Matsue, and S. Shimada, "TLR3-, TLR7-, and TLR9-Mediated Production of Proinflammatory Cytokines and Chemokines from Murine Connective Tissue Type Skin-Derived Mast Cells but Not from Bone Marrow-Derived Mast Cells," *The Journal of Immunology*, vol. 173, no. 1, pp. 531–541, 2004.

[20]   S.E. Girardin, I.G. Boneca, J. Viala, et al., "Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection," *Journal of Biological Chemistry*, vol. 278, no. 11, pp. 8869–8872, 2003.

[21]   M. Chichlowski, G.S. Westwood, S.N. Abraham, and L.P. Hale, "Role of Mast Cells in Inflammatory Bowel Disease and Inflammation-Associated Colorectal Neoplasia in IL-10-Deficient Mice," *PLoS ONE*, vol. 5, no. 8, pp. e12220, 2010.

[22]   J.S. Marshall, I. Leal-Berumen, L. Nielsen, M. Glibetic, and M. Jordana, "Interleukin (IL)-10 inhibits long-term IL-6 production but not preformed mediator release from rat peritoneal mast cells," *Journal of Clinical Investigation*, vol. 97, no. 4, pp. 1122–1128, 1996.

[23]   V. Supajatura, H. Ushio, a. Nakao, et al., "Protective Roles of Mast Cells Against Enterobacterial Infection Are Mediated by Toll-Like Receptor 4," *The Journal of Immunology*, vol. 167, no. 4, pp. 2250–2256, 2001.

[24]   J.S. Marshall, C.A. King, and J.D. McCurdy, "Mast cell cytokine and chemokine responses to bacterial and viral infection," *Curr Pharm Des*, vol. 9, no. 1, pp. 11–24, 2003.

[25]   S. Okumura, K. Yuki, R. Kobayashi, et al., "Hyperexpression of NOD2 in intestinal mast cells of Crohn's disease patients: Preferential expression of inflammatory cell-recruiting molecules via NOD2 in mast cells," *Clinical Immunology*, vol. 130, no. 2, pp. 175–185, 2009.

[26] Y.A. Mekori, and D.D. Metcalfe, "Mast cell − T cell interactions," *Journal of Allergy and Clinical Immunology*, vol. 104, no. 3, pp. 517–523, 1999.

[27] N.A. Nagalingam, C.J. Robinson, I.L. Bergin, et al., "The effects of intestinal microbial community structure on disease manifestation in IL-10$^{-/-}$ mice infected with Helicobacter hepaticus," *Microbiome*, vol. 1, no. 1, pp. 15, 2013.

[28] M.L. Hart, A.C. Ericsson, and C.L. Franklin, "Differing complex microbiota alter disease severity of the IL-10$^{-/-}$ mouse model of inflammatory bowel disease," *Frontiers in Microbiology*, vol. 8, no. MAY, pp. 1–15, 2017.

[29] M.C. Arrieta, K. Madsen, J. Doyle, and J. Meddings, "Reducing small intestinal permeability attenuates colitis in the IL10 gene-deficient mouse.," *Gut*, vol. 58, no. 1, pp. 41–48, 2009.

[30] H. Zhang, Y. Xue, H. Wang, et al., "Mast cell deficiency exacerbates inflammatory bowel symptoms in interleukin-10-deficient mice.," *World Journal of Gastroenterology : WJG*, vol. 20, no. 27, pp. 9106–15, 2014.

[31] S.H. Wright, J. Brown, P.A. Knight, E.M. Thornton, and H.R.P. Miller, "Transforming growth factor-b1 mediates coexpression of the integrin subunit a," *Clin Exp All*, vol. 31, pp. 315–324, 2002.

[32] T.D. Schmittgen, and K.J. Livak, "Analyzing real-time PCR data by the comparative CT method," *Nature Protocols*, vol. 3, no. 6, pp. 1101–1108, 2008.

[33] J. Kalesnikoff, and S.J. Galli, "New developments in mast cell biology.," *Nature Immunology*, vol. 9, no. 11, pp. 1215–23, 2008.

[34] B. Frossi, G. Gri, C. Tripodo, and C. Pucillo, "Exploring a regulatory role for mast cells: 'MCregs'?," *Trends in Immunology*, vol. 31, no. 3, pp. 97–102, 2010.

[35] C. Yu, A.B. Cantor, H. Yang, et al., "Targeted Deletion of a High-Affinity GATA-binding Site in the GATA-1 Promoter Leads to Selective Loss of the Eosinophil Lineage In Vivo," *The Journal of Experimental Medicine*, vol. 195, no. 11, pp. 1387–1395, 2002.

[36] S.K. Meurer, M. Ne??, S. Weiskirchen, et al., "Isolation of mature (Peritoneum-Derived) mast cells and immature (Bone Marrow- Derived) mast cell precursors from mice," *PLoS ONE*, vol. 11, no. 6, pp. 1–16, 2016.

[37] S. Wernersson, and G. Pejler, "Mast cell secretory granules: armed for battle.," *Nature Reviews. Immunology*, vol. 14, no. 7, pp. 478–94, 2014.

[38] Y. Kitamura, K. Oboki, and A. Ito, "Development of mast cells.," *Proceedings of the Japan Academy. Series B, Physical and Biological Sciences*, vol. 83, no. 6, pp. 164–74, 2007.

[39] C. Godfraind, J. Louahed, H. Faulkner, et al., "Intraepithelial infiltration by mast cells with both connective tissue-type and mucosal-type characteristics in gut, trachea, and kidneys of IL-9 transgenic mice.," *Journal of Immunology (Baltimore, Md. : 1950)*, vol. 160, no. 8, pp. 3989–3996, 1998.

[40] J.A. Fernández-Blanco, J. Estévez, T. Shea-Donohue, V. Martínez, and P. Vergara, "Changes in Epithelial Barrier Function in Response to Parasitic Infection: Implications for IBD Pathogenesis.," *Journal of Crohn's & Colitis*, vol. 9, no. 6, pp. 463–76, 2015.

[41] W. Xing, K.F. Austen, M.F. Gurish, and T.G. Jones, "Protease phenotype of constitutive connective tissue and of induced mucosal mast cells in mice is regulated by the tissue.," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 34, pp. 14210–14215, 2011.

[42] Y. Nakamura, N. Kambe, M. Saito, et al., "Mast cells mediate neutrophil recruitment and vascular leakage through the NLRP3 inflammasome in histamine-independent urticaria.," *The Journal of Experimental Medicine*, vol. 206, no. 5, pp. 1037–1046, 2009.

[43] I.D. Haidl, S.M. McAlpine, and J.S. Marshall, "Enhancement of mast cell IL-6 production by combined toll-like and nucleotide-binding oligomerization domain-like receptor activation," *International Archives of Allergy and Immunology*, vol. 154, no. 3, pp. 227–235, 2011.

[44] H. Sandig, and S. Bulfone-Paus, "TLR signaling in mast cells: Common and unique features," *Frontiers in Immunology*, vol. 3, no. JUL, pp. 1–13, 2012.

[45] M. Renshaw, J. Rockwell, C. Engleman, et al., "Cutting Edge: Impaired Toll-Like Receptor Expression and Function in Aging," *The Journal of Immunology*, vol. 169, no. 9, pp. 4697–4701, 2002.

[46] E.Z.M. da Silva, M.C. Jamur, and C. Oliver, Mast Cell Function: A New Vision of an Old Cell., 2014.

[47] S.H. Polukort, J. Rovatti, L. Carlson, et al., "IL-10 Enhances IgE-Mediated Mast Cell Responses and Is Essential for the Development of Experimental Food Allergy in IL-10–Deficient Mice," *The Journal of Immunology*, vol. 196, no. 12, pp. 4865–4876, 2016.

[48] J.D. Mccurdy, T. Lin, and J.S. Marshall, "Toll-like receptor 4-mediated activation of murine mast cells," vol. 70, no. December, pp. 977–984, 2001.

[49]   A. Ozinsky, D.M. Underhill, J.D. Fontenot, et al., "The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors," *Proceedings of the National Academy of Sciences*, vol. 97, no. 25, pp. 13766–13771, 2000.

[50] R. Lang, D. Patel, J.J. Morris, R.L. Rutschman, and P.J. Murray, "Shaping Gene Expression in Activated and Resting Primary Macrophages by IL-10," *The Journal of Immunology*, vol. 169, no. 5, pp. 2253–2263, 2002.

[51] E. Bijjiga, and A.T. Martino, "Interleukin 10 (IL-10) Regulatory Cytokine and its Clinical Consequences," *Journal of Clinical & Cellular Immunology*, pp. 1–6, 2011.

[52] J. Cyktor, and J. Turner, "Interleukin-10 and immunity against prokaryotic and

eukaryotic intracellular pathogens," *Infection and Immunity*, 2011.

[53]   G. Paul, V. Khare, and C. Gasche, "Inflamed gut mucosa: downstream of interleukin-10.," *European Journal of Clinical Investigation*, vol. 42, no. 1, pp. 95–109, 2012.

[54]   S.J. Galli, and M. Tsai, "Mast cells: Versatile regulators of inflammation, tissue remodeling, host defense and homeostasis," *Journal of Dermatology, The (Tokyo)*, vol. 49, no. 1, pp. 7–19, 2009.

CAPÍTULO 2

Il-10 Modulates Intestinal Microbiota and Antibiotic-Induced Dysbiosis in Mice

Roberto Riquelme-Neira,[1] Paola Lopez Colom,[2] Marina Ferrer Clotas,[1] Joan Antoni Fernández-Blanco[1] and Patrocinio Vergara[1]

[1] Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, 08193, Spain.

[2] Animal Nutrition and Welfare Service, Animal and Food Science Departament, Universitat Autònoma de Barcelona, 08193 Spain.

Abstract

Background: Inflammatory bowel disease (IBD) involves a complex interaction among environmental factors, host genetics and immune responses to gut microbiota. IL-10 deficient (IL-10$^{-/-}$) mice have been useful to study this interaction. However, changes in the intestinal immune system and microbiota associated to this model of IBD are far to be fully understood.

Methods: We studied effects of IL-10 deletion on caecal microbiota composition in 6 and 20-week-old mice, before and after the progression of colitis, and on antibiotic-induced dysbiosis. We also characterized alterations in intestinal immune system by assessing colonic expression of IL-1β, IL-6, TNFα, toll-like receptors (TLRs) 2, 4, 6, 7 and 8 and the production of luminal IgA.

Results: At 6 weeks of age, IL-10$^{-/-}$ mice presented the phyla Verrucomicrobia and a lower abundance of Rikenellaceae and Lachnospiraceae taxa without alterations in the immune response. In contrast, 20-week-old IL-10$^{-/-}$ mice showed the presence of phyla TM7, a lower expression of IL-1β, IL-6, TLR6, TLR7, TLR8 and increased levels of TNFα and IgA. Antibiotic therapy reduced microbial diversity and richness and partially attenuated the inflammatory response associated with IL-10 deletion, reducing TNFα expression.

Conclusions: Likely coinciding with the onset of inflammation, changes in gut microbiota and dysregulation of TLRs, TNFα and IgA would favor colitis development in IL-10$^{-/-}$ mice. In addition, IL-10 would modulate antibiotic-mediated alterations in microbiota and intestinal immune system response, suggesting that early microbiota intervention alters colitis progression in IBD.

Key Words: IBD, IL-10 deficient, TLR, IgA.

Introduction

Inflammatory bowel disease (IBD) comprises two major disorders, Crohn's disease (CD) and ulcerative colitis (UC). These are characterized by chronic, relapsing inflammation affecting the gastrointestinal (GI) tract [1,2]. Although, the precise etiology of IBD is not fully understood, it is widely accepted that its development involves a complex interaction between host genetics, environmental factors and aberrant immune responses against gut microbiota [3–6]. Indeed, there is evidence that an altered composition of intestinal microbiota contributes to the incidence of IBD [4,7].

Gut microbiota includes a large number of microorganisms of which the bacterial community is composed of at least 500 different bacterial species [8]. Through the analysis of intestinal communities using 16S ribosomal DNA (16S rDNA) sequencing, the dominant bacterial phyla in the mammalian intestine have been characterized [9]. These include Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Verrucomicrobia and Cyanobacteria both in humans and mice and Fusobacteria in humans but not in mice [9].

Intestinal microbiota contributes to the regulation of physiological processes such as digestion, epithelial function and immune system responses [2,8–11]. In consequence, deregulation of microbiota-host interactions could lead to the development of intestinal disorders, including IBD [2,10]. In that respect, an altered composition of the gut microbiota, with a lower diversity and an increased abundance of Enterobacteriaceae, is related to intestinal inflammation [1–3,5,6,12]. In addition, antibiotic therapy leads to an attenuation or prevention of colitis both in animal models and humans [5,13,14]. This could be related to direct effects of antibiotics on epithelial functions and immune responses but also to the ability of antibiotics to alter intestinal microbial communities (Hill & Artis 2010; Nagalingam et al. 2013; Grasa et al. 2015). That said, changes in the microbial composition associated with the development of intestinal inflammation and their modulation by antibiotic therapies is far from being fully understood.

Experimental animal models of intestinal inflammation such as interleukin (IL)-10 deficient mice (IL-10$^{-/-}$) have been crucial in our understanding of the pathogenesis of IBD [5]. They are particularly useful in separating the importance of interactions

between the immune system and microbiota. Indeed, IL-10^{-/-} mice maintained under germ-free conditions do not develop intestinal inflammation [6,7,15]. Meanwhile, when IL-10 deficient mice are raised under conventional conditions, these spontaneously develop chronic intestinal inflammation after 3 months of age [4,7,15,16]. These data corroborate that the composition of the gut microbiota is a key factor in the development of intestinal inflammation.

The interaction between the host and gut microorganisms is known to be mediated through pattern recognition receptors (PRRs). PRRs include toll-like receptors (TLRs), which recognize microbial-associated molecular patterns (MAMPs) and play a central role in the recognition of gut microbiota and the initiation of immune responses [11,17]. TLRs are expressed throughout the whole intestinal tract by a variety of cell types, including intestinal epithelial cells (IECs) and immune cells (mast cells, dendritic cells, macrophages, B cells and T cells). As a result of TLRs activation, cells release immune mediators, such as cytokines, to modulate inflammatory responses [11]. Interestingly, loss of function and changes in the expression of TLR2 and TLR4 modify the composition of the microbiota and are associated with IBD [17,18].

In parallel, immunoglobulin A (IgA) is another key modulator of host-microbiome interactions. IgA is the most abundant immunoglobulin on mucosal surfaces and contributes to intestinal homeostasis by excluding the penetration of microorganisms inhibiting bacterial motility and neutralizing toxins [12,19,20]. Furthermore, in intestinal inflammation related to IBD, IgA levels are increased [12,21].

Taking this into consideration, the aim of the present study was to characterize changes in the gut microbiota composition and host-microbiota interactions related to the IL-10-deficient mouse model of IBD. Firstly, we used Illumina-based sequencing of bacterial 16S rRNA to characterize differences in caecal microbiota between IL-10^{-/-} and wild-type mice at 6 and 20 weeks of age. In addition, we evaluated how IL-10 deficiency conditions the modulation of the microbiota induced by oral antibiotic therapy. Finally, we assessed the host response to changes in microbiota through the analysis of TLR expression and the production of cytokines and luminal IgA in the intestine.

Material and methods

Animals

Female B6.129P2-*Il10^{tm1Cgn}*/J (IL-10$^{-/-}$) and C57BL/6J wild type (WT) mice, 5-week-old upon arrival, were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Animals were housed in individual ventilated cages under conventional conditions in an environment under controlled temperature (20-22°C) and photoperiod (12:12 h light-dark cycle). Mice had unrestricted access to tap water and Teklad Global 14% protein rodent maintenance diet (2014; ENVIGO CRS S.A., Cambridgeshire, UK). Mice were allowed to acclimatize to these conditions for at least 5 days prior to any experimentation. All procedures were approved by the Ethical Committee of the Universitat Autònoma de Barcelona and the Generalitat de Catalunya (protocols 2773 and 8814, respectively).

Experimental Design

In a first experiment, mice were randomly divided into four experimental groups: (i) 6-week-old WT (n=8; WT6); (ii) 6-week-old IL-10$^{-/-}$ (n=8; IL-10$^{-/-}$6); (iii) 20-week-old WT (n=12; WT20); and (iv) 20-week-old IL-10$^{-/-}$ (n=10; IL-10$^{-/-}$20).

In a second experiment, mice were randomly divided into four experimental groups: (i) vehicle-treated wild type mice (WT VEHICLE); (ii) vehicle-treated IL-10$^{-/-}$ mice (IL-10$^{-/-}$ VEHICLE); (iii) antibiotic-treated wild type mice (WT ATB); and (iv) antibiotic-treated IL-10$^{-/-}$ mice (IL-10$^{-/-}$ ATB). Animals were treated with vehicle or antibiotics for 28 days, as described below. At the end, animals were euthanized proceeding to sample collection.

Antibiotic therapy

Antibiotics were administered to conscious mice by oral gavage as previously described [22,23] with some modifications. Antibiotics were purchased from Sigma-Aldrich CO. (St. Louis, USA) and diluted in deionized water. Two hundred microliters of an antibiotic cocktail containing ampicillin (1 g/L), vancomycin (500 mg/L), neomycin trisulfate (1 g/L) and metronidazole (1 g/L) or water (vehicle-treated) were administered

daily for 28 days. A fresh antibiotic concoction was mixed every three days. Mice were weighed twice a week and the dose administered was adjusted accordingly.

Samples collection

At 6 or 20 weeks or once the antibiotics therapy had finished (11 weeks), mice were deeply anesthetized with isoflurane (Isoflo; Esteve, Barcelona, Spain) and euthanatized by exsanguination through intracardiac puncture followed by cervical dislocation. Afterwards, a laparotomy was performed. Small intestine samples were used to obtain the gut wash (supernatants obtained after the incubation and centrifugation of intestinal tissue) to quantify luminal IgA, as previously described [24]. Caecal content and proximal colon tissue samples were collected and then immediately frozen in liquid nitrogen. Samples were stored at -80°C until analysis.

RNA extraction and reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from frozen tissue samples using RNeasy® Mini Kit (Qiagen, Hilden, Germany). Thereafter, a two-step quantitative real-time PCR (RT-qPCR) was performed. RNA was quantified by Nanodrop (NanoDrop Technologies, Rockland, DE, USA) and 1 μg of RNA was reverse-transcribed in a 20-μl reaction volume for cDNA synthesis using iScript™ cDNA Synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The temperature profile for reverse transcription was 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min.

Validated TaqMan® gene expression assays with hydrolysis probes (Applied Biosystems, Foster City, CA, USA) were used for the following: interleukin 1 beta (IL-1β; Mm00434228_m1); interleukin 6 (IL-6; Mm00446190_m1): tumor necrosis factor a (TNFα; m00443258_m1) and toll-like receptors TLR2 (Mm00442346_m); TLR4 (Mm00445273_m1); TLR6 (Mm02529782_s1); TLR7 (Mm00446590_m1); TLR8 (Mm04209873_m1) and reference genes beta-2 microglobulin (B2m; Mm00437762_m1); and actin beta (Actb; Mm00607939_s1)

PCR reaction mixtures were transferred to clear 384-well reaction plates (Bio-Rad Laboratories, Inc., Hercules, CA, USA); sealed by adhesives and C1000 Touch™ Thermal Cycler platform (Bio-Rad Laboratories, Inc.) for 40 cycles (95°C for 15s, 60°C

for 1 min). Fluorescence signals measured during amplification were processed after amplification. Bio-Rad CFX Manager 2.1 software was used to obtain the cycle threshold (CT) for each sample. All samples, as well as the negative controls, were assayed in triplicates and data were analyzed by the comparative Ct method [$2^{-\Delta\Delta CT}$] with the 6 week-old wild type or the vehicle-treated wild type groups serving as the calibrators [25]. Actb and B2m were tested as reference genes. B2m expression levels were used for normalizing the mRNA levels of the target genes because of their constancy across the different experimental groups.

DNA extraction and 16S rRNA gene amplicon sequencing

Total DNA was isolated from frozen caecal content using QIAamp® DNA Stool Mini Kit (Qiagen), following the manufacturer's instructions.

The quantity of DNA was assessed using a NanoDrop (ND-1000 spectrophotometer, Nano-Drop Technologies, Wilmington, DE, USA).

The amplicon libraries were prepared using the Nextera XT index kits 16S V3-V4 Amplicon-Seq kit. Metagenomic DNA (5 ng/μl) was used as template for the first PCR (PCR-I) amplification, which amplified the variable V3 and V4 of 16S rRNA region, with the following conditions: initial denaturation at 95°C for 3 min plus 25 cycles (95°C for 30s, 55°C for 30s, 72°C for 30s, 72°C for 5 min, and hold at 4 °C). The PCR product was purified from free primers and primer dimer species with AMPure XP beads and eluted with nuclease free water. Five microliters of resuspended PCR product were used as a template for the second PCR (PCR-II), which added the Illumina sequencing adapters and dual-index barcodes to the amplicon target. PCR-II was performed using the following conditions: initial denaturation at 95°C for 3 min plus 8 cycles (95°C for 30s, 55°C for 30s, 72°C at 30s, 72°C for 5 min, and hold at 4°C). The final product was cleaned up using the AMPure XP Beads and the library was analyzed using the Bioanalyzer DNA 1000 (Agilent Technologies, Santa Clara, CA, USA). The concentrated final libraries were diluted using Resuspension Buffer. Five microliters of aliquoted diluted DNA from each library were mixed for pooling libraries. Pooled libraries were denatured with NaOH, diluted with hybridization buffer and then heat denatured before MiSeq sequencing. Five % PhiX served as an internal control. For sequencing on the MiSeq instrument, the generated libraries were placed in the reagent

cartridge and loaded on the instrument along with the flow cell. With the V3-V4 Miseq sequencing kit, 2 x 250 bp paired-end reads were obtained.

Bioinformatic analysis

The sequence reads generated by the 16S rRNA were processed on Quantitative Insights Into Microbial Ecology (QIIME) version 1.9.1 [26]. For the 16S rRNA amplicon analysis, MiSeq read files were paired using *join_paired_ends.py* in QIIME. Quality filtering of MiSeq reads was performed using *split_libraries_fastq.py* also in QIIME allowing the maximum unacceptable Phred quality score of Q20. Resulting reads were clustered to operational taxonomic units (OTUs) using uclust [27] with 97% sequence similarity against bacterial 16S GreenGenes version 13.8 reference database [28] by subsampling pick open reference method (Rideout et al., 2014) with *pick_open_reference_otus.py* script and the percent of failure sequences to include in the subsample to cluster *de novo* set at 0.1. Chimeric sequences were removed via *identify_chimeric_seqs.py* with ChimeraSlayer [29] as default. Further filtering was performed using *filter_otus_from_otu_table.py* setting the minimum total OTU observation count at 0.005% to discard those OTUs with a number of sequences <0.005% of the total number of sequences as recommended by Bokulich et al. [30].

Biostatistics

Sequencing analysis was performed in open source R version 3.4.0. Support for QIIME in R was achieved through the phyloseq package [31]. The package includes functions for importing data from biom format files.

Absolute OTU abundances were used to calculate the alpha diversity of microorganisms per each sample [32,33] and the richness (number of OTUs detected per sample). To calculate the alpha diversity, the diversity() function of the vegan package [34] was used and computed with Shannon [35], Simpson [36] and Simpson inverse indices. To compare any differential effect between groups, an ANOVA analysis was performed for alpha diversity by anova() function in stats package [37]. To evaluate richness, a rarefaction analysis was performed with rarefy() function in vegan package [34] based on the minimum number of reads/sample. The beta diversity, which represents the differences in diversities across samples [32,33], was based on the Whittaker index.

Measurements were calculated using the betadisper() function of the vegan package [34] using the relative OUT abundances.

Differential phyla, family and genus abundance analysis were performed with a deseq2 package [38] based on raw counts. To correct false discovery rate associated with multiple testing, DESeq2 uses the Benjamini-Hochberg (BH) adjustment [39]. Taxon abundances are presented as relative abundance mean ± standard error of the mean (SEM).

Deseq2 package [38] was used to cluster the heatmap using normalized and log-transformed ($log2(n+1)$) of the OTU raw counts followed by *Post-hoc* statistical testing. Relative abundances of bacterial taxa were used to plot taxon abundances. Bars chart were generated with Microsoft Excel (Microsoft Corp., Redmond, WA, USA)

Data from relative gene expression and IgA quantification are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 6.0 software (Graph Prism Software Inc., La Jolla, CA, USA). Comparisons between multiple groups were performed using one-way ANOVA, two-way ANOVA or Kruskal-Wallis test, followed, when necessary, by a Tukey's multiple-comparison test. *P*-values <0.05 were considered statistically significant.

Results

Colonic expression of inflammatory markers and toll-like receptors in wild-type and IL-10$^{-/-}$ mice at 6 and 20 weeks of age

No differences were found for colonic mRNA expression of cytokines or TLRs between 6-week-old wild-type and IL-10 knockout mice (Fig. 1). On the other hand, at 20 weeks of age, IL-10$^{-/-}$ mice showed a lower expression of IL-1β, IL-6, TLR6, TLR7 and TLR8 and an overexpression of TNFα, while TLR2 and TLR4 levels of expression were similar to those observed in control animals (Fig. 1).
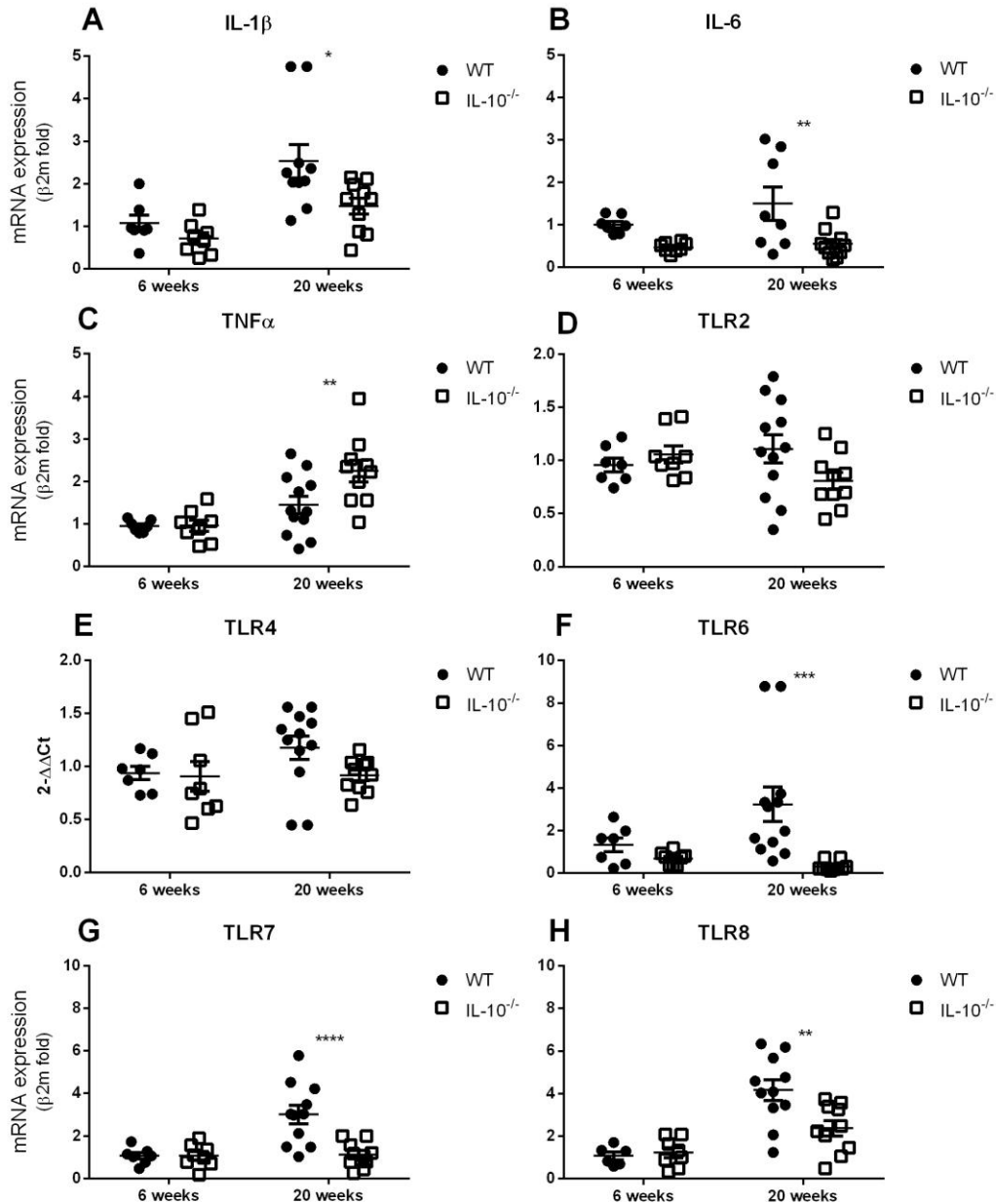
Figure 1. Effects of IL-10 deletion and age on cytokines and toll-like receptors (TLRs) gene expression in the colon. Relative expression of IL-1β (A), IL-6 (B), TNFα (C), TLR2 (D), TLR4 (E), TLR6 (F), TLR7 (G) and TLR8 (H) mRNA in colonic samples from wild-type (WT; filled circles) and IL-10$^{-/-}$ (empty squares) mice at 6 and 20 weeks of age measured by RT-qPCR. Dots represent individual values and vertical lines indicate standard error of the mean. N= 8-12 mice per group. *P<0.05; **P<0.01; ***P<0.001 compared to WT mice (2-way ANOVA with Tukey's post-hoc test).

Effects of antibiotic therapy on colonic expression of inflammatory markers and toll-like receptors in wild-type and IL-10$^{-/-}$ mice

We observed that antibiotic therapy reduced colonic mRNA levels of expression of all cytokines and TLRs analyzed in both wild-type and IL-10$^{-/-}$ mice (Two-way ANOVA; $P < 0.05$; Fig. 2).

Regarding vehicle-treated mice, we found a significantly lower expression of IL-1β and TLR4 in IL-10$^{-/-}$ than in wild-type mice (Fig. 2). Similarly, after antibiotic therapy, IL-10 deletion was related to reduced levels of expression of TLR4 (Fig. 2).
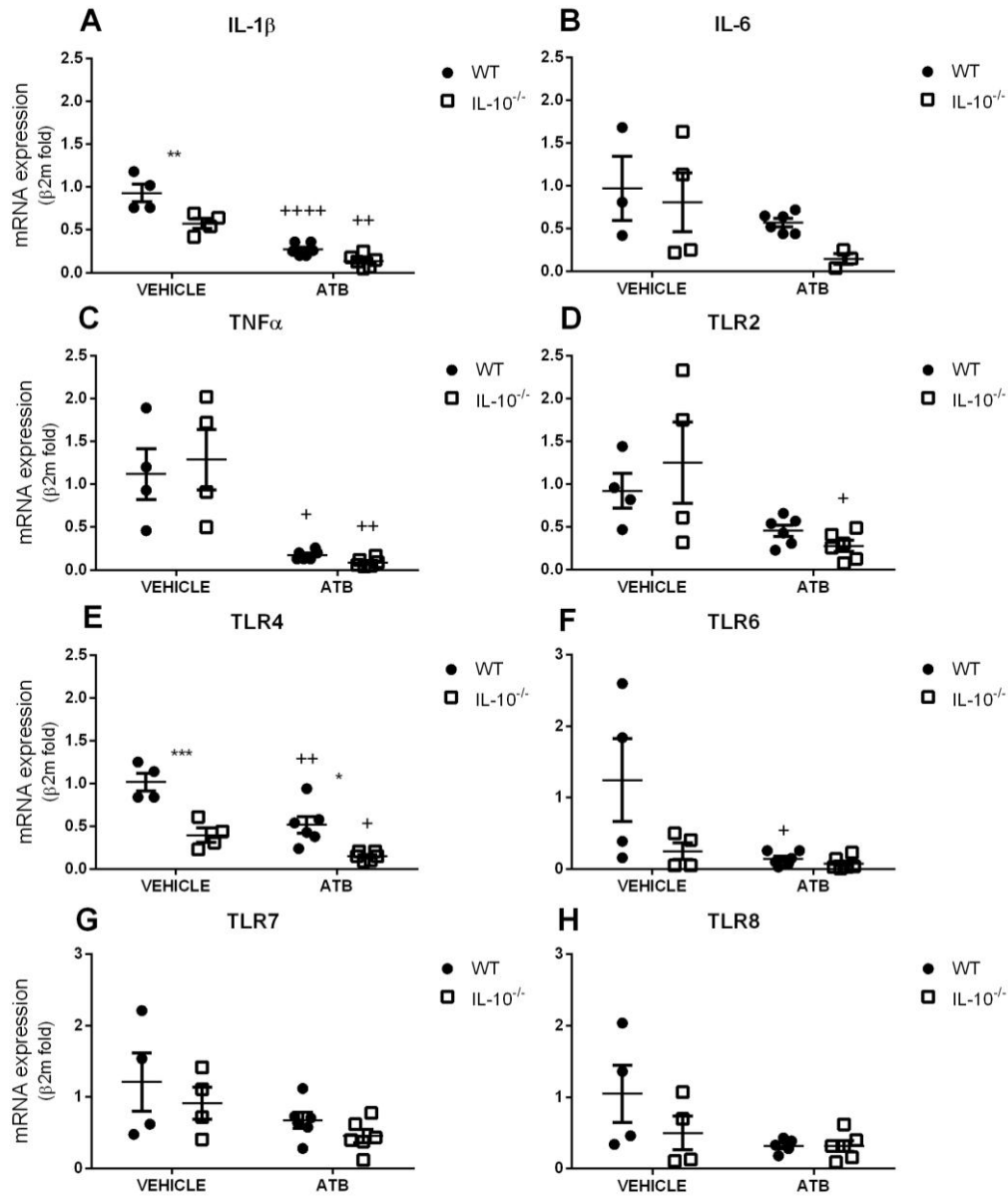
Figure 2. Effects of antibiotic therapy on cytokines and toll-like receptors (TLRs) gene expression in the colon in presence and absence of IL-10. Relative expression of IL-1β (A), IL-6 (B), TNFα (C), TLR2 (D), TLR4 (E), TLR6 (F), TLR7 (G) and TLR8 (H) mRNA in colonic samples from wild-type (WT; filled circles) and IL-10$^{-/-}$ (empty squares) mice treated or not with antibiotics (ATB). Dots represent individual values and vertical lines indicate standard error of the mean. N= 8-12 mice per group. *P<0.05; **P<0.01; ***P<0.001 compared to WT mice; +P<0.05; ++P<0.01; +++P<0.001; ++++ P<0.0001 compared vehicle-treated animals with the same genotype (2-way ANOVA with Tukey's post-hoc test).

Effects of age and antibiotic therapy on luminal concentration of immunoglobulin A (IgA) in wild-type and IL-10$^{-/-}$ mice

No difference in IgA concentration in intestinal lavages was detected between both genotypes at 6 weeks of age (Fig. 3A). Interestingly, 20-week-old IL-10$^{-/-}$ mice showed a higher concentration of IgA than age-matched wild-type animals (Fig. 3A). Concerning antibiotic therapy, taken as a whole, it trended to reduce IgA levels (Two-way ANOVA; P= 0.053; Fig. 3B). However, this effect did not have any relationship with IL-10 expression as no differences were observed between both genotypes (Fig. 3B).
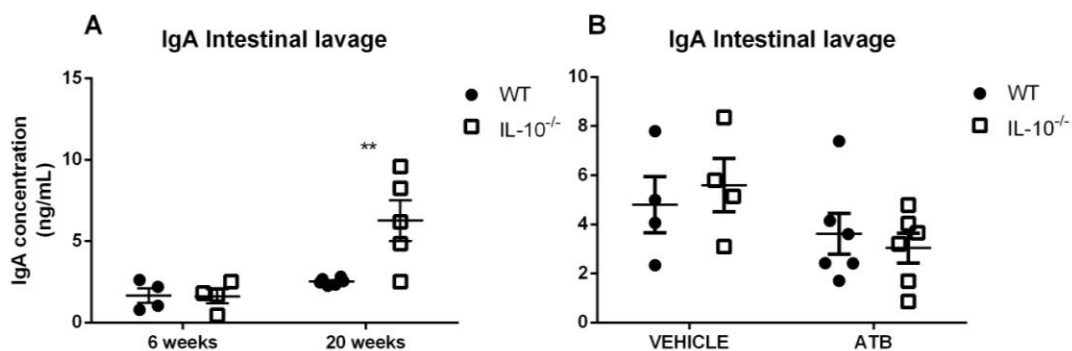
Figure 3. Effects of IL-10 deletion and antibiotic therapy on luminal immunoglobulin A (IgA) concentration. IgA concentration in small intestine lavages obtained from wild-type (WT; filled circles) and IL-10$^{-/-}$ (empty squares) mice at 6 and 20 weeks of age (A) and treated or not with antibiotics (ATB; B). Dots represent individual values and vertical lines indicate standard error of the mean. N= 4-6 mice per group. **P<0.01 compared to WT mice (2-way ANOVA with Tukey's post-hoc test).

Microbiota diversity and composition at phylum, family and genus levels in caecal samples from wild-type and IL-10$^{-/-}$ mice at 6 and 20 weeks of age.

We generated a total of 1,260,562 sequences. After a series of quality checks, which included removal of low-quality sequences and PCR chimeras, 1,068,834 (84.8%) sequences were retained. The number of reads per sample was an average of 28,887 ± 12,258 and a total of 540 OTUs were detected. Despite the variability in the number of reads, the rarefaction curves reported a uniform distribution of the abundance of OTUs among groups and reached the plateau phase meaning a saturated richness (data not shown).

To observe the variety for each individual population, we measured the alpha diversity. The four experimental groups did not show differences in alpha diversity as measured by the Shannon, Simpson and invSimpson index (P> 0.10; Fig. S1). Therefore, microbiota diversity was similar regardless of age or IL-10 expression relating to their genotype. Beta diversity was not either significantly different between groups (P= 0.196; Fig. S2).

Regarding taxon analysis, seven phyla, 27 families and 28 genera were detected among most of the samples. Among the seven phyla, four were the most abundant ($\geq$ 1% all taxa) in wild-type mice at 6 weeks, including Firmicutes, Bacteroidetes, Tenericutes and Proteobacteria (Fig. 4). In 20-week-old wild-type mice and IL-10$^{-/-}$ animals at both 6 and 20 weeks, it was also possible to observe the phyla Verrucomicrobia as highly represented. On the other hand, Actinobacteria was present in all groups as a low-abundant phylum (< 1% of community) and TM7 was only detected in 20-week-old IL-10 knockouts (5/10 animals).
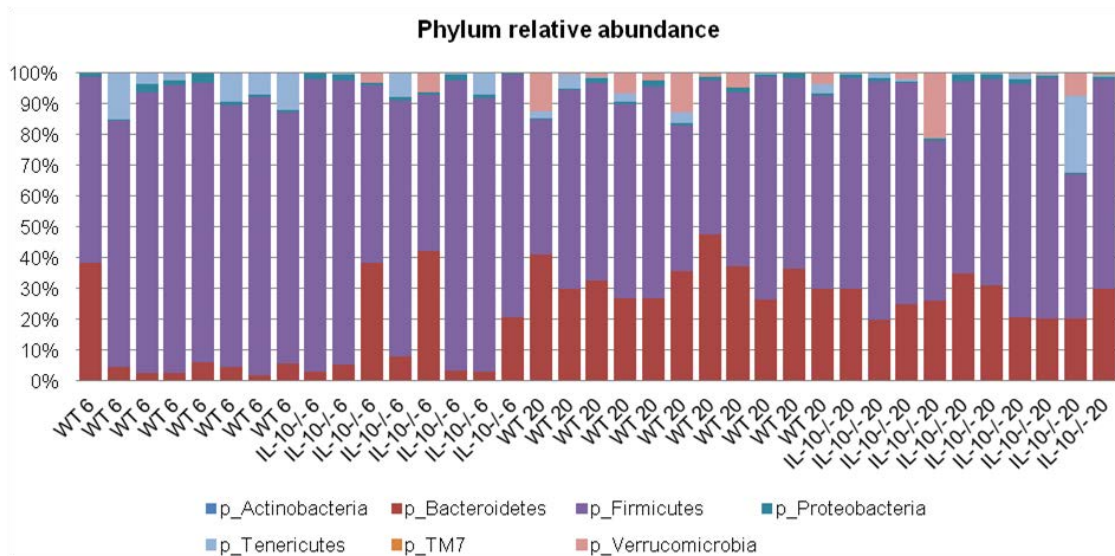
Figure 4. Effects of IL-10 deletion and age on relative abundance of caecal microbiota at taxonomic level of phylum. Bar charts show the bacterial composition of the caecal microbial community in wild-type (WT) and IL-10$^{-/-}$ mice at 6 and 20 weeks of age, annotated to the taxonomic level of phylum. N = 4-6 mice per group. Legend of phylum is shown at the bottom.

The differential taxon abundance analysis presented did not show significant changes in bacterial phyla of IL-10$^{-/-}$ mice between 6 and 20 weeks of age (Table 1). In the wild-type group, we detected a significant decrease in the levels of Firmicutes (P< 0.001; -24.4%) and Proteobacteria (P= 0.003; -0.4%), whereas an increase of Bacteroidetes (P=

0.001; +25%) and Verrucomicrobia (P< 0.001; +4%) at 20 weeks compared to 6 weeks of age. When both genotypes were compared, we observed a higher level of Verrucomicrobia (P< 0.001; +1%) in 6-week-old IL-10$^{-/-}$ mice than in age-matched wild-type animals while no differences were detected at 20 weeks of age (Table 1; only significant differences are showed).

At family level, we observed an increase in the level of Rikenellaceae (P< 0.001; +5%) in IL-10$^{-/-}$ mice at 20 weeks compared to 6 weeks of age. In contrast we observed increased levels of Rikenellaceae (P= 0.008; +3.75%), Verrucomicrobiaceae (P< 0.001; +4%) and S24-7 (P= 0.011; +22%) in wild-type mice from 6 weeks to 20 weeks of age. Comparing both genotypes at week 6, we detected a higher level of Verrucomicrobiaceae (P< 0.001; +1%) together with lower abundances of Rikenellaceae (P< 0.001; -0.62%) and Lachnospiraceae (P= 0.005; -5.64%) in IL-10$^{-/-}$ than in wild-type mice. In line with the results observed at phylum level, no significant differences were found between 20-week-old wild-type and knockout mice (Table 1, only significant differences are showed).

Even though around 75% of the genera was classified as unknown in all groups, we detected that 20-week-old IL-10$^{-/-}$ mice had an increase in the genus *Ruminococcus* (P= 0.006) and *Dorea* (P= 0.026) compared to 6-week-old knockout animals. We also observed changes in wild-type mice, which at 20 weeks showed an increase of *Akkermansia* (P< 0.001) and the candidate genus *Ruminococcus* (P= 0.001) compared to week 6. Interestingly, 6-week-old IL-10 deficient mice showed a higher presence of *Akkermansia* (P< 0.001) than age-matched wild-type mice. However, this difference was not maintained at 20 weeks (Table 1, only significant differences are showed).

Table 1. Effects of IL-10 deletion and age on differential abundance in specific taxa according to genotype and week comparisons (DESeq2)

TAXA		WT20 relative to WT6			IL10$^{-/-}$ 20 relative to IL10$^{-/-}$ 6			IL10$^{-/-}$ 6 relative to WT6		IL10$^{-/-}$ 20 relative to WT20	
PHYLUM		Log$_2$ fold change	*p*- adj		Log$_2$ fold change	*p*- adj		Log$_2$ fold change	*p*- adj	Log$_2$ fold change	*p*- adj
Actinobacteria		-0,426	0,668		0,529	0,482		-0,345	0,773	0,610	0,688
Bacteroidetes	↑	1,895	**0,001**		0,458	0,469		0,882	0,194	-0,555	0,687
Firmicutes	↓	-0,615	**0,0002**		-0,229	0,401		-0,330	0,150	0,057	0,874
Proteobacteria	↓	-0,552	**0,003**		-0,246	0,401		-0,279	0,194	0,027	0,874
Tenericutes		-1,999	0,113		1,250	0,401		-2,120	0,194	1,129	0,687
TM7		0,250	0,949		6,438	0,401		-0,053	0,990	6,135	0,623
Verrucomicrobia	↑	12,675	**2,65E-15**		1,500	0,401	↑	10,864	**3,19E-10**	-0,311	0,874
FAMILY											
Lachnospiraceae		-1,288	0,074		1,177	0,207	↓	-1,896	**0,005**	0,569	0,697
Rikenellaceae	↑	3,994	**0,008**	↑	10,937	**2,13E-15**	↓	-7,010	**4,29E-06**	-0,068	0,981
S24-7	↑	1,979	**0,011**		0,203	0,892		0,938	0,589	-0,838	0,554
Verrucomicrobiaceae	↑	12,890	**1,82E-15**		1,568	0,513	↑	10,751	**1,37E-09**	-0,572	0,967
GENUS											
[Ruminococcus]	↑	2,464	**0,001**		1,243	0,257		0,079	0,986	-1,142	0,347
Akkermansia	↑	12,734	**4,39E-15**		1,541	0,613	↑	10,896	**7,67E-10**	-0,298	0,985
Dorea		-0,689	0,858	↑	3,550	**0,026**		-2,283	0,540	1,955	0,347
Ruminococcus		0,844	0,329	↑	1,609	**0,006**		-0,748	0,596	0,017	0,985

P-value adj and Log$_2$ fold change are shown. Bold face values indicate statistical significance (P< 0.05). Brackets indicate putative taxonomy based upon phylogenetic placement as given in the Greengenes taxonomy. padj is the Benjamini–Hochberg adjusted P value. ↑upwards arrow indicate increase, ↓downwards arrow indicate decrease. WT6: 6-week-old wild-type mice; WT20: 20-week-old wild-type mice; IL10$^{-/-}$ 6: 6-week-old IL-10 deficient mice; IL10$^{-/-}$ 20: 20-week-old IL-10 deficient mice.

Effects of antibiotic therapy on microbiota diversity and composition in caecal samples from wild-type and IL-10$^{-/-}$ mice

We generated a total of 354,026 sequences. After a series of quality checks, which included removal of low-quality sequences and of PCR chimeras, 299,116 (84.5%) sequences were retained. The numbers of reads per sample was an average of 14,956 ± 8,794 and a total of 506 OTUs were detected. Despite the variability in the number of reads, the rarefaction curves reported a uniform distribution of the abundance of OTUs between both treatments and reached the plateau phase meaning a saturated richness (data not shown).

Alpha diversity differed significantly among experimental groups as measured by Shannon, Simpson and inverse Simpson indices (P< 0.001). Both IL-10$^{-/-}$ and wild-type

mice treated with antibiotics showed lower alpha diversity than animals that received the vehicle (Fig 5). In particular, antibiotic-treated wild-type mice presented significantly lower diversity than the rest of the groups. Species abundance distribution was uneven amongst experimental groups. Beta diversity was significantly different between groups (P<0.001) (Fig 6). Particularly, whereas beta diversities of vehicle-treated IL-10^{-/-} and wild-type mice remained similar, antibiotic-treated wild-type mice showed higher beta diversity (i.e. heterogeneity) than antibiotic-treated IL-10^{-/-} mice.
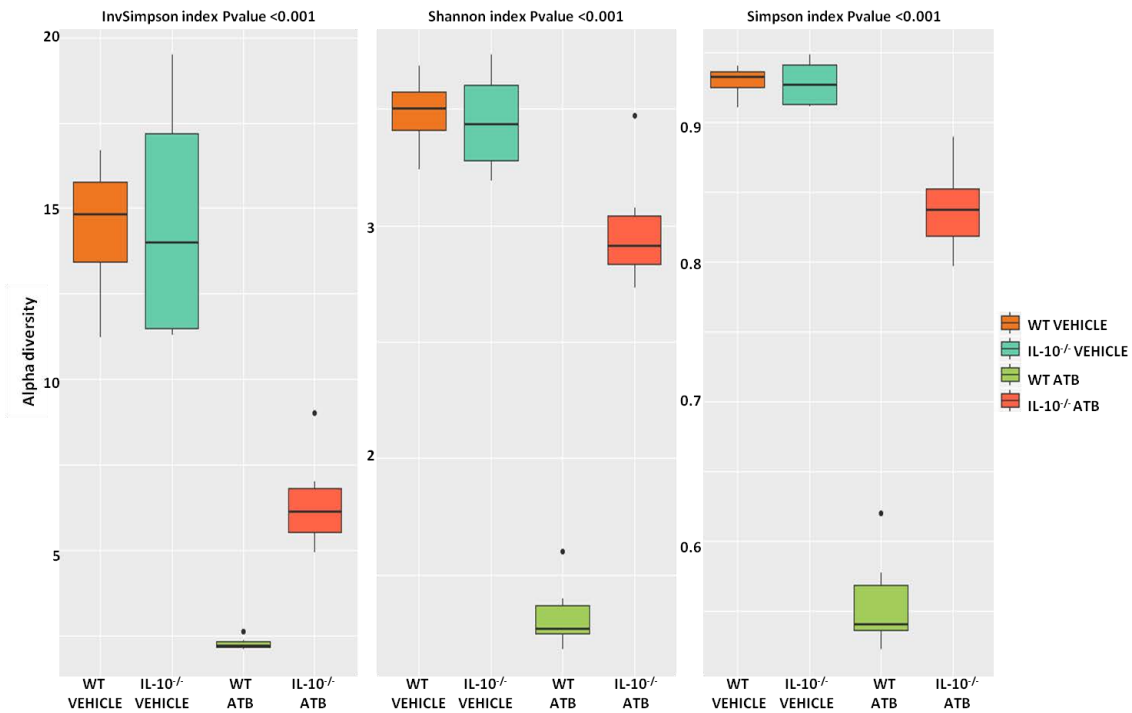
Figure 5. Effects of antibiotic therapy on caecal microbiota alpha diversity in wild-type (WT) and IL-10^{-/-} mice. Shannon, Simpson and Inv-Simpson from WT and IL-10^{-/-} mice treated with antibiotics or vehicle are plotted as interquarile range with median for each experimental group. N= 4-6 mice per group (one-way ANOVA with Tukey's post-hoc test).
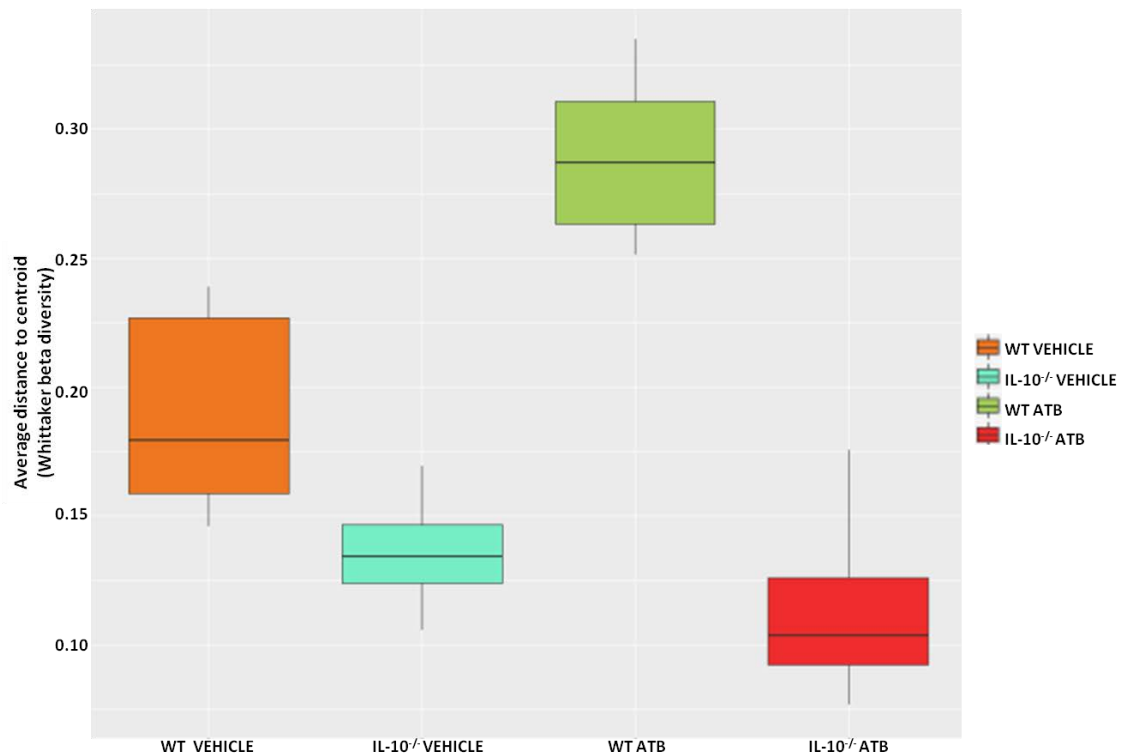
Figure 6. Effects of antibiotic therapy on caecal microbiota beta diversity in wild-type (WT) and IL-10$^{-/-}$ mice. Whittaker distance to centroid from WT and IL-10$^{-/-}$ mice treated with antibiotics (ATB) or vehicle is plotted as interquarile range with median for each experimental group. N= 4-6 mice per group. Labeled groups without a common letter differ P<0.05 (one-way ANOVA with Tukey's post-hoc test).

Regarding taxon analysis, 8 phyla, 34 families and 51 genera were detected among most of the samples. The main changes in relative abundances of phyla were induced by antibiotic therapy (Fig. 7). Regarding wild-type mice, we found significant changes associated with the treatment with antibiotics in almost all phyla. In particular, we detected a large reduction in Bacteroidetes (P< 0.001; -34%), Tenericutes (P< 0.001; -11%) and Verrucomicrobia (P= 0.003; -3.2%) and an increase in Cyanobacteria (P< 0.001; +0.05), Actinobacteria (P< 0.001; -0.004%) and overall in Proteobacteria (P< 0.001; +94) in antibiotic-treated animals. In IL-10-deficient mice, antibiotic therapy induced a high reduction of Bacteroidetes (P< 0.001; -24%) and Verrucomicrobia (P< 0.001; -12%), along with a greater abundance of Proteobacteria (P< 0.001; +33%), Actinobacteria (P< 0.001; +0.72%) and Cyanobacteria (P< 0.001; +0.46%). We also detected that in vehicle-treated mice, IL-10 deficiency was related to a lower abundance of Tenericutes phylum (P= 0.005). Notably, when subjected to antibiotic therapy, IL-10-

102

deficient animals showed lower levels of Proteobacteria than wild-type mice (P< 0.001; -60%) (Table 2).
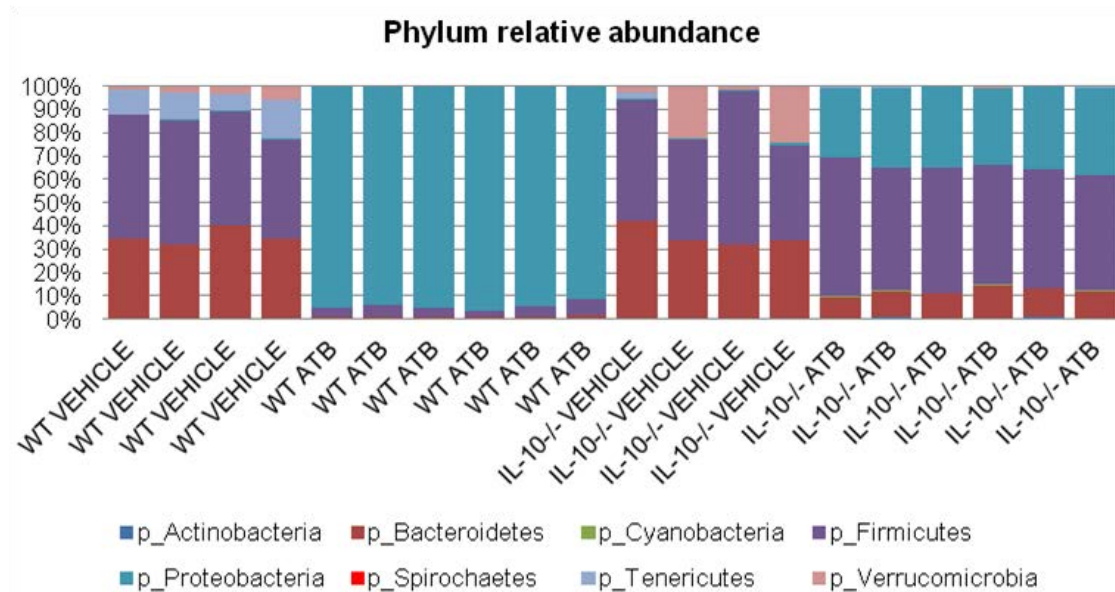
Figure 7. Effects of antibiotic therapy on relative abundance of caecal microbiota at taxonomic level of phylum. Bar charts show the bacterial composition of the caecal microbial community in wild-type (WT) and IL-10$^{-/-}$ mice treated with antibiotics (ATB) or vehicle, annotated to the taxonomic level of phylum. N = 4-6 mice per group. Legend of phylum is shown at the bottom.

In relation to the family level, 34 families were identified of which 18 with the most significant differences among the four groups analyzed are presented in Table 2. It is interesting to note that, as previously described for the phylum level, the main differences were induced by antibiotic therapy. In wild-type mice treated with antibiotics, we observed a reduction in unknown families belonging to the phylum Bacteroidetes (-32%), as well as Anaeroplasmataceae (-11.5%), Bifidobacteriaceae (-0.04%), Dehalobacteriaceae (-0.24%), Lachnospiraceae (-1.96%), Rikenellaceae (-7.53%), Ruminococcaceae (-13%), S24-7 (-27%), Turicibacteraceae (-0.52%) and Verrucomicrobiaceae (-3.26%) (P< 0.001). In contrast, bacteria belonging to the family candidate Weeksellaceae (phylum Bacteroidetes; +0.38%), Campylobacteraceae (+0.02%), Clostridiaceae (+2.5%), Enterobacteriaceae (+92%), Moraxellaceae (+1.5%), Prevotellaceae (+0.26%) and Streptococcaceae (+0.3%) were increased (P< 0.05) compared with vehicle-treated wild-type mice. At the same time, antibiotic therapy induced in IL-10-deficient mice a reduction in the same taxa as in wild-type animals with the exception of Rikenellaceae and Turicibacteraceae (P> 0.05). In addition, and in

contrast to wild-type animals, IL-10 deficient mice treated with antibiotics showed a reduction in the Enterobacteriaceae (-0.15%) and Erysipelotrichaceae (-0.1%) groups. Furthermore, the Weeksellaceae (candidate), Clostridiaceae and Moraxellaceae families were significantly enhanced in antibiotic-treated IL-10$^{-/-}$ mice (P< 0.05; +5.57%, +39.8%, +26.2% respectively). Comparing vehicle-treated animals from both genotypes, we observed a higher abundance of Enterobacteriaceae (P= 0.001; +0.2%) and lower abundances of Rikenellaceae (-7.54%) and Turicibacteraceae (P< 0.001; -0.52%) in the IL-10 knockout group. However, when animals were subjected to antibiotic therapy, Enterobacteriaceae (-92%), Prevotellaceae (-2.8%) and Turicibacteraceae (-0.001%) families were diminished in IL-10-deficient mice (P< 0.05).

Finally, 51 genera were identified with significant differences in 15 of them (Table 2). Again, the major effects on microbiota populations shifts were induced by antibiotic therapy. In wild-type mice, antibiotics reduced the abundance of *Akkermansia, Anaeroplasma, Bifidobacterium, Coprococcus, Dehalobacterium, Oscillospira, Ruminococcus, Turicibacter, Ruminococcus* (candidate) and unknown genera (P< 0.001). Antibiotic therapy also resulted in a greater presence of *Clostridium* and *Erwinia* (P< 0.001). Likewise, the study of antibiotic-related effects on IL-10-deficient mice displayed a similar pattern but with additional decreases in *Acinetobacter* and *Allobaculum* genera (P< 0.01) and no incidence in the genera *Erwinia* or *Turicibacter* (P> 0.10). In line with these results, when comparing the effect of antibiotic therapy between both genotypes, few differences were detected, showing in IL-10 knockout mice lower levels of *Erwinia*, *Turicibacter* and one unknown genera (P< 0.05) and higher levels of *Actinobacillus* genus (P= 0.040).

Table 2. Effects of antibiotic and IL-10 on differential abundance in specific taxa according to antibiotic therapy and genotype comparisons (DESeq2)

TAXA		IL10$^{-/-}$ VEHICLE relative to WT VEHICLE			WT ATB relative to WT VEHICLE			IL10$^{-/-}$ ATB relative to IL10$^{-/-}$ VEHICLE			IL10$^{-/-}$ ATB relative to WT ATB	
PHYLUM		Log$_2$ fold change	*p*-adj		Log$_2$ fold change	*p*-adj		Log$_2$ fold change	*p*-adj		Log$_2$ fold change	*p*-adj
Actinobacteria		-0,808	0,443	↑	3,409	**2,00E-09**	↑	4,591	**1,33E-18**		0,374	0,706
Bacteroidetes		0,019	1,000	↓	-1,460	**2,37E-13**	↓	-1,782	**3,20E-21**		-0,303	0,357
Cyanobacteria		0,789	0,655	↑	6,546	**6,58E-14**	↑	5,439	**3,11E-13**		-0,318	0,785
Firmicutes		0,000	1,000		-0,010	0,774		0,000	1,000		0,010	0,881
Proteobacteria		0,426	0,457	↑	10,863	**7,50E-251**	↑	5,256	**4,47E-60**	↓	-5,181	**7,74E-75**
Spirochaetes		0,000	1,000		3,669	0,059		3,731	0,059		0,083	0,963
Tenericutes	↓	-3,876	**0,005**	↓	-5,714	**9,12E-07**		-0,908	0,443		0,931	0,706
Verrucomicrobia		1,977	0,097	↓	-2,669	**0,003**	↓	-5,409	**4,40E-11**		-0,763	0,706
FAMILY												
unknown		-0,063	0,957	↓	-8,138	**6,40E-68**	↓	-8,773	**1,32E-83**		-0,698	0,406
[Weeksellaceae]		-0,187	0,957	↑	0,939	**7,32E-05**	↑	1,024	**3,73E-05**		-0,102	0,792
Anaeroplasmataceae		-4,014	0,078	↓	-13,921	**6,36E-17**	↓	-7,836	**1,17E-07**		2,071	0,584
Bifidobacteriaceae		-1,729	0,644	↓	-5,306	**4,19E-05**	↓	-4,851	**2,38E-05**		-1,274	0,730
Campylobacteraceae		1,688	0,890	↑	2,910	**0,034**	↑	0,437	0,807		-0,785	0,729
Clostridiaceae		-0,058	0,957	↑	1,089	**2,49E-09**	↑	1,137	**3,99E-10**		-0,010	0,986
Dehalobacteriaceae		1,216	0,890	↓	-6,492	**1,16E-08**	↓	-10,369	**5,88E-25**		-2,661	0,108
Enterobacteriaceae	↑	5,554	**0,001**	↑	12,946	**3,12E-23**	↓	-7,268	**9,21E-11**	↓	-14,660	**3,27E-50**
Erysipelotrichaceae		1,630	0,890		-2,886	0,056	↓	-4,905	**0,0003**		-0,389	0,986
Lachnospiraceae		-0,671	0,957	↓	-5,805	**6,38E-13**	↓	-6,229	**2,02E-15**		-1,095	0,471
Moraxellaceae		0,050	0,957	↑	0,305	**0,027**	↑	0,273	**0,048**		0,018	0,986
Prevotellaceae		-0,117	0,957	↑	0,895	**0,002**		0,506	0,120	↓	-0,506	**0,019**
Rikenellaceae	↓	-12,904	**1,42E-24**	↓	-11,950	**8,04E-35**		0,796	0,667		-0,158	0,986
Ruminococcaceae		0,623	0,804	↓	-7,978	**5,11E-71**	↓	-8,955	**6,90E-103**		-0,353	0,767
S24-7		0,495	0,957	↓	-8,085	**8,79E-32**	↓	-9,853	**3,74E-48**		-1,273	0,181
Streptococcaceae		-0,068	0,957	↑	0,600	**0,034**		0,508	0,096		-0,161	0,733
Turicibacteraceae	↓	-9,059	**1,09E-13**	↓	-8,139	**5,75E-19**		-2,610	0,097	↓	-3,531	**0,018**
Verrucomicrobiaceae		2,247	0,101	↓	-7,873	**1,51E-17**	↓	-11,045	**7,89E-38**		-0,925	0,730
GENUS												
unknown		0,200	1,000	↓	-1,830	**3,18E-10**	↓	-7,556	**1,63E-161**	↓	-5,526	**7,72E-108**
[Ruminococcus]		-1,206	1,000	↓	-5,083	**7,40E-05**	↓	-5,377	**1,25E-05**		-1,499	0,774
Acinetobacter		1,913	0,549		0,958	0,750	↓	-1,998	**0,004**		-1,043	0,313
Actinobacillus		0,172	1,000		-0,385	0,750		0,270	0,882	↑	0,827	**0,040**
Akkermansia		2,349	0,134	↓	-7,909	**1,75E-17**	↓	-11,033	**1,51E-37**		-0,774	0,994
Allobaculum		2,488	0,614		-2,295	0,343	↓	-4,868	**0,002**		-0,086	1,000
Anaeroplasma		-3,829	0,134	↓	-13,134	**8,69E-17**	↓	-7,415	**3,31E-07**		1,890	0,785
Bifidobacterium		-1,506	0,751	↓	-5,187	**3,13E-05**	↓	-4,844	**1,25E-05**		-1,163	0,973
Clostridium		0,087	1,000	↑	0,933	**0,0001**	↑	0,953	**0,0001**		0,107	1,000
Coprococcus		-0,607	1,000	↓	-6,718	**1,58E-09**	↓	-6,090	**8,23E-13**		0,021	1,000
Dehalobacterium		1,378	0,614	↓	-6,477	**3,18E-10**	↓	-10,288	**1,83E-31**		-2,432	0,143
Erwinia		0,000	1,000	↑	7,106	**2,54E-08**		0,000	1,000	↓	-11,309	**8,71E-26**
Oscillospira		1,161	0,134	↓	-7,880	**6,19E-69**	↓	-9,517	**8,35E-127**		-0,476	0,973
Ruminococcus		-0,694	1,000	↓	-8,910	**8,49E-22**	↓	-8,073	**5,87E-34**		0,143	1,000
Turicibacter	↓	-8,742	**9,70E-12**	↓	-8,215	**1,33E-16**		-2,760	0,126	↓	-3,287	**0,040**

P-value adj and Log$_2$ fold change are shown. Bold face values indicate statistical significance (P< 0.05). Brackets indicate putative taxonomy based on phylogenetic placement as given in the Greengenes taxonomy. padj is the Benjamini–Hochberg adjusted P value. ↑upwards arrows indicate increase, ↓downwards arrows indicate decrease. WT: Wild-type; ATB: treated with antibiotics.

Discussion

In the present study, we have described how IL-10 deficiency modulates modifications in gut microbiota associated with antibiotic therapy. To do so, we used the IL-10 deficient mouse model of IBD. In the same model, we studied changes related to aging and antibiotic therapy in the colonic expression of proinflammatory cytokines, microbial sensing TLRs and luminal levels of immunoglobulin A.

It is well stablished that IL-10 deficient mice develop colitis when housed in conventional conditions. However, changes that take place during the initiation and chronification of the disease remain a subject of study. In that respect, we characterized changes in intestinal immune system and microbiota related to IL-10 deletion in mice at 6 and 20 weeks of age, before and after the establishment of colitis, respectively [40–42].

Regarding the expression of proinflammatory cytokines in the colon, no differences between both genotypes were detected at 6 weeks of age. In contrast, in 20-week-old IL10$^{-/-}$ mice, we observed a lower expression of IL-1β, IL-6, TLR6, TLR7 and TLR8, together with an increase in TNFα expression. In this regard, other groups have described an increased expression of TNFα both in IBD patients and in mouse models of the disease. However, this was generally associated with enhanced levels of other proinflammatory cytokines including IL-1β and IL-6 [43–48]. In our case, the lower IL-1β and IL-6 detected in IL-10 knockout mice could be related to the lower levels of TLRs expression observed in these animals. Taken together, these results suggest that IL-10 deletion affects the mechanisms for recognizing microorganisms in addition to condition inflammatory responses. In particular, this could be related to a dysregulation in the expression of inhibitory proteins of TLRs in IL-10 deficient animals [49,50]. In that sense, it has been previously reported that Tollip (Toll interacting protein) deficiency aggravated spontaneous disease onset in IL-10$^{-/-}$ mice [51].

In line with what we observed for cytokines and TLRs expression, changes in luminal IgA levels related to IL-10 deletion were detected at 20 but not at 6 weeks of age. Interestingly, while the development of IBD has been associated with intestinal IgA deficiency and reduction, this was not the case in the IL-10 knockout model [52–54]. In agreement with our results, IgA levels are increased in supernatants from cultures of

colonocytes isolated from IL-10 deficient animals [55–57]. Likewise, this could be related to the greater expression of TNFα observed in IL-10 knockout animals, which would favor the induction of IgA-producing B cells [58,59]. IgA secretion is part of the host defense mechanism as a component of the intestinal barrier function, contributing to prevent bacterial adhesion and translocation [60–63]. Therefore, increased levels of IgA observed in IL-10$^{-/-}$ mice would be another sign of the dysregulation of the immune system and its interaction with luminal microorganisms that would appear in aging animals.

In this context, it was of great interest to study if changes observed in the immune system components were related to modifications in luminal microbiota. Firstly, we analyzed the effects of aging and IL-10 deletion on bacterial richness and diversity as alterations in these parameters has been reported in patients and animal models of IBD [1,7,64–71]. More specifically, Knock *et al.* described how bacterial richness is reduced in the IL-10 deficient model before the onset of colitis and the number and diversity of caecal bacteria are increased after colitis initiation [42]. Partially in agreement, we did not detect differences in microbial diversity but we did observe that bacterial richness trended to be lower in IL-10 deficient mice at 6 weeks of age. However, in our case, the reduction did not reach statistical significance, probably due to data variability (Fig. S1). This lower bacterial richness observed at an early age could suggest a possible association with the genotype, which would contribute to the subsequent development of colitis. In addition, we identified an increase in both richness and diversity between 6- and 20-week-old mice, without differences between wild-type and IL-10 knockout. However, we cannot rule out the possibility that a decrease in richness and diversity could have taken place in IL-10$^{-/-}$ mice in later phases of the colitis development process, as suggested by others [4].

Thereafter, we analyzed changes in microbiota composition. Interestingly, 6-week-old IL-10 deficient mice showed a lower relative abundance of Rikenellaceae and Lachnospiraceae taxa compared to wild-type animals and presented the phyla Verrucomicrobia (family Verrucomicrobiaceae; genus *Akkermansia*). Previous reports have consistently established a decrease in the Rikenellaceae and Lachnospiraceae taxa in patients and models of IBD [5,72–76]. Remarkably, the *Akkermansia* genus has previously shown to be positively correlated with the development of colitis [6,73]. This

could be related, for instance, to the highly immunostimulatory properties of the lipopolysaccharide expressed on its surface or to the ability of this genus to degrade mucins [77,78]. In consequence, the presence of this genus would contribute to create a favorable environment for host-microbiome interactions and subsequent inflammatory responses in IL-10 knockout mice. Additionally, at 20 weeks of age, the main difference between both genotypes was that representatives of the phyla TM7 were only detected in IL-10 knockout mice. Additionally, in IL-10$^{-/-}$ animals, the phyla Proteobacteria and *Dorea* genus (family Lachnospiraceae) were increased with aging. As these taxa have shown some degree of association with the development of IBD, it is feasible that they contribute to the initiation of the inflammatory process leading to a colitogenic state described in IL-10 deficient mice of this age [4,5,86,72,79–85]. That said, further investigations are needed to address the specific role of each of this taxa in the IL-10 knockout model and IBD.

At this point, we decided to explore the role of IL-10 in the immune-mediated control of intestinal dysbiosis. With that purpose, we assessed changes in microbiota induced by a broad-spectrum antibiotic therapy. We detected that the expression of TLRs and proinflammatory cytokines and luminal IgA levels was generally reduced by antibiotic therapy in both genotypes. In agreement with our results, others have described that using a similar antibiotic therapy the expression of TLR4 and number of IgA producing cells in the gut were reduced [87,88]. Again, this could be related to the antibiotic therapy-induced reduction in microbial load [59,89,90]. However, in contrast to our findings, others have shown that a different antibiotic therapy with a shorter duration increases the expression of TLR4, TLR6, TLR7 and TLR8 and downregulates the expression of TLR2 in wild-type animals [11]. Therefore, the conditions of the antibiotic therapy used are crucial when changes in the immune system associated with antibiotic-induced dysbiosis are studied. Furthermore, this should be taken into consideration when establishing antibiotic therapy regimes for treating patients.

In addition, in these experiments, we again observed genotype-dependent differences in the immune system. For instance, IL-10 deficient mice showed a lower expression of IL-1β and TLR4 both in the absence and/or presence of antibiotics. At the same time, we detected that antibiotic therapy reduced the expression of TNFα and TLR2 more severely in knockout mice. Therefore, antibiotics could partially attenuate the

108

inflammatory response associated with IL-10 deletion. These and other studies showing anti-inflammatory effects of antibiotics in IBD patients and models prove the importance of the microbiota in colitis [15,91–93]. In fact, antibiotic therapy has been shown to relief symptoms of IBD, such as abdominal pain, induce remission and reduce the rate of recurrence of the disease [92,94–97].

In order to better understand the role of IL-10 on dysbiosis and host-microbiome interactions, we then assessed changes in microbiota induced by antibiotic therapy. Instead of focusing our research on some particular taxa, we considered it interesting and prudent to study the overall effect on the microbiota produced by antibiotics. In this respect, we observed that wild-type animals treated with antibiotics showed a decrease in both diversity and microbial richness. Interestingly, at the level of microbial composition, it was possible to observe a great antibiotic-induced restructuring of the caecal microbial communities. These observations are supported by other studies showing similar results in terms of reduction of diversity and changes in composition of microbial communities after antibiotic therapy [89,93,98–100]. Indeed, some studies indicate that antibiotic therapy can result in long-term changes in the microbiota both in humans and animals [15,101,102]. Therefore, an intervention that involves the early alteration of gut microbiota in individuals genetically susceptible to developing colitis could alter the progression of the disease in later life. With that idea in mind, we studied the effects of antibiotics on gut microbiota in the IL-10 knockout mouse model.

Compared with wild-type mice, in IL-10 deficient animals, antibiotic therapy did not seem to affect microbial richness and decreased diversity in a more moderate way. These differences could be related to the influence of IL-10 on the effects produced by antibiotics on tissues and the immune system. For instance, IL-10 could participate in the modulation of antibiotic-mediated mitochondrial damage, changes in the expression of cell cycle genes and reduction in T cells, IgA producing cells, antimicrobial peptides and cytokines [23,88,89,103–109]. That said, new studies will be needed in the future to investigate the potential role of IL-10 in all these effects mediated by antibiotic therapy. In this regard, the construction of transkingdom networks could be an interesting approach to study microbiota and host mechanisms involved in the regulation of the microbial composition [88,110].

In summary, we found that in IL-10 deficient mice there is a dysregulation in the expression of TLRs together with increased levels of TNFα and IgA and changes in the microbiota at 20 weeks of age that could favor the development of colitis. In addition, we were able to characterize how IL-10 deficiency conditions antibiotic therapy-mediated alterations in gut microbiota. This would suggest that the susceptibility of patients to antibiotic-induced dysbiosis would be conditioned by IL-10. Therefore, our findings provide a framework for the analysis of the composition and manipulation of intestinal microbiota in patients and animal models of colitis. Finally, this work contributes to a better characterization of the IL-10 knockout mouse model, which is crucial for its use as a tool to develop new therapies for IBD in the future.

SUPPLEMENTARY DATA
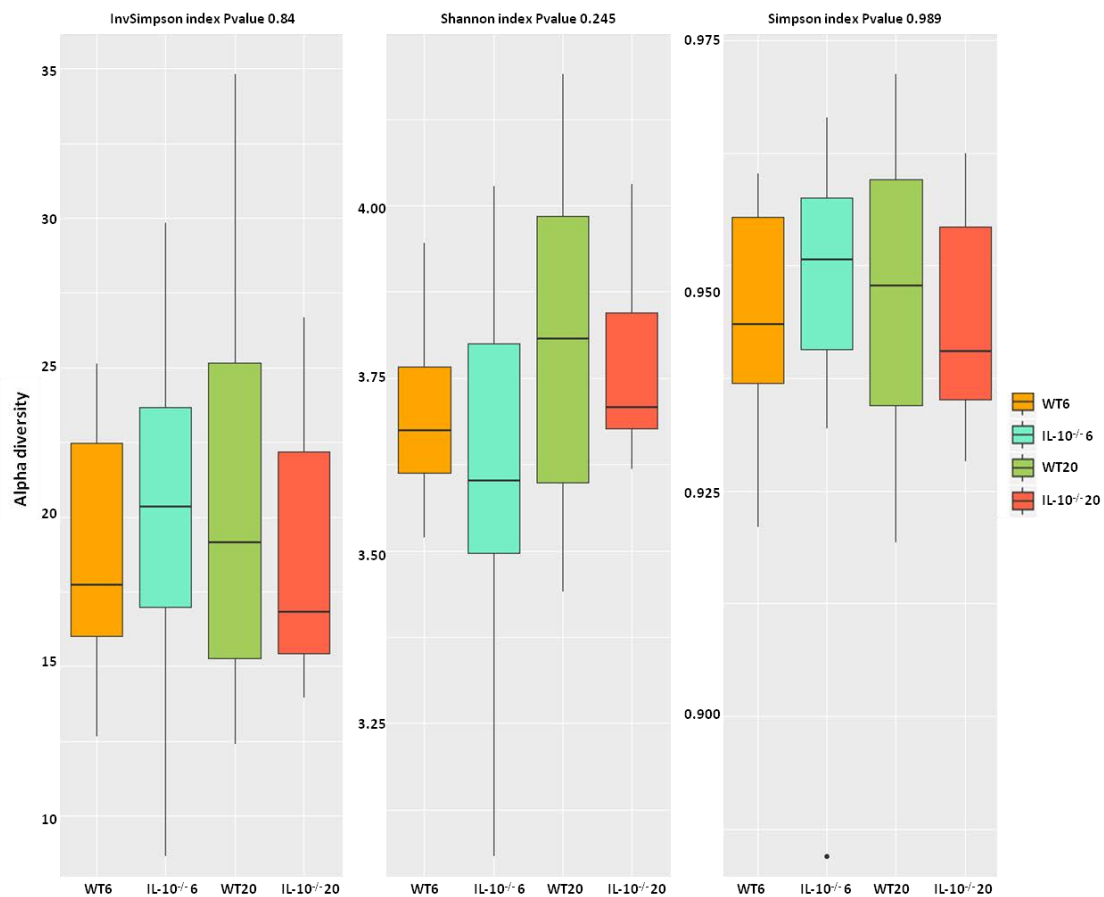
Figure S1. Effects of IL-10 deletion and age on caecal microbiota alpha diversity. Shannon, Simpson and Inv-Simpson from wild-type (WT) and IL-10^{-/-} mice at 6 and 20 weeks of age are plotted as interquarile range with median for each experimental group. N= 8-12 mice per group. P values were calculated using one-way ANOVA.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

References

[1]   P.M. Munyaka, M.F. Rabbi, E. Khafipour, and J.E. Ghia, "Acute dextran sulfate sodium (DSS)-induced colitis promotes gut microbial dysbiosis in mice," *Journal of Basic Microbiology*, vol. 56, no. 9, pp. 986–998, 2016.

[2]   H. Nagao-Kitamoto, A.B. Shreiner, M.G. Gillilland, et al., "Functional Characterization of Inflammatory Bowel Disease-Associated Gut Dysbiosis in Gnotobiotic Mice," *CMGH Cellular and Molecular Gastroenterology and Hepatology*, vol. 2, no. 4, pp. 468–481, 2016.

[3]   N.A. Nagalingam, C.J. Robinson, I.L. Bergin, et al., "The effects of intestinal microbial community structure on disease manifestation in IL-10-/- mice infected with Helicobacter hepaticus," *Microbiome*, vol. 1, no. 1, pp. 15, 2013.

[4]   N. Maharshak, C.D. Packey, M. Ellermann, et al., "Altered enteric microbiota ecology in interleukin 10-deficient mice during development and progression of intestinal inflammation," *Gut Microbes*, vol. 4, no. 4, pp. 316–324, 2014.

[5]   M.L. Hart, A.C. Ericsson, and C.L. Franklin, "Differing complex microbiota alter disease severity of the IL-10-/- mouse model of inflammatory bowel disease," *Frontiers in Microbiology*, vol. 8, no. MAY, pp. 1–15, 2017.

[6]   S.S. Seregin, N. Golovchenko, B. Schaf, et al., "NLRP6 Protects Il10−/− Mice from Colitis by Limiting Colonization of Akkermansia muciniphila," *Cell Reports*, vol. 19, no. 4, pp. 733–745, 2017.

[7]   S.A. Bassett, W. Young, M.P.G. Barnett, et al., "Changes in composition of caecal microbiota associated with increased colon inflammation in interleukin-10 gene-deficient mice inoculated with Enterococcus species.," *Nutrients*, vol. 7, no. 3, pp. 1798–816, 2015.

[8]   C. Lupp, M.L. Robertson, M.E. Wickham, et al., "Host-Mediated Inflammation Disrupts the Intestinal Microbiota and Promotes the Overgrowth of Enterobacteriaceae," *Cell Host and Microbe*, vol. 2, no. 2, pp. 119–129, 2007.

[9]   D. Hill, and D. Artis, "Intestinal bacteria and the regulation of immune cell homeostasis.," *Annual Review of Immunology*, vol. 28, pp. 623–67, 2010.

[10]   F. Hildebrand, T.L.A. Nguyen, B. Brinkman, et al., "Inflammation-associated enterotypes, host genotype, cage and inter-individual effects drive gut microbiota variation in common laboratory mice," *Genome Biology*, vol. 14, no. 1, pp. R4, 2013.

[11]   L. Grasa, L. Abecia, R. Forcén, et al., "Antibiotic-Induced Depletion of Murine Microbiota Induces Mild Inflammation and Changes in Toll-Like Receptor Patterns and Intestinal Motility," *Microbial Ecology*, vol. 70, no. 3, pp. 835–848, 2015.

[12]   T. Zhang, C. Ding, M. Zhao, et al., "Sodium Butyrate Reduces Colitogenic

Immunoglobulin A-Coated Bacteria and Modifies the Composition of Microbiota in IL-10 Deficient Mice," *Nutrients*, vol. 8, no. 12, 2016.

[13]   B.C. Sydora, M.M. Tavernini, A. Wessler, L.D. Jewell, and R.N. Fedorak, "Lack of interleukin-10 leads to intestinal inflammation, independent of the time at which luminal microbial colonization occurs.," *Inflammatory Bowel Diseases*, vol. 9, no. 2, pp. 87–97, 2003.

[14]   R.B. Sartor, "Microbial influences in inflammatory bowel diseases.," *Gastroenterology*, vol. 134, no. 2, pp. 577–94, 2008.

[15]   K.L. Madsen, J.S. Doyle, M.M. Tavernini, et al., "Antibiotic therapy attenuates colitis in interleukin 10 gene–deficient mice," *Gastroenterology*, vol. 118, no. 6, pp. 1094–1105, 2000.

[16]   H. Zhang, Y. Xue, H. Wang, et al., "Mast cell deficiency exacerbates inflammatory bowel symptoms in interleukin-10-deficient mice.," *World Journal of Gastroenterology : WJG*, vol. 20, no. 27, pp. 9106–15, 2014.

[17]   E. Terán-Ventura, M. Roca, M.T. Martin, et al., "Characterization of housing-related spontaneous variations of gut microbiota and expression of toll-like receptors 2 and 4 in rats.," *Microbial Ecology*, vol. 60, no. 3, pp. 691–702, 2010.

[18]   E. Cario, "Toll-like receptors in inflammatory bowel diseases: A decade later," *Inflammatory Bowel Diseases*, vol. 16, no. 9, pp. 1583–1597, 2010.

[19]   F.J. Pérez-Cano, C. Ramírez-Santana, M. Molero-Luís, et al., "Mucosal IgA increase in rats by continuous CLA feeding during suckling and early infancy.," *Journal of Lipid Research*, vol. 50, no. 3, pp. 467–76, 2009.

[20]   D. Rios, M.B. Wood, J. Li, et al., "Antigen sampling by intestinal M cells is the principal pathway initiating mucosal IgA production to commensal enteric bacteria," *Mucosal Immunology*, vol. 9, no. April, pp. 1–10, 2015.

[21]   A.J. Macpherson, Y. Köller, and K.D. McCoy, "The bilateral responsiveness between intestinal microbes and IgA.," *Trends in Immunology*, vol. 36, no. 8, pp. 460–70, 2015.

[22]   S. Rakoff-Nahoum, J. Paglino, F. Eslami-Varzaneh, S. Edberg, and R. Medzhitov, "Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis," *Cell*, vol. 118, no. 2, pp. 229–241, 2004.

[23]   D.H. Reikvam, A. Erofeev, A. Sandvik, et al., "Depletion of murine intestinal microbiota: Effects on gut mucosa and epithelial gene expression," *PLoS ONE*, vol. 6, no. 3, pp. 1–13, 2011.

[24]   E. Ramiro-Puig, F.J. Pérez-Cano, S. Ramos-Romero, et al., "Intestinal immune system of young rats influenced by cocoa-enriched diet," *The Journal of Nutritional Biochemistry*, vol. 19, no. 8, pp. 555–565, 2008.

[25]   K.J. Livak, and T.D. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.," *Methods (San Diego, Calif.)*, vol. 25, no. 4, pp. 402–408, 2001.

[26] J.G. Caporaso, J. Kuczynski, J. Stombaugh, et al., "correspondence QIIME allows analysis of high- throughput community sequencing data Intensity normalization improves color calling in SOLiD sequencing," *Nature Publishing Group*, vol. 7, no. 5, pp. 335–336, 2010.

[27] R.C. Edgar, "Search and clustering orders of magnitude faster than BLAST.," *Bioinformatics (Oxford, England)*, vol. 26, no. 19, pp. 2460–1, 2010.

[28] T.Z. DeSantis, P. Hugenholtz, N. Larsen, et al., "Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB," *Applied and Environmental Microbiology*, vol. 72, no. 7, pp. 5069–5072, 2006.

[29] B.J. Haas, D. Gevers, A.M. Earl, et al., "Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons.," *Genome Research*, vol. 21, no. 3, pp. 494–504, 2011.

[30] N.A. Bokulich, S. Subramanian, J.J. Faith, et al., "Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing.," *Nature Methods*, vol. 10, no. 1, pp. 57–9, 2013.

[31] P.J. McMurdie, and S. Holmes, "phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data.," *PloS One*, vol. 8, no. 4, pp. e61217, 2013.

[32] R.H. Whittaker, "Vegetation of the Siskiyou Mountains, Oregon and California," *Ecological Monographs*, vol. 30, no. 3, pp. 279–338, 1960.

[33] R.H. Whittaker, "Evolution and Measurement of Species Diversity," *Taxon*, vol. 21, no. 2/3, pp. 213, 1972.

[34] J. Oksanen, F.G. Blanchet, M. Friendly, et al., "Package ' vegan '— Community Ecology Package , Package ' vegan ,'" no. December 2016, pp. 0–291, 2016.

[35] C.E. Shannon, "A mathematical theory of communication," *The Bell System Technical Journal*, vol. 27, no. July 1928, pp. 379–423, 1948.

[36] E.H. Simpson, "Measurement of Diversity," *Nature*, vol. 163, no. 4148, pp. 688–688, 1949.

[37] R Core Team, R: A Language and Environment for Statistical Computing, 2017.

[38] M.I. Love, W. Huber, and S. Anders, "Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2.," *Genome Biology*, vol. 15, no. 12, pp. 550, 2014.

[39] Y. Benjamini, and Y. Hochberg, "Controlling the False Discovery Rate : A Practical and Powerful Approach to Multiple Testing Author (s): Yoav Benjamini and Yosef Hochberg Source : Journal of the Royal Statistical Society . Series B (Methodological), Vol . 57 , No . 1 Published by : Wi," *Journal of the Royal Statistical Society Series B*, vol. 57, no. 1, pp. 289–300, 1995.

[40] R.J. Kennedy, M. Hoper, K. Deodhar, et al., "Interleukin 10-deficient colitis: new similarities to human inflammatory bowel disease.," *The British Journal of*

Surgery, vol. 87, no. 10, pp. 1346–51, 2000.

[41]   J. McCarthy, L. O'Mahony, L. O'Callaghan, et al., "Double blind, placebo controlled trial of two probiotic strains in interleukin 10 knockout mice and mechanistic link with cytokine balance.," *Gut*, vol. 52, no. 7, pp. 975–80, 2003.

[42]   B. Knoch, K. Nones, M.P.G. Barnett, W.C. McNabb, and N.C. Roy, "Diversity of caecal bacteria is altered in interleukin-10 gene-deficient mice before and after colitis onset and when fed polyunsaturated fatty acids.," *Microbiology (Reading, England)*, vol. 156, no. Pt 11, pp. 3306–16, 2010.

[43]   A. Rijnierse, A.S. Koster, F.P. Nijkamp, and A.D. Kraneveld, "TNF-a is crucial for the development of mast cell-dependent colitis in mice," *Am J Physiol Gastrointest Liver Physiol*, vol. 291, pp. 969–976, 2006.

[44]   R. Atreya, and M.F. Neurath, "New therapeutic strategies for treatment of inflammatory bowel disease.," *Mucosal Immunology*, vol. 1, no. 3, pp. 175–82, 2008.

[45]   A.J. Yarur, S.G. Strobel, A.R. Deshpande, and M.T. Abreu, "Predictors of aggressive inflammatory bowel disease," *Gastroenterology and Hepatology*, vol. 7, no. 10, pp. 652–659, 2011.

[46]   M.J. Waldner, and M.F. Neurath, "Master regulator of intestinal disease: IL-6 in chronic inflammation and cancer development.," *Seminars in Immunology*, vol. 26, no. 1, pp. 75–9, 2014.

[47]   J.A. Uranga, V. López-Miranda, F. Lombó, and R. Abalo, "Food, nutrients and nutraceuticals affecting the course of inflammatory bowel disease," *Pharmacological Reports*, vol. 68, no. 4, pp. 816–826, 2016.

[48]   Y.-T. Xiao, W.-H. Yan, Y. Cao, J.-K. Yan, and W. Cai, "Neutralization of IL-6 and TNF-α ameliorates intestinal permeability in DSS-induced colitis," *Cytokine*, vol. 83, pp. 189–192, 2016.

[49]   O. Shibolet, and D.K. Podolsky, "TLRs in the Gut. IV. Negative regulation of Toll-like receptors and intestinal homeostasis: addition by subtraction.," *American Journal of Physiology. Gastrointestinal and Liver Physiology*, vol. 292, no. 6, pp. G1469-73, 2007.

[50]   P. Fernandes, J. MacSharry, T. Darby, et al., "Differential expression of key regulators of Toll-like receptors in ulcerative colitis and Crohn's disease: a role for Tollip and peroxisome proliferator-activated receptor gamma?," *Clinical and Experimental Immunology*, vol. 183, no. 3, pp. 358–68, 2016.

[51]   M.H. Maillard, H. Bega, H.H. Uhlig, et al., "Toll-interacting protein modulates colitis susceptibility in mice," *Inflammatory Bowel Diseases*, vol. 20, no. 4, pp. 660–670, 2014.

[52]   P. Brandtzaeg, H.S. Carlsen, and T.S. Halstensen, "The B-cell system in inflammatory bowel disease.," *Advances in Experimental Medicine and Biology*, vol. 579, pp. 149–67, 2006.

[53] J.F. Ludvigsson, M. Neovius, and L. Hammarström, "Association between IgA deficiency & other autoimmune conditions: a population-based matched cohort study.," *Journal of Clinical Immunology*, vol. 34, no. 4, pp. 444–51, 2014.

[54] J. Ni, G.D. Wu, L. Albenberg, and V.T. Tomov, "Gut microbiota and IBD: causation or correlation?," *Nature Reviews. Gastroenterology & Hepatology*, vol. 14, no. 10, pp. 573–584, 2017.

[55] R. Kühn, J. Löhler, D. Rennick, K. Rajewsky, and W. Müller, "Interleukin-10-deficient mice develop chronic enterocolitis.," *Cell*, vol. 75, pp. 263–274, 1993.

[56] D.J. Berg, N. Davidson, R. Kühn, et al., "Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses.," *The Journal of Clinical Investigation*, vol. 98, no. 4, pp. 1010–20, 1996.

[57] R.K. Sellon, S. Tonkonogy, M. Schultz, et al., "Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice," *Infection and Immunity*, vol. 66, no. 11, pp. 5224–5231, 1998.

[58] H. Tezuka, Y. Abe, M. Iwata, et al., "Regulation of IgA production by naturally occurring TNF/iNOS-producing dendritic cells.," *Nature*, vol. 448, no. 7156, pp. 929–33, 2007.

[59] N. Kamada, G.Y. Chen, N. Inohara, and G. Núñez, "Control of pathogens and pathobionts by the gut microbiota," *Nature Immunology*, vol. 14, no. 7, pp. 685–690, 2013.

[60] D.A. Peterson, N.P. McNulty, J.L. Guruge, and J.I. Gordon, "IgA response to symbiotic bacteria as a mediator of gut homeostasis.," *Cell Host & Microbe*, vol. 2, no. 5, pp. 328–39, 2007.

[61] A. Mathias, and B. Corthésy, "N-Glycans on secretory component: mediators of the interaction between secretory IgA and gram-positive commensals sustaining intestinal homeostasis.," *Gut Microbes*, vol. 2, no. 5, pp. 287–93, 2011.

[62] M. Aguilera, P. Vergara, and V. Martínez, "Stress and antibiotics alter luminal and wall-adhered microbiota and enhance the local expression of visceral sensory-related systems in mice.," *Neurogastroenterology and Motility : The Official Journal of the European Gastrointestinal Motility Society*, vol. 25, no. 8, pp. e515-29, 2013.

[63] L. Lin, and J. Zhang, "Role of intestinal microbiota and metabolites on gut homeostasis and human diseases," *BMC Immunology*, vol. 18, no. 1, pp. 2, 2017.

[64] S.J. Ott, M. Musfeldt, D.F. Wenderoth, et al., "Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease.," *Gut*, vol. 53, no. 5, pp. 685–93, 2004.

[65] C. Manichanh, L. Rigottier-Gois, E. Bonnaud, et al., "Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach," *Gut*, vol.

55, no. 2, pp. 205–211, 2006.

[66] B.P. Willing, J. Dicksved, J. Halfvarson, et al., "A Pyrosequencing Study in Twins Shows That Gastrointestinal Microbial Profiles Vary With Inflammatory Bowel Disease Phenotypes," *Gastroenterology*, vol. 139, no. 6, pp. 1844–1854.e1, 2010.

[67] N.A. Nagalingam, J.Y. Kao, and V.B. Young, "Microbial ecology of the murine gut associated with the development of dextran sodium sulfate-induced colitis.," *Inflammatory Bowel Diseases*, vol. 17, no. 4, pp. 917–26, 2011.

[68] A.W. Walker, J.D. Sanderson, C. Churcher, et al., "High-throughput clone library analysis of the mucosa-associated microbiota reveals dysbiosis and differences between inflamed and non-inflamed regions of the intestine in inflammatory bowel disease.," *BMC Microbiology*, vol. 11, pp. 7, 2011.

[69] H. Nemoto, K. Kataoka, H. Ishikawa, et al., "Reduced diversity and imbalance of fecal microbiota in patients with ulcerative colitis.," *Digestive Diseases and Sciences*, vol. 57, no. 11, pp. 2955–64, 2012.

[70] A.K. Samanta, V.A. Torok, N.J. Percy, S.M. Abimosleh, and G.S. Howarth, "Microbial fingerprinting detects unique bacterial communities in the faecal microbiota of rats with experimentally-induced colitis.," *Journal of Microbiology (Seoul, Korea)*, vol. 50, no. 2, pp. 218–25, 2012.

[71] M. Alipour, D. Zaidi, R. Valcheva, et al., "Mucosal Barrier Depletion and Loss of Bacterial Diversity are Primary Abnormalities in Paediatric Ulcerative Colitis.," *Journal of Crohn's & Colitis*, vol. 10, no. 4, pp. 462–71, 2016.

[72] D.N. Frank, A.L. St Amand, R.A. Feldman, et al., "Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases.," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 34, pp. 13780–5, 2007.

[73] D. Berry, C. Schwab, G. Milinovich, et al., "Phylotype-level 16S rRNA analysis reveals new bacterial indicators of health state in acute murine colitis.," *The ISME Journal*, vol. 6, no. 11, pp. 2091–106, 2012.

[74] E. Papa, M. Docktor, C. Smillie, et al., "Non-invasive mapping of the gastrointestinal microbiota identifies children with inflammatory bowel disease.," *PloS One*, vol. 7, no. 6, pp. e39242, 2012.

[75] W. Walters, Z. Xu, and R. Knight, "Meta-analyses of human gut microbes associated with obesity and IBD.," *FEBS Letters*, vol. 588, no. 22, pp. 4223–4233, 2014.

[76] L. Chen, J.E. Wilson, M.J. Koenigsknecht, et al., "NLRP12 attenuates colon inflammation by maintaining colonic microbial diversity and promoting protective commensal bacterial growth.," *Nature Immunology*, vol. 18, no. 5, pp. 541–551, 2017.

[77] M. Derrien, E.E. Vaughan, C.M. Plugge, and W.M. de Vos, "Akkermansia

muciniphila gen. nov., sp. nov., a human intestinal mucin-degrading bacterium.," *International Journal of Systematic and Evolutionary Microbiology*, vol. 54, no. Pt 5, pp. 1469–76, 2004.

[78] G.C. Zella, E.J. Hait, T. Glavan, et al., "Distinct microbiome in pouchitis compared to healthy pouches in ulcerative colitis and familial adenomatous polyposis.," *Inflammatory Bowel Diseases*, vol. 17, no. 5, pp. 1092–100, 2011.

[79] U. Gophna, K. Sommerfeld, S. Gophna, W.F. Doolittle, and S.J.O. Veldhuyzen van Zanten, "Differences between tissue-associated intestinal microfloras of patients with Crohn's disease and ulcerative colitis.," *Journal of Clinical Microbiology*, vol. 44, no. 11, pp. 4136–41, 2006.

[80] T. Kuehbacher, A. Rehman, P. Lepage, et al., "Intestinal TM7 bacterial phylogenies in active inflammatory bowel disease," *Journal of Medical Microbiology*, vol. 57, no. 12, pp. 1569–1576, 2008.

[81] A. Rehman, P. Lepage, A. Nolte, et al., "Transcriptional activity of the dominant gut mucosal microbiota in chronic inflammatory bowel disease patients," *Journal of Medical Microbiology*, vol. 59, no. 9, pp. 1114–1122, 2010.

[82] E. Elinav, T. Strowig, J. Henao-Mejia, and R.A. Flavell, "Regulation of the Antimicrobial Response by NLR Proteins," *Immunity*, vol. 34, no. 5, pp. 665–679, 2011.

[83] X.C. Morgan, T.L. Tickle, H. Sokol, et al., "Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment.," *Genome Biology*, vol. 13, no. 9, pp. R79, 2012.

[84] C. sung Kang, M. Ban, E.J. Choi, et al., "Extracellular Vesicles Derived from Gut Microbiota, Especially Akkermansia muciniphila, Protect the Progression of Dextran Sulfate Sodium-Induced Colitis," *PLoS ONE*, vol. 8, no. 10, 2013.

[85] I. Yang, D. Eibach, F. Kops, et al., "Intestinal microbiota composition of interleukin-10 deficient C57BL/6J mice and susceptibility to Helicobacter hepaticus-induced colitis.," *PloS One*, vol. 8, no. 8, pp. e70783, 2013.

[86] A. Gupta, S. Kang, and J. Wagner, "Analysis of Mucosal Microbiota in Inflammatory Bowel Disease using a Custom Phylogenetic Microarray," *Austin Journal of Gastroenterology*, vol. 1, no. 4, pp. 1–6, 2014.

[87] X. Chen, B.-S. Feng, P.-Y. Zheng, et al., "Fc gamma receptor signaling in mast cells links microbial stimulation to mucosal immune inflammation in the intestine.," *The American Journal of Pathology*, vol. 173, no. 6, pp. 1647–56, 2008.

[88] A. Morgun, A. Dzutsev, X. Dong, et al., "Uncovering effects of antibiotics on the host and microbiota using transkingdom gene networks.," *Gut*, vol. 64, no. 11, pp. 1732–43, 2015.

[89] D.A. Hill, C. Hoffmann, M.C. Abt, et al., "Metagenomic analyses reveal antibiotic-induced temporal and spatial changes in intestinal microbiota with

associated alterations in immune cell homeostasis.," *Mucosal Immunology*, vol. 3, no. 2, pp. 148–58, 2010.

[90] A.J. Macpherson, and K.D. McCoy, "Independence Day for IgA," *Immunity*, vol. 43, no. 3, pp. 416–418, 2015.

[91] F. Hoentjen, H.J.M. Harmsen, H. Braat, et al., "Antibiotics with a selective aerobic or anaerobic spectrum have different therapeutic activities in various regions of the colon in interleukin 10 gene deficient mice.," *Gut*, vol. 52, no. 12, pp. 1721–7, 2003.

[92] P. Muniyappa, R. Gulati, F. Mohr, and V. Hupertz, "Use and safety of rifaximin in children with inflammatory bowel disease.," *Journal of Pediatric Gastroenterology and Nutrition*, vol. 49, no. 4, pp. 400–4, 2009.

[93] N.L. Ward, C.D. Phillips, D.D. Nguyen, et al., "Antibiotic Treatment Induces Long-lasting Changes in the Fecal Microbiota that Protect Against Colitis.," *Inflammatory Bowel Diseases*, vol. 22, no. 10, pp. 2328–40, 2016.

[94] R.B. Sartor, "Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: Antibiotics, probiotics, and prebiotics," *Gastroenterology*, vol. 126, no. 6, pp. 1620–1633, 2004.

[95] K.J. Khan, T.A. Ullman, A.C. Ford, et al., "Antibiotic therapy in inflammatory bowel disease: A systematic review and meta-analysis," *American Journal of Gastroenterology*, vol. 106, no. 4, pp. 661–673, 2011.

[96] C. Prantera, H. Lochs, M. Grimaldi, et al., "Rifaximin-extended intestinal release induces remission in patients with moderately active crohn's disease," *Gastroenterology*, vol. 142, no. 3, pp. 473–481, 2012.

[97] G. Cammarota, G. Ianiro, R. Cianci, et al., "The involvement of gut microbiota in inflammatory bowel disease pathogenesis: Potential for therapy," *Pharmacology and Therapeutics*, vol. 149, pp. 191–212, 2015.

[98] D.A. Antonopoulos, S.M. Huse, H.G. Morrison, et al., "Reproducible community dynamics of the gastrointestinal microbiota following antibiotic perturbation.," *Infection and Immunity*, vol. 77, no. 6, pp. 2367–75, 2009.

[99] I. Cho, S. Yamanishi, L. Cox, et al., "Antibiotics in early life alter the murine colonic microbiome and adiposity.," *Nature*, vol. 488, no. 7413, pp. 621–6, 2012.

[100] A.M. Schubert, H. Sinani, and P.D. Schloss, "Antibiotic-Induced Alterations of the Murine Gut Microbiota and Subsequent Effects on Colonization Resistance against Clostridium difficile.," *MBio*, vol. 6, no. 4, pp. e00974, 2015.

[101] C. Ubeda, Y. Taur, R.R. Jenq, et al., "Vancomycin-resistant Enterococcus domination of intestinal microbiota is enabled by antibiotic treatment in mice and precedes bloodstream invasion in humans.," *The Journal of Clinical Investigation*, vol. 120, no. 12, pp. 4332–41, 2010.

[102] A.E. Perez-Cobas, M.J. Gosalbes, A. Friedrichs, et al., "Gut microbiota disturbance during antibiotic therapy: a multi-omic approach," *Gut*, vol. 62, no.

11, pp. 1–11, 2012.

[103] K. Brandl, G. Plitas, C.N. Mihu, et al., "Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits.," *Nature*, vol. 455, no. 7214, pp. 804–7, 2008.

[104] J.A. Hall, N. Bouladoux, C.M. Sun, et al., "Commensal DNA Limits Regulatory T Cell Conversion and Is a Natural Adjuvant of Intestinal Immune Responses," *Immunity*, vol. 29, no. 4, pp. 637–649, 2008.

[105] I.I. Ivanov, R. de L. Frutos, N. Manel, et al., "Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine.," *Cell Host & Microbe*, vol. 4, no. 4, pp. 337–49, 2008.

[106] S. Vaishnava, C.L. Behrendt, A.S. Ismail, L. Eckmann, and L. V Hooper, "Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface.," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 52, pp. 20858–20863, 2008.

[107] A.S. Ismail, K.M. Severson, S. Vaishnava, et al., "Gammadelta intraepithelial lymphocytes are essential mediators of host-microbial homeostasis at the intestinal mucosal surface.," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 21, pp. 8743–8, 2011.

[108] A.E. Barnhill, M.T. Brewer, and S.A. Carlson, "Adverse effects of antimicrobials via predictable or idiosyncratic inhibition of host mitochondrial components.," *Antimicrobial Agents and Chemotherapy*, vol. 56, no. 8, pp. 4046–51, 2012.

[109] S. Kalghatgi, C.S. Spina, J.C. Costello, et al., "Bactericidal antibiotics induce mitochondrial dysfunction and oxidative damage in Mammalian cells.," *Science Translational Medicine*, vol. 5, no. 192, pp. 192ra85, 2013.

[110] E.A. Franzosa, X.C. Morgan, N. Segata, et al., "Relating the metatranscriptome and metagenome of the human gut.," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 111, no. 22, pp. E2329-38, 2014.

DISCUSIÓN GENERAL

Durante los últimos años, se han llevado a cabo diversas investigaciones que buscan profundizar en el rol que cumplen los mastocitos (MC) y la microbiota en el desarrollo de procesos inflamatorios intestinales.

En lo referente a los mastocitos, se ha demostrado que, además de estar involucrados en el desarrollo de procesos alérgicos, participan en el reconocimiento de microorganismos a través de receptores de reconocimiento de patrones (PRRs) (Marshall 2004; Mrabet-Dahbi et al. 2009; Sandig & Bulfone-Paus 2012; Xie et al. 2012).

Por otra parte, el análisis de la microbiota intestinal a través de métodos de secuenciación masiva ha permitido relacionar el desarrollo de diversas patologías con la diversidad y composición de las comunidades microbianas intestinales (Mayer et al. 2014; Petersen & Round 2014).

Con el fin de estudiar más en profundidad el papel de los mastocitos en el reconocimiento microbiano y en la modulación de la inflamación intestinal, se empleó un modelo de ratones deficientes en interleucina 10 (IL-10$^{-/-}$). Se consideró este modelo de especial interés debido a la actividad inmunomoduladora de la IL-10 y la regulación que ésta ejerce sobre la expresión de proteasas mastocitarias (Godfraind et al. 1998; Xing et al. 2011). Otro punto a destacar que nos hizo decantarnos por este modelo es el hecho de que los animales IL-10$^{-/-}$ desarrollan colitis únicamente en ambientes convencionales y de manera dependiente de la microbiota intestinal (Sellon et al. 1998; Madsen et al. 2000). En este contexto, nos propusimos caracterizar el efecto de la carencia de IL-10 sobre el fenotipo mastocitario y su activación vía PRRs en etapas previas y posteriores al desarrollo de la colitis. Adicionalmente, caracterizamos los efectos de la ausencia de IL-10 sobre los cambios en la microbiota luminal, la expresión de receptores de tipo toll (TLRs) y la producción de mediadores proinflamatorios e inmunoglobulina A (IgA) luminal asociados la administración de antibióticos vía oral.

Influencia de IL-10 en la diferenciación del fenotipo mastocitario

En cuanto a la diferenciación de mastocitos de tipo mucosa (MLMC) y mastocitos cultivados derivados de peritoneo (PCMC), previamente se han caracterizado ambas poblaciones por expresar un patrón de proteasas distinto (Wernersson & Pejler 2014). En concordancia con estudios previos, hemos observado que MLMC expresan la

proteasa mastocitaria de ratón-1 (mMCP-1), que por el contrario no se detectó en los PCMC. Por otra parte, la mMCP-6 característica de mastocitos de tipo conectivo fue detectada en ambas poblaciones mastocitarias. No obstante, cabe destacar que algunos estudios han demostrado que proteasas asociadas a mastocitos de tipo conectivo (CTMC), pueden estar presentes en mastocitos de mucosa o mastocitos en transición (Godfraind et al. 1998; Rönnberg et al. 2013; Fernández-Blanco et al. 2015).

La ausencia de IL-10 se vio relacionada con una menor expresión de la proteasa mastocitaria mMCP-6 en mastocitos de tipo conectivo derivados de células peritoneales (PCMC). Aunque no fue posible detectar IL-10 en el sobrenadante de los mastocitos de los animales no modificados (posiblemente debido a los tiempos de obtención de las muestras), la IL-10 podría mediar sus efectos de forma autocrína o estar contenida en el interior de la célula, como sugieren estudios previos (Godfraind et al. 1998; Xing et al. 2011).

En lo referente a la morfología, la observación de los mastocitos por microscopia óptica de campo claro tras su tinción con azul de toluidina no reveló diferencias morfológicas asociadas a la carencia en IL-10. Dicho esto, sería interesante emplear técnicas de mayor resolución como la microscopia electrónica de transmisión, para llevar a cabo una evaluación morfológica más minuciosa de estas células, incluyendo el análisis del número y el tamaño de los gránulos.

Expresión diferencial de receptores de reconocimiento de patrones en mastocitos de tipo mucosa y conectivo

En los mastocitos de tipo mucosa (MLMC) detectamos la expresión de los cuatro PRRs analizados (TLR2, TLR4, TLR7 y NOD2). Por el contrario, en los mastocitos cultivados derivados de peritoneo (PCMC) no fue posible detectar ni TLR7 ni NOD2. En consonancia con nuestros resultados, estudios previos indican que la expresión de los PRRs depende tanto del fenotipo mastocitario como del origen y diferenciación de las células (Mrabet-Dahbi et al. 2009; Nakamura et al. 2009; Okumura et al. 2009; Haidl et al. 2011; Sandig & Bulfone-Paus 2012).

Adicionalmente, observamos cómo la expresión de los PRRs puede variar en función de la edad de los animales de los que derivarán los mastocitos. Por lo general, se detectaron

mayores niveles de expresión de estos receptores a edades tempranas, previamente a las fases de desarrollo de colitis. No tenemos constancia de que los cambios en la expresión de los PRRs asociados a la edad hubieran sido descritos con anterioridad al presente trabajo. Sin embargo, se han observado cambios similares en macrófagos (Renshaw et al. 2002). Dichos cambios en la expresión de PRRs en estas células del sistema inmune innato comportarían variaciones en las interacciones del huésped frente a microorganismos, incluyendo la microbiota comensal.

En cuanto al rol que ejerce la IL-10 sobre la expresión de PRRs, detectamos que varía según el tipo mastocitario, el receptor estudiado y la edad de los animales de los cuales fueron aislados los mastocitos. Esto podría estar directamente relacionado con la capacidad de esta citocina de regular la sobrevivencia y expansión de los mastocitos, así como también, con su capacidad inhibitoria (da Silva et al. 2014; Gabryšová & Howes 2014; Polukort et al. 2016). Cabe destacar que el efecto de la ausencia de IL-10 depende del tipo específico de receptor. En este sentido, vale mencionar que podrían existir mecanismos de regulación cruzada entre receptores, o incluso compensatorios frente a la carencia de IL-10 (Cao 2016). Sin embargo, a partir de nuestros resultados no es posible inferir el mecanismo por el cual la IL-10 regularía la expresión de estos receptores. Con dicha finalidad, sería de interés evaluar la posible regulación por parte de la IL-10 de las vías de señalización asociadas a la activación de los PRRs. Estos datos serían de gran interés a la hora de complementar estudios en que se describen algunas vías a través de la cuales la IL-10 inhibe la producción de mediadores inflamatorios (Gabryšová & Howes 2014).

Subtipo mastocitario, edad y deficiencia de IL-10 condicionan la respuesta frente a estímulo específico.

En términos generales, la secreción de citocinas asociada a la activación de PRRs de los mastocitos con ligandos específicos depende del tipo mastocitario (MLMC o PCMC), de la edad de los animales en el momento en que se realizó el aislamiento celular y de la carencia constitutiva o no de IL-10.

Pudimos observar cómo tanto los MLMC como los PCMC responden al estímulo vía TLR2, mientras que no se detectó respuesta de ninguno de los tipos mastocitarios para el ligando NOD2. Por otra parte, las respuestas a los agonistas para TLR4 y TLR7 se

mostraron altamente dependientes del tipo mastocitario. En ambos casos, estos indujeron la secreción de TNFα e IL-6 por parte de los MLMC, pero no de los PCMC. En consonancia con nuestros resultados, otros grupos han descrito que la respuesta mastocitaria tras la estimulación de los PRRs varía en función del fenotipo de los mastocitos (McCurdy et al. 2001; Supajatura et al. 2002; Mrabet-Dahbi et al. 2009; Nakamura et al. 2009).

En lo referente a los efectos de la edad, observamos como la secreción de mediadores asociada a la estimulación de TLR2 y TLR4 era menor cuando los animales empleados tenían 20 semanas en comparación con los ratones de 6 semanas. Por una parte, esta variación podría ser atribuida a la diminución de la expresión de los receptores discutida previamente. Por otro lado, podría estar relacionada con una disminución general de la respuesta inmune con la edad. Ello condicionaría la interacción microbiota-huésped y podría explicar, al menos en parte, porque la colitis asociada al modelo murino deficiente en IL-10 es dependiente de la edad (Renshaw et al. 2002; Arrieta et al. 2009; Chichlowski et al. 2010).

De manera similar a lo descrito para la expresión de los PRRs, la deficiencia de IL-10 se traduce en cambios en la secreción de citocinas que son dependientes del receptor estimulado. Por un lado, la ausencia de IL-10 disminuye la respuesta proinflamatoria asociada a la activación de TLR2. En contraposición, la activación de la secreción de citocinas desencadenada por ligandos de TLR4 se ve incrementada en ausencia de IL-10. Ello indicaría una acción inmunomoduladora de la IL-10 sobre los mastocitos, de modo similar a lo previamente descrito para otros tipos celulares (Marshall et al. 1996; Bijjiga & Martino 2011; Cyktor & Turner 2011; Paul et al. 2012). Estas diferencias relacionadas con el tipo de estímulo refuerzan lo señalado anteriormente, en cuanto a la posible existencia de vías de señalización cruzadas entre receptores.

Los cambios en los niveles intestinales de TLRs, citocinas proinflamatorias e IgA asociados a la deficiencia de IL-10 son dependientes de la edad

De acuerdo a nuestros resultados, en etapas previas al desarrollo de colitis los animales deficientes en IL-10 no manifiestan alteraciones a nivel de expresión de TLRs, citocinas pro-inflamatorias y producción de IgA. Por el contrario, conforme avanza la edad de los animales, se manifiestan diferencias en estos aspectos en comparación con los ratones

que expresan constitutivamente IL-10. En concreto, observamos un incremento en los niveles de TNFα e IgA, característico de este modelo y de la enfermedad inflamatoria intestinal (IBD) (Kühn et al. 1993; Davidson et al. 1996; Sellon et al. 1998). A su vez, a las 20 semanas de edad, los animales deficientes en IL-10 mostraron una menor expresión de algunos TLRs (TLR6, TLR7 y TLR8), comparado con los procedentes de animales control. Dicha menor expresión de TLRs comprometería el reconocimiento de los microorganismos luminales y la capacidad del sistema inmune de mantener la homeostasis a nivel intestinal. Ello explicaría la activación del proceso inflamatorio asociado a un aumento de la expresión de TNFα y de la secreción de IgA.

Cambios en la microbiota podrían estar relacionados con el inicio y desarrollo del proceso inflamatorio

Se han descrito múltiples factores asociados al desarrollo de la IBD. Entre ellos, se ha descrito tanto en pacientes como en modelos de la enfermedad su asociación a una disbiosis a nivel intestinal. Dicho esto, a día de hoy no se ha podido concluir cual es su relación causa-efecto y por tanto si dicha disbiosis es causa o consecuencia de la patología.

En concreto, la disbiosis descrita en humanos y en modelos murinos consiste en una disminución de la diversidad y riqueza microbiana una vez la enfermedad está establecida (Ott et al. 2004; Manichanh et al. 2006; Willing et al. 2010; Nagalingam et al. 2011; Walker et al. 2011; Nemoto et al. 2012; Samanta et al. 2012; Bassett et al. 2015; Munyaka et al. 2016). Sin embargo, no están tan bien caracterizados los cambios acontecidos previamente al inicio del proceso inflamatorio. El uso de modelos animales, como los ratones deficientes en IL-10, nos brinda una oportunidad única a la hora de intentar esclarecer las diferencias a nivel de la microbiota previos al inicio de la patología.

En nuestro caso, observamos como los animales carentes de IL-10 tendían a presentar una menor riqueza bacteriana en fases previas a las asociadas con el desarrollo de colitis y un incremento de dicha riqueza en edades más avanzadas, de manera similar a lo descrito por otros investigadores (Knoch et al. 2010). Los cambios en la riqueza y composición microbianas detectados en los ratones deficientes para IL-10 podrían contribuir al posterior desarrollo de colitis. De particular interés son la mayor

abundancia de agentes potencialmente dañinos como los del filo Verrucomicrobia, que contribuirían a la degradación de la capa de moco, así como la menor abundancia de microorganismos considerados beneficiosos incluidos en las familias Rikenellaceae y Lachnospiraceae (Derrien et al. 2004; Frank et al. 2007; Berry et al. 2012; Papa et al. 2012; Walters et al. 2014; Chen et al. 2017; Hart et al. 2017). En conjunto, estos cambios podrían favorecer un ambiente pro-inflamatorio en las fases previas al desarrollo de la colitis que se vería agravado posteriormente por el aumento en la abundancia de Proteobacterias y TM7, ambos taxas han sido asociados con disbiosis y el desarrollo de IBD (Kuehbacher et al. 2008; Maharshak et al. 2014; Litvak et al. 2017).

La administración de antibióticos induce cambios en el sistema inmunitario intestinal

El régimen de antibióticos utilizados produjo una disminución generalizada en la expresión de TLRs y citocinas proinflamatorias y en la concentración luminal de IgA, independientemente de si los ratones eran o no deficientes en IL-10. Éste y otros estudios dan soporte a la idea de la participación de los diferentes microorganismos intestinales a la hora de regular la expresión y producción de los citados componentes del sistema inmunitario (Chen et al. 2008; Morgun et al. 2015). En este contexto, la disminución de estos factores relacionados con la respuesta inmune estaría relacionada con la reducción de la carga bacteriana, más allá de la predominancia de algún taxa en particular.

La alteración de la microbiota intestinal asociada a la antibioterapia está condicionada por la IL-10

La alteración de la microbiota inducida mediante la utilización de antibióticos fue claramente diferente en el caso de los animales deficientes para IL-10. En primer lugar, la magnitud de la disminución de la riqueza y diversidad asociada a la antibioterapia fue menor en los ratones IL-10$^{-/-}$. En la misma línea, estudios previos han reportado cómo la riqueza y diversidad microbiana disminuyen tras la administración de antibióticos (Antonopoulos et al. 2009; Hill et al. 2010; Cho et al. 2012; Schubert et al. 2015; Ward et al. 2016). Al mismo tiempo, se observó un menor incremento en la abundancia de enterobacterias en los animales carentes de IL-10. Dentro del grupo enterobacterias se

encuentra una amplia gama de patógenos oportunistas, tales como *Escherichia*, *Enterobacter* y *Salmonella* (Antonopoulos et al. 2009; Hill et al. 2010). Por lo tanto, su menor abundancia podría estar asociada con la mejoría de la enfermedad observada tras la administración de antibióticos en los ratones IL-10$^{-/-}$, tal y como sucede en otros modelos de IBD y en pacientes (Madsen et al. 2000; Hoentjen et al. 2003; Muniyappa et al. 2009; Ward et al. 2016).

Con los resultados del presente estudio no es posible inferir cual es la relación existente entre la carencia de IL-10 y los cambios en la susceptibilidad de la microbiota intestinal a ser modificada por la acción de los antibióticos. No se pueden excluir en este sentido que vengan condicionados por cambios en los efectos directos de los antibióticos sobre los tejidos (Brandl et al. 2008; Hall et al. 2008; Ivanov et al. 2008; Vaishnava et al. 2008; Hill et al. 2010; Ismail et al. 2011; Reikvam et al. 2011; Barnhill et al. 2012; Kalghatgi et al. 2013; Morgun et al. 2015). Por tanto, cabe remarcar la necesidad de estudiar la modulación de la IL-10 sobre la respuesta tisular a los antibióticos en el futuro.

En resumen, estos hallazgos proveen nuevas perspectivas sobre la función de los mastocitos y la IL-10 en la interacción microorganismo-huésped. Muestran cómo la ausencia de IL-10 puede afectar la composición de la microbiota y la expresión de factores asociados a la respuesta inmune. Y sugieren que la modificación temprana de la microbiota mediante la utilización de antibióticos en individuos genéticamente susceptibles podría alterar la progresión de la colitis.

CONCLUSIONES

1. En el modelo murino, tanto en ausencia como en presencia de IL-10, la diferenciación de células derivadas de medula ósea resulta en células que muestran características típicas de células mastocitarias correspondientes con los fenotipos de mucosa (MLMC) y de peritoneo (PCMC), cuando son sometidos a cultivos específicos.

2. La ausencia de IL-10 en mastocitos de tipo mucosa (MLMC) no implica diferencias en la expresión de la proteasa mastocitaria mMCP-1, característica de mastocitos de mucosa. Por otra parte, la carencia de IL-10 en mastocitos cultivados derivados de peritoneo (PCMC) implica una regulación a la baja en la expresión de la proteasa mastocitaria mMCP-6, característica de mastocitos de tipo conectivo.

3. Tanto en MLMC como en PCMC la ausencia de IL-10 no se traduce en diferencias en la expresión de TLR2, sin embargo, el aumento de la edad implica una reducción en la expresión de TLR2 en ambos fenotipos y genotipos mastocitarios. Además, al ser sometidos a estímulo específico en edad más avanzada, la deficiencia de IL-10 implica una menor expresión de TLR2 en ambas poblaciones mastocitarias.

4. La carencia de IL-10 en MLMC estimulados con agonista TLR2 implica una menor secreción de IL-6 a edad temprana, junto con una menor secreción de IL-6 y TNFα a edad más avanzada. Por su parte, en PCMC la deficiencia de IL-10 implica una menor secreción de IL-6 y TNFα a edad temprana.

5. En MLMC la ausencia de IL-10 implica una menor expresión de TLR4 en edad temprana, revirtiéndose en edad más avanzada. Por su parte, en PCMC la carencia de IL-10 no representa diferencias en la expresión de TLR4. Adicionalmente, en edad avanzada no fue posible detectar el receptor en ambos genotipos. La estimulación con LPS en MLMC en ausencia de IL-10 implica una menor expresión de TLR4. Por el contrario, en PCMC la ausencia de IL-10 parece no afectar la expresión del receptor TLR4.

6. La carencia de IL-10 en MLMC estimulados con agonista TLR4 implica una mayor secreción de IL-6 en edad temprana. Por otra parte, la estimulación de PCMC con el mismo agonista no se traduce en una respuesta secretora de las citocinas IL-6 y TNFα.

7. En MLMC la ausencia de IL-10 implica una menor expresión de TLR7 en edad avanzada. Por el contrario, en PCMC no fue posible detectar el receptor en ninguno de

los genotipos. Adicionalmente, en MLMC sometidos a estímulo en edad más avanzada, la deficiencia de IL-10 implica una menor expresión de TLR7.

8. La carencia de IL-10 en MLMC estimulados con agonista TLR7 implica una mayor secreción de IL-6 en edad temprana, junto con una menor secreción de TNFα en edad más avanzada. Por su parte, no se detectó respuesta en PCMC frente a la estimulación de este receptor en ambos genotipos.

9. En MLMC la ausencia de IL-10 implica una menor expresión de NOD2 en edad temprana. Sin embargo, la estimulación específica de este receptor en ambos genotipos implica una reducción de su expresión. Por otra parte, en PCMC no fue posible detectar el receptor NOD2 en ninguno de los genotipos. Adicionalmente, la estimulación de ambas poblaciones y genotipos mastocitarios no se traduce en una respuesta secretora de las citocinas IL-6 y TNFα.

10. En ausencia de IL-10, la respuesta mediada por FcεRI en MLMC implica una menor secreción de IL-6 y TNFα en edad más avanzada. Por su parte en PCMC, frente al mismo estímulo, la ausencia de IL-10 indujo una menor secreción de IL-6 y TNFα en edad temprana, junto con una mayor secreción de TNFα en edad más avanzada.

11. Mientras que en edad temprana la deficiencia de IL-10 no afecta la expresión de TLRs en el colon, en edad más avanzada la carencia de IL-10 implica una menor expresión de IL-1β, IL-6, TLR6, TLR7 y TLR8; y una mayor expresión de TNFα. Por otra parte, la administración del tratamiento antibiótico induce una reducción general de la expresión de citocinas y TLRs tanto en animales deficientes o no de IL-10.

12. En edad temprana la deficiencia de IL-10 no afecta la concentración de IgA luminal. No obstante, en edad más avanzada la carencia de IL-10 implica una mayor concentración de esta inmunoglobulina. Por otra parte, la administración del tratamiento antibiótico tiende a disminuir la concentración de IgA independiente del genotipo.

13. En edad temprana la deficiencia de IL-10 se asocia a una mayor abundancia del filo Verrucomicrobia, y una menor abundancia de las familias Rikenellaceae y Lachnospiraceae. Adicionalmente, solo en ausencia de IL-10 fue posible observar representantes del filo TM7 en edad más avanzada. Por otra parte, la administración del tratamiento antibiótico se traduce en una reducción de la diversidad y riqueza

microbiana, así como también en una reestructuración general de la composición de la microbiota independiente del genotipo. Además, la carencia de IL-10 conlleva a un menor aumento del filo Proteobacteria tras la administración de antibióticos.

Conclusión final.

La ausencia de IL-10 modifica la expresión de PRRs en mastocitos y ello conlleva a diferencias en la expresión de citocinas respecto de lo que se observa en animales con genotipo no modificado. Estos cambios también se observan en la pared intestinal y son compatibles con la susceptibilidad a la inflamación de este modelo murino. La ausencia de IL-10 también condiciona la microbiota intestinal y la respuesta a antibióticos.

REFERENCIAS

Abraham, S.N. & St John, A.L., 2010. Mast cell-orchestrated immunity to pathogens. *Nature reviews. Immunology*, 10(6), pp.440–52.

Abreu, M.T., 2010. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. *Nature reviews. Immunology*, 10(2), pp.131–44.

Adair, K.L. & Douglas, A.E., 2017. Making a microbiome: the many determinants of host-associated microbial community composition. *Current opinion in microbiology*, 35, pp.23–29.

Aguilera, M., Vergara, P. & Martínez, V., 2013. Stress and antibiotics alter luminal and wall-adhered microbiota and enhance the local expression of visceral sensory-related systems in mice. *Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society*, 25(8), pp.e515-29.

Akira, S., Uematsu, S. & Takeuchi, O., 2006. Pathogen recognition and innate immunity. *Cell*, 124(4), pp.783–801.

Antonopoulos, D.A. et al., 2009. Reproducible community dynamics of the gastrointestinal microbiota following antibiotic perturbation. *Infection and immunity*, 77(6), pp.2367–75.

Arrieta, M.C. et al., 2009. Reducing small intestinal permeability attenuates colitis in the IL10 gene-deficient mouse. *Gut*, 58(1), pp.41–8.

Ashelford, K.E. et al., 2005. At least 1 in 20 16S rRNA sequence records currently held in public repositories is estimated to contain substantial anomalies. *Applied and environmental microbiology*, 71(12), pp.7724–36.

Atreya, R. & Neurath, M.F., 2010. Chemokines in inflammatory bowel diseases. *Digestive diseases (Basel, Switzerland)*, 28(3), pp.386–94.

Bąbolewska, E. et al., 2012. Different potency of bacterial antigens TLR2 and TLR4 ligands in stimulating mature mast cells to cysteinyl leukotriene synthesis. *Microbiology and immunology*, 56(3), pp.183–90.

Baert, F.J. & Rutgeerts, P.R., 1999. Anti-TNF strategies in Crohn's disease: mechanisms, clinical effects, indications. *International journal of colorectal disease*, 14(1), pp.47–51.

Barnhill, A.E., Brewer, M.T. & Carlson, S.A., 2012. Adverse effects of antimicrobials via predictable or idiosyncratic inhibition of host mitochondrial components. *Antimicrobial agents and chemotherapy*, 56(8), pp.4046–51.

Bassett, S.A. et al., 2015. Changes in composition of caecal microbiota associated with increased colon inflammation in interleukin-10 gene-deficient mice inoculated with Enterococcus species. *Nutrients*, 7(3), pp.1798–816.

Beaven, M.A., 2009. Our perception of the mast cell from Paul Ehrlich to now. *European journal of immunology*, 39(1), pp.11–25.

Belkaid, Y. & Harrison, O.J., 2017. Homeostatic Immunity and the Microbiota.

Immunity, 46(4), pp.562–576.

Bendtsen, K.M. et al., 2015. The influence of the young microbiome on inflammatory diseases--Lessons from animal studies. *Birth defects research. Part C, Embryo today : reviews*, 105(4), pp.278–95.

Bernstein, C.N. & Shanahan, F., 2008. Disorders of a modern lifestyle: reconciling the epidemiology of inflammatory bowel diseases. *Gut*, 57(9), pp.1185–91.

Berry, D. et al., 2012. Phylotype-level 16S rRNA analysis reveals new bacterial indicators of health state in acute murine colitis. *The ISME journal*, 6(11), pp.2091–106.

Bijjiga, E. & Martino, A.T., 2011. Interleukin 10 (IL-10) Regulatory Cytokine and its Clinical Consequences. *Journal of Clinical & Cellular Immunology*, pp.1–6.

Billiet, T. et al., 2014. Targeting TNF-α for the treatment of inflammatory bowel disease. *Expert opinion on biological therapy*, 14(1), pp.75–101.

Bischoff, S.C. et al., 1999. Mast cells are an important cellular source of tumour necrosis factor alpha in human intestinal tissue. *Gut*, 44(5), pp.643–52.

Bischoff, S.C., 2007. Role of mast cells in allergic and non-allergic immune responses: comparison of human and murine data. *Nature reviews. Immunology*, 7(2), pp.93–104.

Biswas, A., Petnicki-Ocwieja, T. & Kobayashi, K.S., 2012. Nod2: a key regulator linking microbiota to intestinal mucosal immunity. *Journal of molecular medicine (Berlin, Germany)*, 90(1), pp.15–24.

Bouma, G. & Strober, W., 2003. The immunological and genetic basis of inflammatory bowel disease. *Nature reviews. Immunology*, 3(7), pp.521–33.

Bouskra, D. et al., 2008. Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. *Nature*, 456(7221), pp.507–510.

Bradding, P., Walls, A.F. & Holgate, S.T., 2006. The role of the mast cell in the pathophysiology of asthma. *The Journal of allergy and clinical immunology*, 117(6), pp.1277–84.

Braegger, C.P. et al., 1992. Tumour necrosis factor alpha in stool as a marker of intestinal inflammation. *Lancet (London, England)*, 339(8785), pp.89–91.

Brandl, K. et al., 2008. Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits. *Nature*, 455(7214), pp.804–7.

Breese, E.J. et al., 1994. Tumor necrosis factor alpha-producing cells in the intestinal mucosa of children with inflammatory bowel disease. *Gastroenterology*, 106(6), pp.1455–66.

Buffie, C.G. & Pamer, E.G., 2013. Microbiota-mediated colonization resistance against intestinal pathogens. *Nature Reviews Immunology*, 13(11), pp.790–801.

Camilleri, M. et al., 2012. Intestinal barrier function in health and gastrointestinal

disease. *Neurogastroenterology and Motility*, 24(6), pp.503–512.

Cao, X., 2016. Self-regulation and cross-regulation of pattern-recognition receptor signalling in health and disease. *Nature Reviews Immunology*, 16(1), pp.35–50.

Cario, E., 2008. Barrier-protective function of intestinal epithelial Toll-like receptor 2. *Mucosal immunology*, 1 Suppl 1(November), pp.S62-6.

Cario, E., 2010. Toll-like receptors in inflammatory bowel diseases: A decade later. *Inflammatory Bowel Diseases*, 16(9), pp.1583–1597.

Cario, E., Gerken, G. & Podolsky, D.K., 2007. Toll-Like Receptor 2 Controls Mucosal Inflammation by Regulating Epithelial Barrier Function. *Gastroenterology*, 132(4), pp.1359–1374.

Chamaillard, M. et al., 2003. An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nature Immunology*, 4(7), pp.702–707.

Chang, C. & Lin, H., 2016. Dysbiosis in gastrointestinal disorders. *Best Practice and Research: Clinical Gastroenterology*, 30(1), pp.3–15.

Chang, J.Y. et al., 2008. Decreased Diversity of the Fecal Microbiome in Recurrent *Clostridium difficile* –Associated Diarrhea. *The Journal of Infectious Diseases*, 197(3), pp.435–438.

Chen, J., Pitmon, E. & Wang, K., 2017. Microbiome, inflammation and colorectal cancer. *Seminars in Immunology*, 32(October), pp.43–53.

Chen, X. et al., 2008. Fc gamma receptor signaling in mast cells links microbial stimulation to mucosal immune inflammation in the intestine. *The American journal of pathology*, 173(6), pp.1647–56.

Chichlowski, M. et al., 2010. Role of Mast Cells in Inflammatory Bowel Disease and Inflammation-Associated Colorectal Neoplasia in IL-10-Deficient Mice D. Unutmaz, ed. *PLoS ONE*, 5(8), p.e12220.

Chistiakov, D. a D.A. et al., 2014. Intestinal mucosal tolerance and impact of gut microbiota to mucosal tolerance. *Frontiers in microbiology*, 5(January), pp.1–9.

Cho, I. et al., 2012. Antibiotics in early life alter the murine colonic microbiome and adiposity. *Nature*, 488(7413), pp.621–6.

Cho, J.H., 2008. The genetics and immunopathogenesis of inflammatory bowel disease. *Nature Reviews Immunology*, 8(6), pp.458–466.

Chung, H. et al., 2012. Gut immune maturation depends on colonization with a host-specific microbiota. *Cell*, 149(7), pp.1578–1593.

Couper, K.N., Blount, D.G. & Riley, E.M., 2008. IL-10: the master regulator of immunity to infection. *Journal of immunology (Baltimore, Md. : 1950)*, 180(9), pp.5771–7.

Creagh, E.M. & O'Neill, L.A.J., 2006. TLRs, NLRs and RLRs: a trinity of pathogen

sensors that co-operate in innate immunity. *Trends in Immunology*, 27(8), pp.352–357.

Croswell, A. et al., 2009. Prolonged impact of antibiotics on intestinal microbial ecology and susceptibility to enteric Salmonella infection. *Infection and Immunity*, 77(7), pp.2741–2753.

Cryan, J.F. & Dinan, T.G., 2012. Mind-altering microorganisms: The impact of the gut microbiota on brain and behaviour. *Nature Reviews Neuroscience*, 13(10), pp.701–712.

Cyktor, J. & Turner, J., 2011. Interleukin-10 and immunity against prokaryotic and eukaryotic intracellular pathogens. *Infection and immunity*, 79(8), pp.2964–73.

Davidson, N.J. et al., 1996. T helper cell 1-type CD4+ T cells, but not B cells, mediate colitis in interleukin 10-deficient mice. *The Journal of experimental medicine*, 184(1), pp.241–51.

Derrien, M. et al., 2004. Akkermansia muciniphila gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. *International journal of systematic and evolutionary microbiology*, 54(Pt 5), pp.1469–76.

Dethlefsen, L., McFall-Ngai, M. & Relman, D.A., 2007. An ecological and evolutionary perspective on humang-microbe mutualism and disease. *Nature*, 449(7164), pp.811–818.

Diehl, S. & Rincón, M., 2002. The two faces of IL-6 on Th1/Th2 differentiation. *Molecular Immunology*, 39(9), pp.531–536.

Dieleman, L.A. et al., 2000. Helicobacter hepaticus does not induce or potentiate colitis in interleukin-10-deficient mice. *Infection and immunity*, 68(9), pp.5107–13.

Dinoto, A. et al., 2006. Population Dynamics of Bifidobacterium Species in Human Feces during Raffinose Administration Monitored by Fluorescence In Situ Hybridization-Flow Cytometry. *Applied and Environmental Microbiology*, 72(12), pp.7739–7747.

Donaldson, G.P., Lee, S.M. & Mazmanian, S.K., 2015. Gut biogeography of the bacterial microbiota. *Nature Reviews Microbiology*, 14(1), pp.20–32.

Echtenacher, B., Mannel, D.N. & Hultner, L., 1996. Critical protective role of mast cells in a model of acute septic peritonitis. *Nature*, 381(6577), pp.75–77.

Ekoff, M., Strasser, A. & Nilsson, G., 2007. FcεRI aggregation promotes survival of connective tissue-like mast cells but not mucosal-like mast cells. *The Journal of Immunology*, 178, pp.4177–4183.

Elia, P.P. et al., 2015. The role of innate immunity receptors in the pathogenesis of inflammatory bowel disease. *Mediators of inflammation*, 2015, p.936193.

Feng, B.-S. et al., 2007. Mast Cells Play a Crucial Role in Staphylococcus aureus Peptidoglycan-Induced Diarrhea. *The American Journal of Pathology*, 171(2), pp.537–547.

Fernández-Blanco, J.A. et al., 2015. Changes in Epithelial Barrier Function in Response to Parasitic Infection: Implications for IBD Pathogenesis. *Journal of Crohn's & colitis*, 9(6), pp.463–76.

Fox, C.C. et al., 1990. Enhancement of human intestinal mast cell mediator release in active ulcerative colitis. *Gastroenterology*, 99(1), pp.119–24.

Frank, D.N. et al., 2007. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proceedings of the National Academy of Sciences of the United States of America*, 104(34), pp.13780–5.

Frosali, S. et al., 2015. How the Intricate Interaction among Toll-Like Receptors, Microbiota, and Intestinal Immunity Can Influence Gastrointestinal Pathology. *Journal of Immunology Research*, 2015, p.489821.

Gabryšová, L. et al., 2014. The regulation of IL-10 expression. *Current topics in microbiology and immunology*, 380, pp.157–90.

Galli, S., Grimbaldeston, M. & Tsai, M., 2008. Immunomodulatory mast cells: negative, as well as positive, regulators of immunity. *Nature Reviews Immunology*, 8(Box 1).

Galli, S.J. et al., 2005. Mast cells as "tunable" effector and immunoregulatory cells: Recent Advances. *Annual Review of Immunology*, 23(1), pp.749–786.

Galli, S.J., Borregaard, N. & Wynn, T.A., 2011. Phenotypic and functional plasticity of cells of innate immunity: macrophages, mast cells and neutrophils. *Nature Immunology*, 12(11), pp.1035–1044.

Galli, S.J. & Tsai, M., 2012. IgE and mast cells in allergic disease. *Nature medicine*, 18(5), pp.693–704.

Garner, C.D. et al., 2009. Perturbation of the small intestine microbial ecology by streptomycin alters pathology in a Salmonella enterica serovar typhimurium murine model of infection. *Infection and Immunity*, 77(7), pp.2691–2702.

Gent, A.E. et al., 1994. Inflammatory bowel disease and domestic hygiene in infancy. *The Lancet*, 343(8900), pp.766–767.

Geremia, A. et al., 2014. Innate and adaptive immunity in inflammatory bowel disease. *Autoimmunity Reviews*, 13(1), pp.3–10.

Gevers, D. et al., 2014. The treatment-naive microbiome in new-onset Crohn's disease. *Cell Host and Microbe*, 15(3), pp.382–392.

Di Gioia, M. & Zanoni, I., 2015. Toll-like receptor co-receptors as master regulators of the immune response. *Molecular immunology*, 63(2), pp.143–52.

Girardin, S.E., Boneca, I.G., et al., 2003. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *Journal of Biological Chemistry*, 278(11), pp.8869–8872.

Girardin, S.E., Hugot, J.P. & Sansonetti, P.J., 2003. Lessons from Nod2 studies:

Towards a link between Crohn's disease and bacterial sensing. *Trends in Immunology*, 24(12), pp.652–658.

Godfraind, C. et al., 1998. Intraepithelial infiltration by mast cells with both connective tissue-type and mucosal-type characteristics in gut, trachea, and kidneys of IL-9 transgenic mice. *Journal of immunology (Baltimore, Md. : 1950)*, 160(8), pp.3989–3996.

Goodrich, J.K. et al., 2014. Conducting a microbiome study. *Cell*, 158(2), pp.250–262.

Gurish, M.F. & Boyce, J.A., 2002. Mast cell growth, differentiation, and death. *Clinical reviews in allergy & immunology*, 22(2), pp.107–18.

Haidl, I.D., McAlpine, S.M. & Marshall, J.S., 2011. Enhancement of mast cell IL-6 production by combined toll-like and nucleotide-binding oligomerization domain-like receptor activation. *International Archives of Allergy and Immunology*, 154(3), pp.227–235.

Hall, J.A. et al., 2008. Commensal DNA Limits Regulatory T Cell Conversion and Is a Natural Adjuvant of Intestinal Immune Responses. *Immunity*, 29(4), pp.637–649.

Hart, M.L., Ericsson, A.C. & Franklin, C.L., 2017. Differing complex microbiota alter disease severity of the IL-10-/- mouse model of inflammatory bowel disease. *Frontiers in Microbiology*, 8(MAY), pp.1–15.

Hasegawa, M. et al., 2006. Differential release and distribution of Nod1 and Nod2 immunostimulatory molecules among bacterial species and environments. *Journal of Biological Chemistry*, 281(39), pp.29054–29063.

Hering, N.A., Fromm, M. & Schulzke, J.-D., 2012. Determinants of colonic barrier function in inflammatory bowel disease and potential therapeutics. *The Journal of Physiology*, 590(5), pp.1035–1044.

Hill, D.A. et al., 2010. Metagenomic analyses reveal antibiotic-induced temporal and spatial changes in intestinal microbiota with associated alterations in immune cell homeostasis. *Mucosal immunology*, 3(2), pp.148–58.

Hochdörfer, T., Kuhny, M. & Zorn, C., 2011. Activation of the PI3K pathway increases TLR-induced TNF-α and IL-6 but reduces IL-1β production in mast cells. *Cellular signalling*, 23, pp.866–875.

Hoentjen, F. et al., 2003. Antibiotics with a selective aerobic or anaerobic spectrum have different therapeutic activities in various regions of the colon in interleukin 10 gene deficient mice. *Gut*, 52(12), pp.1721–7.

Hofmann, A.M. & Abraham, S.N., 2009. New roles for mast cells in modulating allergic reactions and immunity against pathogens. *Current Opinion in Immunology*, 21(6), pp.679–686.

Inohara, N. et al., 2003. Host recognition of bacterial muramyl dipeptide mediated through NOD2: Implications for Crohn's disease. *Journal of Biological Chemistry*, 278(8), pp.5509–5512.

Ismail, A.S. et al., 2011. Gammadelta intraepithelial lymphocytes are essential mediators of host-microbial homeostasis at the intestinal mucosal surface. *Proceedings of the National Academy of Sciences of the United States of America*, 108(21), pp.8743–8.

Ivanov, I.I. et al., 2008. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell host & microbe*, 4(4), pp.337–49.

Ivanov, I.I. & Honda, K., 2012. Intestinal commensal microbes as immune modulators. *Cell host & microbe*, 12(4), pp.496–508.

Jurjus, A.R., Khoury, N.N. & Reimund, J.-M., 2004. Animal models of inflammatory bowel disease. *Journal of Pharmacological and Toxicological Methods*, 50(2), pp.81–92.

Kai, Y. et al., 2005. Colitis in mice lacking the common cytokine receptor γ chain is mediated by IL-6-producing CD4+T cells. *Gastroenterology*, 128(4), pp.922–934.

Kalghatgi, S. et al., 2013. Bactericidal antibiotics induce mitochondrial dysfunction and oxidative damage in Mammalian cells. *Science translational medicine*, 5(192), p.192ra85.

Kalliomäki, M. et al., 2008. Early differences in fecal microbiota composition in children may. *American Journal of Clinical Nutrition*, 87(1), pp.534–538.

Kamada, N. et al., 2013. Control of pathogens and pathobionts by the gut microbiota. *Nature Immunology*, 14(7), pp.685–690.

Kamdar, K., Nguyen, V. & DePaolo, R.W., 2013. Toll-like receptor signaling and regulation of intestinal immunity. *Virulence*, 4(3), pp.207–12.

Kawai, T. & Akira, S., 2009. The roles of TLRs, RLRs and NLRs in pathogen recognition. *International Immunology*, 21(4), pp.317–337.

Kawai, T. & Akira, S., 2011. Toll-like Receptors and Their Crosstalk with Other Innate Receptors in Infection and Immunity. *Immunity*, 34(5), pp.637–650.

Kawamoto, S. et al., 2014. Foxp3+ T Cells Regulate Immunoglobulin A Selection and Facilitate Diversification of Bacterial Species Responsible for Immune Homeostasis. *Immunity*, 41(1), pp.152–165.

Keita, å. V. & Söderholm, J.D., 2010. The intestinal barrier and its regulation by neuroimmune factors. *Neurogastroenterology & Motility*, 22(7), pp.718–733.

Keubler, L.M. et al., 2015. A Multihit Model: Colitis Lessons from the Interleukin-10-deficient Mouse. *Inflammatory bowel diseases*, 21(8), pp.1967–75.

Khor, B., Gardet, A. & Xavier, R.J., 2011. Genetics and pathogenesis of inflammatory bowel disease. *Nature*, 474(7351), pp.307–317.

Kishimoto, T., Akira, S. & Taga, T., 1992. Interleukin-6 and its receptor: a paradigm for cytokines. *Science (New York, N.Y.)*, 258(5082), pp.593–7.

Knoch, B. et al., 2010. Diversity of caecal bacteria is altered in interleukin-10 gene-deficient mice before and after colitis onset and when fed polyunsaturated fatty acids. *Microbiology (Reading, England)*, 156(Pt 11), pp.3306–16.

Kobayashi, K.S. et al., 2005. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science*, 307(5710), pp.731–4.

Kole, A. & Maloy, K.J., 2014. Control of intestinal inflammation by interleukin-10. S. Fillatreau & A. O'Garra, eds. *Current topics in microbiology and immunology*, 380, pp.19–38.

Komatsu, M. et al., 2001. Tumor necrosis factor-α in serum of patients with inflammatory bowel disease as measured by a highly sensitive immuno-PCR. *Clinical Chemistry*, 47(7), pp.1297–1301.

Kostic, A.D., Xavier, R.J. & Gevers, D., 2014. The microbiome in inflammatory bowel disease: current status and the future ahead. *Gastroenterology*, 146(6), pp.1489–99.

Krishnaswamy, G. & Chi, D.S., 2005. *Mast Cells* G. Krishnaswamy & D. S. Chi, eds., Totowa, NJ: Humana Press.

Kuehbacher, T. et al., 2008. Intestinal TM7 bacterial phylogenies in active inflammatory bowel disease. *Journal of Medical Microbiology*, 57(12), pp.1569–1576.

Kühn, R. et al., 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell*, 75, pp.263–274.

Kunder, C. a et al., 2013. Mast cell modulation of the vascular and lymphatic endothelium Review article Mast cell modulation of the vascular and lymphatic endothelium. *Blood*, 118(20), pp.5383–5393.

Lepage, P. et al., 2005. Biodiversity of the mucosa-associated microbiota is stable along the distal digestive tract in healthy individuals and patients with IBD. *Inflammatory Bowel Diseases*, 11(5), pp.473–480.

Leulier, F. & Lemaitre, B., 2008. Toll-like receptors - Taking an evolutionary approach. *Nature Reviews Genetics*, 9(3), pp.165–178.

Levy, M. et al., 2017. Dysbiosis and the immune system. *Nature Reviews Immunology*, 17(4), pp.219–232.

Ley, R.E. et al., 2008. Worlds within worlds: evolution of the vertebrate gut microbiota. *Nature reviews. Microbiology*, 6(10), pp.776–88.

Ley, R.E., Peterson, D.A. & Gordon, J.I., 2006. Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell*, 124(4), pp.837–48.

Li, J. et al., 2015. Functional Impacts of the Intestinal Microbiome in the Pathogenesis of Inflammatory Bowel Disease. *Inflammatory Bowel Diseases*, 21(1), pp.139–153.

Litvak, Y. et al., 2017. Dysbiotic Proteobacteria expansion: a microbial signature of

epithelial dysfunction. *Current opinion in microbiology*, 39, pp.1–6.

Liu, F.T., Goodarzi, H. & Chen, H.Y., 2011. IgE, mast cells, and eosinophils in atopic dermatitis. *Clinical Reviews in Allergy and Immunology*, 41(3), pp.298–310.

Liu, Q. & Ding, J.L., 2016. The molecular mechanisms of TLR-signaling cooperation in cytokine regulation. *Immunology and Cell Biology*, 94(November 2015), pp.1–5.

MacDermott, R.P. et al., 1986. Altered patterns of secretion of monomeric IgA and IgA subclass 1 by intestinal mononuclear cells in inflammatory bowel disease. *Gastroenterology*, 91(2), pp.379–385.

MacDonald, T.T. et al., 1990. Tumour necrosis factor-alpha and interferon-gamma production measured at the single cell level in normal and inflamed human intestine. *Clinical and Experimental Immunology*, 81(2), pp.301–305.

Macpherson, A.J. et al., 2012. The habitat, double life, citizenship, and forgetfulness of IgA. *Immunological Reviews*, 245(1), pp.132–146.

Macpherson, A.J., Köller, Y. & McCoy, K.D., 2015. The bilateral responsiveness between intestinal microbes and IgA. *Trends in immunology*, 36(8), pp.460–70.

Madsen, K.L. et al., 2000. Antibiotic therapy attenuates colitis in interleukin 10 gene–deficient mice. *Gastroenterology*, 118(6), pp.1094–1105.

Maharshak, N. et al., 2014. Altered enteric microbiota ecology in interleukin 10-deficient mice during development and progression of intestinal inflammation. *Gut Microbes*, 4(4), pp.316–324.

Malbec, O. et al., 2004. Linker for Activation of T Cells Integrates Positive and Negative Signaling in Mast Cells. *The Journal of Immunology*, 173(8), pp.5086–5094.

Malbec, O. et al., 2007. Peritoneal Cell-Derived Mast Cells: An In Vitro Model of Mature Serosal-Type Mouse Mast Cells. *The Journal of Immunology*, 178(10), pp.6465–6475.

Malik, T.A., 2015. Inflammatory Bowel Disease. Historical Perspective, Epidemiology, and Risk Factors. *Surgical Clinics of North America*, 95(6), pp.1105–1122.

Mancuso, G. et al., 2009. Bacterial recognition by TLR7 in the lysosomes of conventional dendritic cells. *Nature Immunology*, 10(6), pp.587–594.

Manichanh, C. et al., 2006. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. *Gut*, 55(2), pp.205–211.

Manichanh, C. et al., 2012. The gut microbiota in IBD. *Nature Reviews Gastroenterology & Hepatology*, 9(10), pp.599–608.

Marshall, J.S. et al., 1996. Interleukin (IL)-10 inhibits long-term IL-6 production but not preformed mediator release from rat peritoneal mast cells. *Journal of Clinical Investigation*, 97(4), pp.1122–1128.

Marshall, J.S., 2004. Mast-cell responses to pathogens. *Nature reviews. Immunology*,

4(10), pp.787–799.

Marshall, J.S., King, C.A. & McCurdy, J.D., 2003. Mast cell cytokine and chemokine responses to bacterial and viral infection. *Current pharmaceutical design*, 9(1), pp.11–24.

Marshall, J.S., McCurdy, J.D. & Olynych, T., 2003. Toll-like receptor-mediated activation of mast cells: implications for allergic disease? *International archives of allergy and immunology*, 132(2), pp.87–97.

Matsushima, H. et al., 2004. TLR3-, TLR7-, and TLR9-Mediated Production of Proinflammatory Cytokines and Chemokines from Murine Connective Tissue Type Skin-Derived Mast Cells but Not from Bone Marrow-Derived Mast Cells. *The Journal of Immunology*, 173(1), pp.531–541.

Mayer, E.A. et al., 2014. Gut Microbes and the Brain: Paradigm Shift in Neuroscience. *Journal of Neuroscience*, 34(46), pp.15490–15496.

Mayer, L., 2010. Evolving paradigms in the pathogenesis of IBD. *Journal of Gastroenterology*, 45(1), pp.9–16.

McClure, R. & Massari, P., 2014. TLR-Dependent Human Mucosal Epithelial Cell Responses to Microbial Pathogens. *Frontiers in immunology*, 5(August), p.386.

McCurdy, J.D., Lin, T.J. & Marshall, J.S., 2001. Toll-like receptor 4-mediated activation of murine mast cells. *Journal of leukocyte biology*, 70(6), pp.977–84.

McDermott, J.R. et al., 2003. Mast cells disrupt epithelial barrier function during enteric nematode infection. *Proceedings of the National Academy of Sciences of the United States of America*, 100(13), pp.7761–7766.

McLachlan, J.B. et al., 2003. Mast cell-derived tumor necrosis factor induces hypertrophy of draining lymph nodes during infection. *Nature Immunology*, 4(12), pp.1199–1205.

McLean, M.H. et al., 2015. Does the microbiota play a role in the pathogenesis of autoimmune diseases? *Gut*, 64(2), pp.332–341.

Meena, N.K. et al., 2015. Association of TLR5 gene polymorphisms in ulcerative colitis patients of North India and their role in cytokine homeostasis. *PLoS ONE*, 10(3), pp.1–12.

Metcalfe, D.D., Baram, D. & Mekori, Y.A., 1997. Mast cells. *Physiol Rev*, 77(4), pp.1033–1079.

Miller, H. & Wright, S., 1999. A novel function for transforming growth factor-β1: upregulation of the expression and the IgE-independent extracellular release of a mucosal mast cell granule-. *Blood*, 93(10), pp.3473–3476.

Miller, H.R.P. & Pemberton, A.D., 2002. Tissue-specific expression of mast cell granule serine proteinases and their role in inflammation in the lung and gut. *Immunology*, 105(4), pp.375–390.

Molodecky, N.A. et al., 2012. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology*, 142(1), p.46–54.e42.

Molodecky, N.A. & Kaplan, G.G., 2010. Environmental risk factors for inflammatory bowel disease. *Gastroenterology & hepatology*, 6(5), pp.339–46.

Morgun, A. et al., 2015. Uncovering effects of antibiotics on the host and microbiota using transkingdom gene networks. *Gut*, 64(11), pp.1732–43.

Mrabet-Dahbi, S. et al., 2009. Murine mast cells secrete a unique profile of cytokines and prostaglandins in response to distinct TLR2 ligands. *Experimental Dermatology*, 18(5), pp.437–444.

Muniyappa, P. et al., 2009. Use and safety of rifaximin in children with inflammatory bowel disease. *Journal of pediatric gastroenterology and nutrition*, 49(4), pp.400–4.

Munyaka, P.M. et al., 2016. Acute dextran sulfate sodium (DSS)-induced colitis promotes gut microbial dysbiosis in mice. *Journal of Basic Microbiology*, 56(9), pp.986–998.

Murch, S.H. et al., 1991. Serum concentrations of tumour necrosis factor alpha in childhood chronic inflammatory bowel disease. *Gut*, 32(8), pp.913–917.

Nagalingam, N.A., Kao, J.Y. & Young, V.B., 2011. Microbial ecology of the murine gut associated with the development of dextran sodium sulfate-induced colitis. *Inflammatory bowel diseases*, 17(4), pp.917–26.

Nakamura, Y. et al., 2009. Mast cells mediate neutrophil recruitment and vascular leakage through the NLRP3 inflammasome in histamine-independent urticaria. *The Journal of experimental medicine*, 206(5), pp.1037–1046.

De Nardo, D., 2015. Toll-like receptors: Activation, signalling and transcriptional modulation. *Cytokine*, 74(2), pp.181–189.

Naugler, W.E. & Karin, M., 2008. The wolf in sheep's clothing: the role of interleukin-6 in immunity, inflammation and cancer. *Trends in Molecular Medicine*, 14(3), pp.109–119.

Nemoto, H. et al., 2012. Reduced diversity and imbalance of fecal microbiota in patients with ulcerative colitis. *Digestive diseases and sciences*, 57(11), pp.2955–64.

Neurath, M.F., 2014. Cytokines in inflammatory bowel disease. *Nature reviews. Immunology*, 14(5), pp.329–42.

Nicholls, S. et al., 1993. Cytokines in stools of children with inflammatory bowel disease or infective diarrhoea. *Journal of clinical pathology*, 46(8), pp.757–60.

Nigrovic, P.A. & Lee, D.M., 2005. Mast cells in inflammatory arthritis. *Arthritis Research and Therapy*, 7(1), pp.1–11.

Nolte, H. et al., 1990. Histamine release from gut mast cells from patients with inflammatory bowel diseases. *Gut*, 31(7), pp.791–4.

O'shea, J., Tato, C.M. & Siegel, R., 2008. Cytokines and cytokine receptors. In *Clinical Immunology*. Elsevier, pp. 139–171.

Okumura, S. et al., 2009. Hyperexpression of NOD2 in intestinal mast cells of Crohn's disease patients: Preferential expression of inflammatory cell-recruiting molecules via NOD2 in mast cells. *Clinical Immunology*, 130(2), pp.175–185.

Ott, S.J. et al., 2004. Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. *Gut*, 53(5), pp.685–93.

Pabst, O. & Bernhardt, G., 2010. The puzzle of intestinal lamina propria dendritic cells and macrophages. *European journal of immunology*, 40(8), pp.2107–11.

Pagliari, D. et al., 2015. The Interactions between Innate Immunity and Microbiota in Gastrointestinal Diseases. *Journal of Immunology Research*, 2015.

Papa, E. et al., 2012. Non-invasive mapping of the gastrointestinal microbiota identifies children with inflammatory bowel disease. *PloS one*, 7(6), p.e39242.

Park, B.S. & Lee, J.O., 2013. Recognition of lipopolysaccharide pattern by TLR4 complexes. *Experimental and Molecular Medicine*, 45(12), pp.e66-9.

Paul, G., Khare, V. & Gasche, C., 2012. Inflamed gut mucosa: downstream of interleukin-10. *European journal of clinical investigation*, 42(1), pp.95–109.

Pedersen, J. et al., 2014. Inflammatory pathways of importance for management of inflammatory bowel disease. *World journal of gastroenterology : WJG*, 20(1), pp.64–77.

Pejler et. al., G., 2010. Mast cell proteases: multfaceted regulators of inflammatory disease. *Blood*, 115(24), pp.4981–4990.

Pejler, G. et al., 2007. Mast Cell Proteases. *Advances in Immunology*, 95(07), pp.167–255.

Peng Yu, A. et al., 2008. The costs of Crohn's disease in the United States and other Western countries: a systematic review. *Current Medical Research and Opinion*, 24(2), pp.319–328.

Perez-Lopez, A. et al., 2016. Mucosal immunity to pathogenic intestinal bacteria. *Nat Rev Immunol*, advance on(3), pp.135–148.

Petersen, C. & Round, J.L., 2014. Defining dysbiosis and its influence on host immunity and disease. *Cellular Microbiology*, 16(7), pp.1024–1033.

Pizarro, T.T. et al., 2003. Mouse models for the study of Crohn's disease. *Trends in Molecular Medicine*, 9(5), pp.218–222.

Plato, A., Hardison, S.E. & Brown, G.D., 2015. Pattern recognition receptors in antifungal immunity. *Seminars in Immunopathology*, 37(2), pp.97–106.

Podolsky, D.K., 2002. Inflammatory Bowel Disease. *New England Journal of Medicine*, 347(6), pp.1982–1984.

Polukort, S.H. et al., 2016. IL-10 Enhances IgE-Mediated Mast Cell Responses and Is Essential for the Development of Experimental Food Allergy in IL-10–Deficient Mice. *The Journal of Immunology*, 196(12), pp.4865–4876.

Qin, J. et al., 2010. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature*, 464(7285), pp.59–65.

Raithel, M. et al., 1995. Mucosal histamine content and histamine secretion in Crohn's disease, ulcerative colitis and allergic enteropathy. *International Archives of Allergy and Immunology*, 108(2), pp.127–133.

Ramakrishna, B.S., 2013. Role of the gut microbiota in human nutrition and metabolism. *Journal of Gastroenterology and Hepatology*, 28, pp.9–17.

Reikvam, D.H. et al., 2011. Depletion of murine intestinal microbiota: Effects on gut mucosa and epithelial gene expression. *PLoS ONE*, 6(3), pp.1–13.

Renshaw, M. et al., 2002. Cutting edge: impaired Toll-like receptor expression and function in aging. *Journal of immunology (Baltimore, Md. : 1950)*, 169(9), pp.4697–701.

Rescigno, M., 2011. The intestinal epithelial barrier in the control of homeostasis and immunity. *Trends in Immunology*, 32(6), pp.256–264.

Rijnierse, A. et al., 2006. TNF-a is crucial for the development of mast cell-dependent colitis in mice. *Am J Physiol Gastrointest Liver Physiol*, 291, pp.969–976.

Rijnierse, A., Nijkamp, F.P. & Kraneveld, A.D., 2007. Mast cells and nerves tickle in the tummy Implications for inflammatory bowel disease and irritable bowel syndrome. *Pharmacology & Therapeutics*, 116(2), pp.207–235.

Robbie-Ryan, M. & Brown, M.A., 2002. The role of mast cells in allergy and autoimmunity. *Current Opinion in Immunology*, 14(6), pp.728–733.

Rogier, E.W. et al., 2014. Secretory IgA is Concentrated in the Outer Layer of Colonic Mucus along with Gut Bacteria. *Pathogens (Basel, Switzerland)*, 3(2), pp.390–403.

Rönnberg, E. et al., 2013. Granzyme D is a novel murine mast cell protease that is highly induced by multiple pathways of mast cell activation. *Infection and immunity*, 81(6), pp.2085–94.

Round, J.L. & Mazmanian, S.K., 2009. The gut microbiota shapes intestinal immune responses during health and disease. *Nature reviews. Immunology*, 9(5), pp.313–23.

Sabat, R. et al., 2010. Biology of interleukin-10. *Cytokine & growth factor reviews*, 21(5), pp.331–44.

Salzman, N.H. & Bevins, C.L., 2013. Dysbiosis-A consequence of Paneth cell dysfunction. *Seminars in Immunology*, 25(5), pp.334–341.

Samanta, A.K. et al., 2012. Microbial fingerprinting detects unique bacterial communities in the faecal microbiota of rats with experimentally-induced colitis. *Journal of microbiology (Seoul, Korea)*, 50(2), pp.218–25.

Sandig, H. & Bulfone-Paus, S., 2012. TLR signaling in mast cells: Common and unique features. *Frontiers in Immunology*, 3(JUL), pp.1–13.

Saraiva, M. & O'Garra, A., 2010. The regulation of IL-10 production by immune cells. *Nature reviews. Immunology*, 10(3), pp.170–81.

Sartor, R.B., 2008. Microbial influences in inflammatory bowel diseases. *Gastroenterology*, 134(2), pp.577–94.

Scheller, J. et al., 2011. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochimica et Biophysica Acta - Molecular Cell Research*, 1813(5), pp.878–888.

Schiller, M. et al., 2006. Immune response modifiers - mode of action. *Experimental Dermatology*, 15(5), pp.331–341.

Schubert, A.M., Sinani, H. & Schloss, P.D., 2015. Antibiotic-Induced Alterations of the Murine Gut Microbiota and Subsequent Effects on Colonization Resistance against Clostridium difficile. *mBio*, 6(4), p.e00974.

Sekirov, I. et al., 2010. Gut microbiota in health and disease. *Physiological reviews*, 90(3), pp.859–904.

Sellon, R.K. et al., 1998. Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. *Infection and Immunity*, 66(11), pp.5224–5231.

Shah, N. et al., 2012. Interleukin-10 and interleukin-10-receptor defects in inflammatory bowel disease. *Current allergy and asthma reports*, 12(5), pp.373–9.

Shanahan, F. & Bernstein, C.N., 2009. The evolving epidemiology of inflammatory bowel disease. *Current Opinion in Gastroenterology*, 25(4), pp.301–305.

Shin, J. et al., 2016. Analysis of the mouse gut microbiome using full-length 16S rRNA amplicon sequencing. *Scientific Reports*, 6(1), p.29681.

da Silva, E.Z.M., Jamur, M.C. & Oliver, C., 2014. Mast cell function: a new vision of an old cell. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society*, 62(10), pp.698–738.

Simreń, M. et al., 2013. Intestinal microbiota in functional bowel disorders: A Rome foundation report. *Gut*, 62(1), pp.159–176.

Sommer, F. & Bäckhed, F., 2013. The gut microbiota--masters of host development and physiology. *Nature reviews. Microbiology*, 11(4), pp.227–38.

Soret, R. et al., 2010. Short-Chain Fatty Acids Regulate the Enteric Neurons and Control Gastrointestinal Motility in Rats. *Gastroenterology*, 138(5), p.1772–1782.e4.

St John, A.L. & Abraham, S.N., 2013. Innate immunity and its regulation by mast cells. *Journal of immunology (Baltimore, Md. : 1950)*, 190(9), pp.4458–63.

Strober, W., 2008. Why study animal models of IBD? *Inflammatory bowel diseases*, 14 Suppl 2, pp.S129-31.

Strober, W., Fuss, I.J. & Blumberg, R.S., 2002. The immunology of mucosal models of inflammation. *Annual review of immunology*, 20, pp.495–549.

Supajatura, V. et al., 2002. Differential responses of mast cell Toll-like receptors 2 and 4 in allergy and innate immunity. *The Journal of clinical investigation*, 109(10), pp.1351–1359.

Supajatura, V. et al., 2001. Protective Roles of Mast Cells Against Enterobacterial Infection Are Mediated by Toll-Like Receptor 4. *The Journal of Immunology*, 167(4), pp.2250–2256.

Suryaprasad, A.G. & Prindiville, T., 2003. The biology of TNF blockade. *Autoimmunity Reviews*, 2(6), pp.346–357.

Sutherland, D.B., Suzuki, K. & Fagarasan, S., 2016. Fostering of advanced mutualism with gut microbiota by Immunoglobulin A. *Immunological Reviews*, 270(1), pp.20–31.

Swidsinski, A. et al., 2005. Spatial organization and composition of the mucosal flora in patients with inflammatory bowel disease. *Journal of clinical microbiology*, 43(7), pp.3380–9.

Takeda, K. & Akira, S., 2005. Toll-like receptors in innate immunity. *International Immunology*, 17(1), pp.1–14.

Takeuchi, O. & Akira, S., 2010. Pattern Recognition Receptors and Inflammation. *Cell*, 140(6), pp.805–820.

Thompson-Chagoyán, O.C., Maldonado, J. & Gil, A., 2005. Aetiology of inflammatory bowel disease (IBD): Role of intestinal microbiota and gut-associated lymphoid tissue immune response. *Clinical Nutrition*, 24(3), pp.339–352.

Thoreson, R. & Cullen, J.J., 2007. Pathophysiology of Inflammatory Bowel Disease: An Overview. *Surgical Clinics of North America*, 87(3), pp.575–585.

Tracey, D. et al., 2008. Tumor necrosis factor antagonist mechanisms of action: A comprehensive review. *Pharmacology and Therapeutics*, 117(2), pp.244–279.

Tsai, M. et al., 1991. Induction of mast cell proliferation, maturation, and heparin synthesis by the rat c-kit ligand, stem cell factor. *Proceedings of the National Academy of Sciences*, 88(14), pp.6382–6386.

Urb, M. & Sheppard, D.C., 2012. The role of mast cells in the defence against pathogens. *PLoS pathogens*, 8(4), p.e1002619.

Vaishnava, S. et al., 2008. Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. *Proceedings of the National*

Academy of Sciences of the United States of America, 105(52), pp.20858–20863.

Valatas, V., Bamias, G. & Kolios, G., 2015. Experimental colitis models: Insights into the pathogenesis of inflammatory bowel disease and translational issues. *European Journal of Pharmacology*, 759, pp.253–264.

Vukman, K. V et al., 2012. Mast cells cultured from IL-3-treated mice show impaired responses to bacterial antigen stimulation. *Inflammation research : official journal of the European Histamine Research Society ... [et al.]*, 61(1), pp.79–85.

Waldner, M.J. & Neurath, M.F., 2014. Master regulator of intestinal disease: IL-6 in chronic inflammation and cancer development. *Seminars in immunology*, 26(1), pp.75–9.

Walker, A.W. et al., 2011. High-throughput clone library analysis of the mucosa-associated microbiota reveals dysbiosis and differences between inflamed and non-inflamed regions of the intestine in inflammatory bowel disease. *BMC microbiology*, 11, p.7.

Walters, W. a, Xu, Z. & Knight, R., 2014. Meta-analyses of human gut microbes associated with obesity and IBD. *FEBS letters*, 588(22), pp.4223–4233.

Wang, M. et al., 2005. Comparison of bacterial diversity along the human intestinal tract by direct cloning and sequencing of 16S rRNA genes. *FEMS microbiology ecology*, 54(2), pp.219–31.

Ward, M.A. et al., 2016. Insights into the pathogenesis of ulcerative colitis from a murine model of stasis-induced dysbiosis, colonic metaplasia, and genetic susceptibility. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, 310(11), pp.G973–G988.

Ward, N.L. et al., 2016. Antibiotic Treatment Induces Long-lasting Changes in the Fecal Microbiota that Protect Against Colitis. *Inflammatory bowel diseases*, 22(10), pp.2328–40.

Wernersson, S. & Pejler, G., 2014. Mast cell secretory granules: armed for battle. *Nature reviews. Immunology*, 14(7), pp.478–94.

Westerberg, C.M., Ullerås, E. & Nilsson, G., 2012. Differentiation of mast cell subpopulations from mouse embryonic stem cells. *Journal of immunological methods*, 382(1–2), pp.160–6.

Williams, C.M. & Galli, S.J., 2000. Mast cells can amplify airway reactivity and features of chronic inflammation in an asthma model in mice. *The Journal of experimental medicine*, 192(3), pp.455–462.

Willing, B.P. et al., 2010. A Pyrosequencing Study in Twins Shows That Gastrointestinal Microbial Profiles Vary With Inflammatory Bowel Disease Phenotypes. *Gastroenterology*, 139(6), p.1844–1854.

De Winter, B.Y., van den Wijngaard, R.M. & de Jonge, W.J., 2012. Intestinal mast cells in gut inflammation and motility disturbances. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, 1822(1), pp.66–73.

Wirtz, S. et al., 2007. Chemically induced mouse models of intestinal inflammation. *Nature Protocols*, 2(3), pp.541–546.

Wright, S.H. et al., 2002. Transforming growth factor-b1 mediates coexpression of the integrin subunit a. *Clin Exp All*, 31, pp.315–324.

Xie, X. et al., 2012. Intracellular Staphylococcus aureus-induced NF-κB activation and proinflammatory responses of P815 cells are mediated by NOD2. *Journal of Huazhong University of Science and Technology. Medical sciences = Hua zhong ke ji da xue xue bao. Yi xue Ying De wen ban = Huazhong keji daxue xuebao. Yixue Yingdewen ban*, 32(3), pp.317–23.

Xing, W. et al., 2011. Protease phenotype of constitutive connective tissue and of induced mucosal mast cells in mice is regulated by the tissue. *Proceedings of the National Academy of Sciences of the United States of America*, 108(34), pp.14210–14215.

Zhang, M. et al., 2017. Interactions between Intestinal microbiota and host immune response in inflammatory bowel disease. *Frontiers in Immunology*, 8(AUG), pp.1–13.