PREDISPOSICIÓN GENÉTICA A LA INFLAMACIÓN Y TRASLOCACIÓN BACTERIANA EN ENFERMEDAD HEPÁTICA E INTESTINAL

TESIS DOCTORAL

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Dr. Rubén Francés Guarinos, Profesor titular de la Universidad Miguel Hernández de Elche,

CERTIFICA que el trabajo de investigación que lleva por título "Predisposición genética a la inflamación y traslocación bacteriana en Enfermedad Hepática e Intestinal", presentado por Dña. Paula Piñero Romero para optar al grado de Doctor, ha sido realizado bajo su dirección en el Instituto de Biología Molecular y Celular de la Universidad Miguel Hernández de Elche. Considerando que la presente tesis se halla concluida, **AUTORIZA** su presentación para que pueda ser juzgada por el tribunal correspondiente.

Y para que así conste a los efectos oportunos, se expide el presente escrito.

El presente trabajo es presentado junto con los artículos:

Autores: Paula Piñero, Oriol Juanola, Esther Caparrós, Pedro Zapater, Paula Giménez, José M González-Navajas, José Such, Francés R.

Título: Toll-like receptor polymorphisms compromise the inflammatory response against bacterial antigen translocation in cirrhosis.

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Empleados como indicio de calidad para la presentación de la tesis doctoral y cumpliendo así con la normativa del RD99/2011 y en la normativa vigente de la Universidad Miguel Hernández.

Dr. Ricardo Mallavia Marin, Catedrático y Coordinador del Programa de Doctorado en Biología Molecular y Celular del Instituto de Investigación, Desarrollo e Innovacion en Biotecnología Sanitaria de Elche de la Universidad Miguel Hernández de Elche.

DA SU CONFORMIDAD a la lectura de tesis doctoral titulada: "*Predisposición genética a la inflamación y la traslocación bacteriana en enfermedad hepática e intestinal*", presentada por Dña. Paula Piñero Romero.

Y para que así conste a los efectos oportunos, se expide el presente escrito

Intenta aprender algo sobre todo y todo sobre algo.
Thomas Huxley

ABREVIATURAS

ADN Ácido Desoxirribonucleico
ARN Ácido Ribonucleico
BSA Bovine Serum Albumin
CD Cluster of Differentiation
CPA Célula Presentadora de Antígenos
CPG Cytosine Phosphate Guanine
E.COLI Escherichia coli
EC Enfermedad de Crohn
EII Enfermedad Inflamatoria Intestinal
ELISA Enzyme- Linked ImmunoSorbent Assay
HRP Horseradish Peroxidase
IFN Interferón
IG Inmunoglobulina
IL Interleucina
IRF Interferon Regulatory Factor
JAK Janus Kinase
K3EDTA Tripotassium Ethylenediaminetetraacetic Acid
KDA Kilodalton
LA Líquido Ascítico
LPS Lipopolisacárido
LRR Leucine-rich Repeat
LTA Ácido Lipoteicoico
MyD88 Myeloid Differentiation primary response protein 88
NF-κB Nuclear Factor Kappa B
NG Nanogramo
NM Nanometro
NOX Nitrogen Oxides
PB Pares de Bases
PBE Peritonitis Bacteriana Espontánea
PBMC Peripheral Blood Mononuclear Cell
PCR Polymerase Chain Reaction
PMN Polimorfonuclear
PVDF Polifluoruro de vinilideno
RS Reference SNP ID number
SDS Sodium dodecyl sulfate
SN Sobrenadante
SNP Single Nucleotide Polymorphism
SST Serum separator tube
STAT Signal Transducer and Activator of Transcription
TB Traslocación bacteriana
TBE Tris-Borate-EDTA buffer
TBS Tris Buffered Saline
TIR Toll/IL-1 Receptor
TRAM TRIF- Related Adaptor Molecule
TIRAP TIR domain Containing Adaptor Protein
TLR Toll- Like Receptor
TNF Tumor Necrosis Factor
Treg Célula T reguladora
TRIF TIR-domain containing adaptor- Inducing Interferon
TYK TyrosineKinase
UFC Unidad Formadora de Colonias
WT Wild Type
MAPK Mitogen Activated Protein Kinase

ÍNDICE

RESUMEN

La traslocación bacteriana (TB) se define como el paso de bacterias o sus productos desde la luz intestinal a los ganglios linfáticos mesentéricos, llegando a la sangre y otros órganos. Los episodios de TB son eventos recurrentes en pacientes con trastornos inflamatorios crónicos desarrollados alrededor del llamado eje hígado-intestino, desde la cirrosis hepática hasta la enfermedad inflamatoria intestinal.

El paso de antígenos bacterianos comensales a la sangre de pacientes con cirrosis descompensada es un fenómeno que desencadena complicaciones clínicas relevantes. La respuesta del huésped a estos antígenos se establece a través de receptores y citoquinas que activan y facilitan una respuesta inmune completa y eficiente. Entre estos receptores, es particularmente relevante la familia de receptores tipo Toll (TLR). Los TLR comprenden diferentes receptores que se unen específicamente a productos bacterianos únicos, activando los mecanismos necesarios para la eliminación de dichos productos.

Se han descrito varios polimorfismos en estos genes TLR asociados con alteraciones inmunes o complicaciones clínicas en la cirrosis y otros trastornos relacionados con la inflamación. Nuestro objetivo fue evaluar el efecto de los polimorfismos en receptores Toll-like en la respuesta soluble frente a los episodios de traslocación bacteriana.

En una primera parte del estudio, distribuimos pacientes, con cirrosis y ascitis, en función de la presencia de los SNPs TLR2 rs4696480, TLR4 rs4986790 y TLR9 rs187084. Se cuantificaron los niveles de diferentes antígenos bacterianos, citocinas proinflamatorias, óxido nítrico y se evaluó la respuesta in vitro de los 3 receptores frente a sus ligandos específicos. Los genotipos variantes de TLR-2, TLR-4 y TLR-9 se asociaron con un aumento significativo de los niveles de antígenos, mientras que las concentraciones de TNF-α, IL-6 y óxido nítrico disminuyeron significativamente en todos los pacientes con genotipos TLR variantes.

De manera similar, la traslocación del ADN bacteriano (ADNbact) a la sangre también es un evento clínicamente relevante en hasta el 40% de los pacientes con enfermedad de Crohn (EC). Esta enfermedad se debe a una interacción desequilibrada entre la microbiota y el sistema inmunitario de la mucosa intestinal en individuos genéticamente predispuestos, lo que lleva al desarrollo de un ambiente inflamatorio sostenido. La traslocación de ADNbact se ha asociado con un aumento de la actividad de esta enfermedad, y constituye un factor de riesgo de brote a corto plazo.

El aclaramiento antigénico se consigue como resultado de una respuesta inflamatoria en cascada, en la que ocupan un papel relevante las citocinas. La IL26, de la familia IL-20, es una citocina destacada en la EC. Esta molécula facilita la destrucción de microbios, detecta el ADNbact extracelular y promueve una potente respuesta proinflamatoria al inducir la secreción de interferón (IFN) -α por parte de las células plasmocitoides dendríticas (pDC). En ausencia de IL26 el aclaramiento de ADNbact podría verse comprometido y promover la aparición de brotes en la enfermedad.

Diversos estudios de asociación genómica han identificado SNPs en el gen de la IL26 asociados con la EII. Con dichos antecedentes, en una segunda parte del estudio, decidimos evaluar la relación entre los niveles séricos de IL26 y la traslocación de ADNbact en la enfermedad de Crohn (EC). Realizamos un estudio prospectivo en pacientes con enfermedad de Crohn en remisión en el que se evaluaron polimorfismos comunes en el gen de la IL26, niveles de citocinas séricas, proteínas del complemento, ADNbact amplificado y anti-TNF-α.

La presencia de ADN bacteriano en suero se correlacionó significativamente con un aumento en los niveles de IL26. Las PBMCs de los pacientes con genotipos variantes mostraron una capacidad bactericida reducida en comparación con los pacientes con genotipo salvaje. La estimulación con una proteína IL26 recombinante redujo la presencia de citocinas proinflamatorias en respuesta a E. coli en las células procedentes de pacientes con genotipos variantes. Los niveles de anti-TNF-α, medidos en el suero de pacientes con genotipo varIL26 frente a wtIL26, fueron significativamente más bajos en presencia de ADNbact. Las células de los pacientes con genotipos polimórficos frente a los de genotipo salvaje, cultivadas con ADN de E. coli e infliximab, mostraron una disminución significativa en la concentración de fármaco libre.

Los antecedentes genéticos pueden ser un potencial modulador de la respuesta inmune en las complicaciones derivadas de las bacterias en los trastornos inflamatorios crónicos que involucran el eje del intestino-hígado. Los episodios de translocación bacteriana han mostrado una gran relevancia en el desarrollo y mantenimiento de la inflamación crónica en el eje hígado-intestino. Las variantes alélicas en genes que interaccionan con estos productos modifican la capacidad de respuesta inmunológica de los pacientes, complicando el curso de la enfermedad inflamatoria.

ABSTRACT

Bacterial translocation (BT) is defined as the passage of bacteria or their products from the intestinal lumen to the mesenteric lymph nodes, reaching the blood and other organs. The episodes of BT are recurrent events in patients with chronic inflammatory disorders developed around the so-called gut-liver axis, from liver cirrhosis to inflammatory bowel disease.

The passage of commensal bacterial antigens to the blood of patients with decompensated cirrhosis is a phenomenon that triggers relevant clinical complications. The host response to these antigens is established through receptors and cytokines that activate a complete and efficient immune response. Among these receptors, the family of Toll-like receptors (TLR) is particularly relevant. The TLRs comprise different receptors that bind specifically to unique bacterial products, activating the necessary mechanisms for the elimination of such products.

Several polymorphisms have been described in these TLR genes associated with immune alterations or clinical complications in cirrhosis and other disorders related to inflammation. In the first study, our aim was to evaluate the effect of polymorphisms on Toll-like receptors in the soluble response against episodes of bacterial translocation.

We distributed patients, with cirrhosis and ascites, depending on the presence of the SNPs TLR2 rs4696480, TLR4 rs4986790 and TLR9 rs187084. The levels of different bacterial antigens, proinflammatory cytokines and nitric oxide were quantified and the in vitro response of the 3 receptors against their specific ligands was evaluated. The variant genotypes of TLR-2, TLR-4 and TLR-9 were associated with a significant increase in antigen levels, while the concentrations of TNF-α, IL-6 and nitric oxide decreased significantly in all patients with genotypes TLR variants.

Similarly, the translocation of bacterial DNA (bactDNA) to the blood is also a clinically relevant event in up to 40% of patients with Crohn's disease (CD). This disease is due to an unbalanced interaction between the microbiota and the immune system of the intestinal mucosa in genetically predisposed individuals, which leads to the development of a sustained inflammatory environment. The translocation of bactDNA has been associated with an increase in the activity of this disease, and constitutes a risk factor for short-term outbreak.

Antigenic clearance is achieved as a result of a cascade inflammatory response, in which cytokines play an important role. IL26, member of the IL-20 family, is a prominent cytokine in CD. This molecule facilitates the destruction of microbes, detects extracellular bactDNA and promotes a potent proinflammatory response by inducing the secretion of interferon (IFN) -α by dendritic plasma cells (pDC). In the absence of IL26, the clearance of bactDNA could be compromised and promote the appearance of outbreaks in the disease.

Several genomic association studies have identified SNPs in the IL26 gene associated with IBD. With this background, in a second part of the study, we decided to evaluate the relationship between the serum levels of IL26 and the translocation of bactDNA in CD. In the second investigation, we conducted a prospective study in CD patients in remission. We evaluated common polymorphisms in the IL26 gene, serum cytokine levels, complement proteins, amplified bactDNA and anti-TNF-α.

The presence of bacterial DNA in serum correlated with an increase in IL26 levels. The PBMCs of patients with variant genotypes showed a reduced bactericidal capacity compared to patients with wild genotype. Stimulation with a recombinant IL26 protein reduced the presence of proinflammatory cytokines in response to E. coli in cells from patients with variant genotypes. The levels of anti-TNF-α, measured in the serum of patients with varIL26 versus wtIL26 genotypes, were significantly lower in the presence of ADNbact. Cells of patients with polymorphic genotypes versus those of wild type genotype, cultured with E. coli DNA and infliximab, showed a significant decrease in free drug concentration.

In clonclusion, the genetic background can be a potential modulator of the immune response in the complications derived from bacteria in chronic inflammatory disorders involving the gut-liver axis. Episodes of bacterial translocation have shown great relevance in the development and maintenance of chronic inflammation in the gut-liver axis.

INTRODUCCIÓN

1.1. Traslocación bacteriana

La traslocación bacteriana (TB) se define como el paso de bacterias o sus productos desde la luz intestinal hacia los ganglios linfáticos mesentéricos (GLM) (1). Esta migración se considera un fenómeno fisiológico poco frecuente en individuos sanos, controlado de forma eficaz por la inmunidad intestinal. Los episodios de TB en pacientes con trastornos inflamatorios crónicos en el entorno del eje hígado-intestino son, sin embargo, más frecuentes y están relacionados con muchas de las complicaciones que sufren estos pacientes (2, 3).

Los cambios en la composición de la microbiota intestinal durante la progresión de la enfermedad, el incremento de la permeabilidad de la barrera intestinal y la alteración de la respuesta inflamatoria se consideran los principales mecanismos que inducen esta tasa aumentada de TB.

La comprensión de la interacción entre la microbiota intestinal y el Sistema Inmunitario ha crecido enormemente en los últimos años. Dos ejemplos de patologías en las que esta interacción se ha revelado fundamental son la cirrosis, como estadio final de la inflamación hepática crónica, y la enfermedad inflamatoria intestinal crónica.

1.1.1. Traslocación bacteriana en la Cirrosis hepática

La cirrosis es el estadio final de todas las enfermedades hepáticas crónicas progresivas. Es un proceso difuso caracterizado por la pérdida de parénquima hepático y la formación de septos fibrosos y

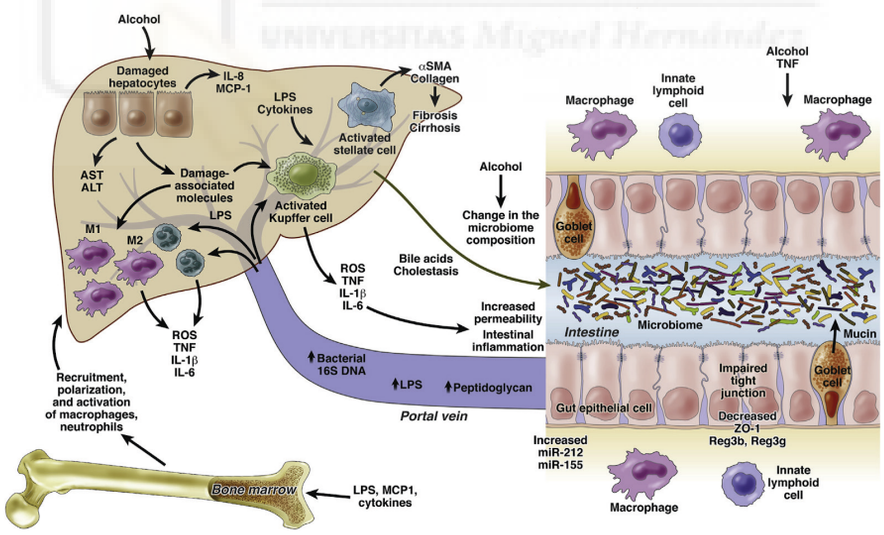
nódulos de regeneración, que alteran la arquitectura y anatomía vascular normal del hígado (4).

En el transcurso de esta enfermedad se distinguen dos fases muy diferenciadas. La primera de ellas se conoce como cirrosis compensada, no manifiesta síntomas destacados y puede prolongarse durante años. Por otra parte, en la fase más avanzada de la enfermedad, se produce un fenómeno conocido como descompensación (cirrosis descompensada), que viene acompañado de una serie de complicaciones graves, entre las cuales se encuentra el desarrollo de infecciones bacterianas.

Las infecciones bacterianas suponen una de las complicaciones más relevantes en los pacientes con cirrosis avanzada, siendo las más frecuentes la PBE, seguida por las infecciones del tracto urinario y la neumonía (5). La PBE consiste en una infección bacteriana del líquido ascítico (LA) que se origina sin que existan focos infecciosos intraperitoneales (fístulas, abscesos...) que la inicien (6). Sin embargo, la llegada de bacterias al LA no siempre ha de desembocar en el desarrollo de una PBE, pues el LA tiene cierta capacidad bactericida para enfrentarse a estos accesos.

Según varios estudios, existe cierta predisposición al desarrollo de una PBE, asociado a aquellos pacientes que presentan niveles bajos de proteínas o fracción C3 del complemento en LA entre otros factores (7, 8), teniendo por tanto mermada la capacidad bactericida.

El mecanismo patogénico clave que inicia la bacteriemia y posteriormente el desarrollo de una PBE es la TB (9), debida mayoritariamente al sobrecrecimiento de bacterias intestinales (10) y la disfunción del sistema inmune, generada principalmente por una disminución en la actividad bactericida por parte de las células fagocíticas (11). La respuesta inmunitaria proinflamatoria del huésped tiene una gran influencia sobre el estado hemodinámico del paciente con cirrosis y ascitis y por tanto un papel decisivo en el pronóstico de la PBE (12).



(44) Figura 1. Principales eventos en el eje intestino-hígado en la cirrosis hepática.

La traslocación de productos bacterianos desde el intestino a la circulación mesentérica induce la producción local y sistémica de TNF y otras citocinas proinflamatorias (13), situación que empeora cuanto más dañada está la función hepática, pues mayor es el nivel de ADN bacteriano y endotoxina en la circulación portal (14, 15) (Figura 1).

A su vez, la activación del óxido nítrico y el incremento en los niveles séricos de citocinas contribuyen a la vasodilatación sistémica, el incremento del gasto cardíaco y la disminución de la presión arterial media, lo que desemboca en la aparición de varices esofágicas, ascitis y síndrome hepatorrenal (16, 17), otras de las complicaciones habituales en estadios avanzados de la enfermedad.

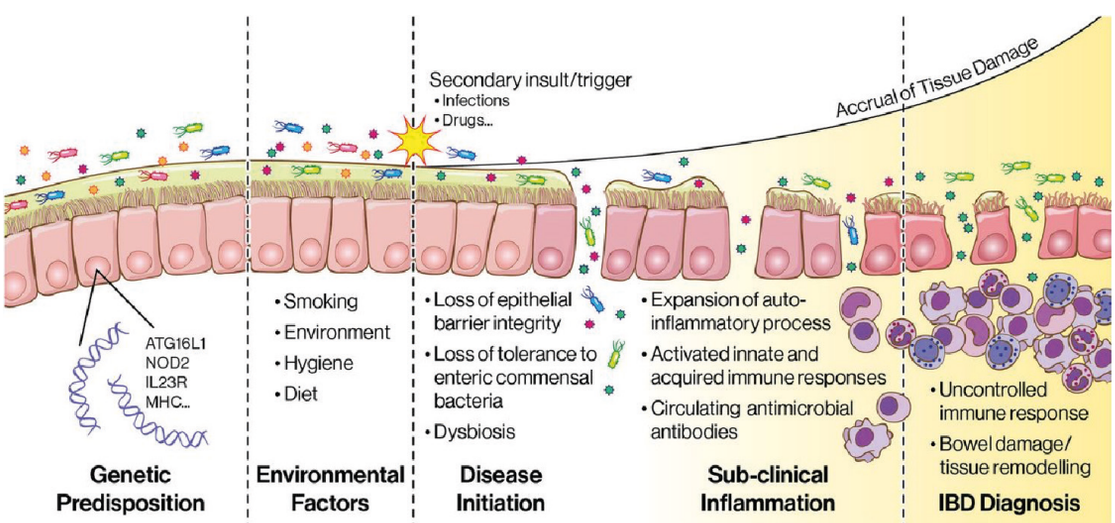
1.1.2. Traslocación bacteriana en la Enfermedad de Crohn

La enfermedad de Crohn (EC), junto con la colitis ulcerosa, se engloba dentro de los trastornos que componen la denominada enfermedad inflamatoria intestinal (EII). Se trata de un trastorno inflamatorio de origen desconocido que puede afectar a cualquier parte del tubo digestivo, desde la boca hasta el ano, de forma segmentaria (18).

Al igual que ocurre con la cirrosis, la presencia de antígenos bacterianos en la sangre de estos pacientes es altamente frecuente. De hecho, se ha demostrado la presencia de ADN bacteriano en sangre periférica hasta en un 40% de individuos con EC (19, 20). Así mismo, la presencia de ADN bacteriano se ha relacionado con una mayor actividad de la enfermedad, llegando a mostrarse como un factor de riesgo independiente en la aparición de brotes a corto plazo (21).

Aún no se conoce la causa exacta de la EII. Sin embargo, parece originarse por una respuesta inmune inadecuada frente a la microbiota intestinal en individuos con cierta predisposición genética (22), probablemente impulsada por variantes alélicas en genes que regulan el reconocimiento microbiano, las vías de señalización del sistema inmune innato o la autofagia (23). De hecho, tanto en ratón como humano, todas las mutaciones en genes que controlan el reconocimiento inmunológico innato, la inmunidad adaptativa y la permeabilidad epitelial se encuentran asociadas con la inflamación intestinal (24), lo que contribuye a la traslocación de productos antigénicos bacterianos, a su vez estrechamente relacionados con una respuesta inflamatoria sostenida en pacientes con EC (25). A la presencia de dichas alteraciones genéticas se suman determinados factores ambientales que parecen contribuir sustancialmente al desencadenamiento de la enfermedad entre los que destacan el tabaco, el uso prolongado de ciertos fármacos y las dietas ricas en azucares refinados (Figura 2).

La producción inicial de citocinas en esta enfermedad se rige por los patrones de diferenciación de las células T imperantes en la misma y se caracteriza por su inclinación hacia el desarrollo de una respuesta de tipo Th1 y Th17, produciéndose IFN-γ e IL-17/ IL-22 (26). La EC parece estar asociada a un desequilibrio en el sistema inmune que lo incline hacia el desarrollo de este tipo de respuesta de forma incontrolada.



(41) Figura 2. Modelo de patogénesis y progresión de la EII.

1.2. Patogenia de la TB

Los factores determinantes en la aparición de traslocación bacteriana son, junto con la aparición de desequilibrios en la microbiota intestinal y el aumento de la permeabilidad intestinal, la presencia de defectos en el sistema inmunitario del hospedador; que pueden ser adquiridos o determinados por susceptibilidad genética (9, 27).

Las alteraciones en la flora intestinal pueden presentarse a nivel tanto cuantitativo (sobrecrecimiento bacteriano) como cualitativo (disbiosis). El sobrecrecimiento bacteriano es característico de los pacientes con cirrosis y, en aquellos pacientes en los que ocurre, es más frecuentemente la aparición de peritonitis bacteriana espontánea (PBE) (28). Del mismo modo, la disbiosis intestinal también ha sido descrita en pacientes con varios trastornos gastrointestinales, entre ellos la enfermedad de Crohn (29).

El aumento de la permeabilidad intestinal observado en los enfermos de cirrosis (30, 31), que ocurre así mismo en pacientes con EC, además de otros trastornos inflamatorios (32, 33), se debe a un conjunto de alteraciones en el epitelio intestinal. Estas alteraciones se encuentran tanto a nivel estructural, quedando demostrado por varios estudios que disminuyen los niveles de expresión de las proteínas ‘’tight junction” (TJ) en ambos trastornos (34-37), como en sus propiedades secretoras y mecánicas, reflejadas en un aumento del grosor de la capa de mucosidad natural en el duodeno (38) y disfunciones en la motilidad intestinal respectivamente (39).

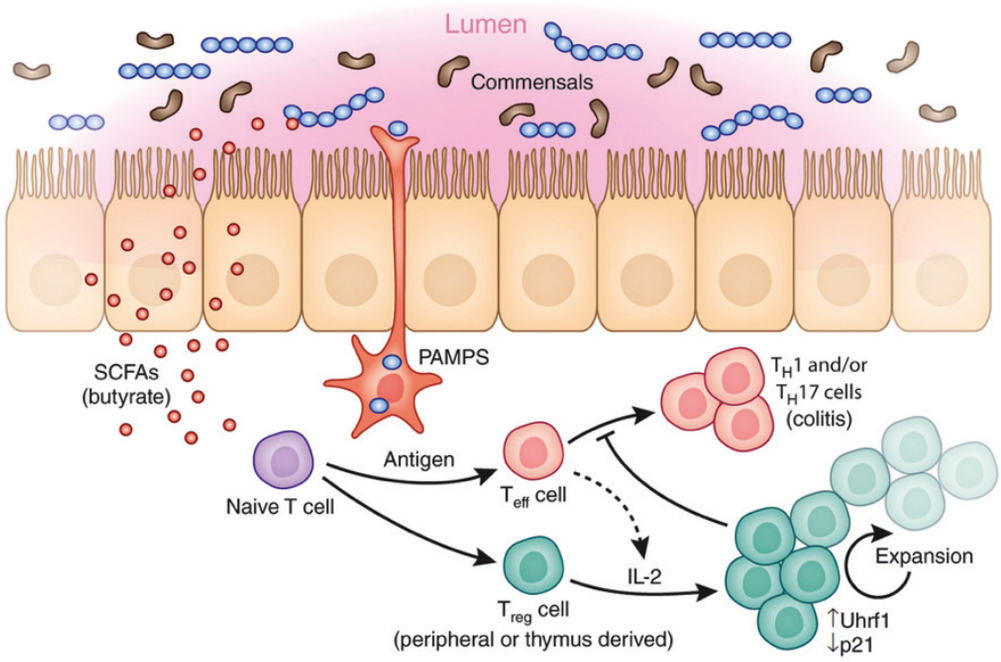
1.3. El sistema inmune en la TB:Respuesta inmunitaria y aclaramiento antigénico

El sistema inmune es el encargado de detectar la presencia de antígenos bacterianos y otras moléculas potencialmente peligrosas para el organismo, así como de generar una respuesta adecuada y eficiente que garantice su eliminación.

La pared intestinal sirve como frontera entre el contenido intestinal, en el que se identifican múltiples moléculas potencialmente inmunogénicas; intrínsecas a la microbiota entérica, y el resto del organismo. El sistema inmune intestinal ha de mantener un estado de tolerancia permanente frente la microbiota comensal, sin perder la habilidad de combatir posibles patógenos y sus productos. Este equilibrio se consigue mediante la regulación reciproca de células CD4+ efectoras y células T reguladoras (Figura 3).

En la EC este equilibrio se pierde y las células T proinflamatorias superan en número a las Treg, con propiedades antiinflamatorias (40).

Del mismo modo, el hígado es un órgano estratégicamente situado que ha de mantener un delicado equilibrio entre inmunidad y tolerancia, necesaria frente a la continua exposición de productos intestinales derivados de la circulación portal. Su morfología interna, configurada por sinusoides, hace que la sangre circule lentamente, permitiendo la interacción entre las sustancias derivadas del intestino y las células hepáticas, posibilitando la inducción de tolerancia antígeno-específica (42).



(57) Figura 3. Interacción entre la microbiota intestinal y las células T colónicas.

Por otra parte, las varias poblaciones de células dendríticas presentes en este órgano se encuentran en estado inmaduro y por tanto tienen una capacidad reducida para impulsar la activación de las células T. Además, en un hígado sano predomina un microambiente antiinflamatorio con altos niveles de IL-10 producida por las mismas células presentadoras de antígeno (CPAs) residentes (43).

La cirrosis, perturba el papel homeostático del hígado en la respuesta inmune sistémica, que podría verse afectada asimismo por la presencia de otras alteraciones agravando aún más la condición de esta enfermedad.

1.3.1. Reconocimiento antigénico

La primera barrera de defensa frente a cualquier tipo de agente infectivo es la constituida por el sistema inmune innato, cuya primera función consiste en distinguir entre el propio organismo y cualquier variedad de patógeno potencialmente peligroso para el mismo. Esta misión se cumple a través de una amplia gama de receptores capaces de detectar de forma específica todo tipo de moléculas antigénicas. Existen varios grupos de receptores encargados de estas funciones, entre los cuales tienen un papel destacado los receptores Toll like (TLRs).

Los TLRs son glicoproteínas integrales de membrana caracterizadas por la presencia de un número variable de motivos ricos en leucina repetidos (LRR) en su dominio extracelular y un dominio de señalización intracelular, homologo al del receptor de la IL-1 (IL-1R), denominado TIR (46).

Se trata de una familia de receptores de superficie e intracelulares con 10 miembros funcionales en humanos y 13 en ratón (45), cada uno de los cuales reconoce de forma específica determinados antígenos microbianos (Tabla 1).

Tabla 1. Localización celular y ligandos específicos de los TLRs.

TLR1 (with TLR2)	Cell membrane	MyD88/TIRAP	Triacyl lipopeptides Soluble factors	Bacteria, mycobacteria *Neisseria meningitidis*
TLR2 (with TLR1 or TLR6)	Cell membrane	MyD88/TIRAP	Lipoproteins, lipopeptides Lipoteichoic acid Peptidoglycan Lipoarabinomannan Phenol-soluble modulin, porins Atypical LPS Glycoinositolphospholipids, glycolipids Beta-glucan, mannan Core and NS3 proteins, dUTPase, glycoproteins HSP70	Various pathogens Gram-positive bacteria Bacteria Mycobacteria *Staphylococcus epidermidis*, *Neisseria* *Leptospira interrogans*, *Porphyromonas gingivalis* *Trypanozoma*, *Toxoplasma*, *Plasmodium* Fungi Hepatitis virus, Epstein–Barr vi Cytomegalovirus Host
TLR3	Endolysosomal	TRIF	Double-stranded RNA	Viruses
TLR4	Cell membrane and endolysosomal	MyD88/TIRAP, TRIF/TRAM	LPS O-linked mannan Taxol Fusion and envelope protein HSP60 HMGB1, HSP70, fibronectin, fibrinogen	Gram-negative bacteria Fungi Plants Respiratory syncytial virus, mo mammary tumor virus *Chlamydia pneumoniae* Host
TLR5	Cell membrane	MyD88	Flagellin	Flagellated bacteria
TLR6 (with TLR2)	Cell membrane	MyD88/TIRAP	Diacyl lipopeptides, lipoteichoic acid, β-glucan	*Mycoplasma*, Gram-positive bacteria, fungi
TLR7	Endolysosomal	MyD88	Single-stranded RNA Imidazoquinoline, loxoribine, bropirimine	Viruses, bacteria Synthetic compounds
TLR8	Endolysosomal	MyD88	Single-stranded RNA Imidazoquinoline	Viruses, bacteria Synthetic compounds
TLR9	Endolysosomal	MyD88	CpG-containing DNA Homozoin	Bacteria, viruses, fungi *Plasmodium falciparum*
TLR10 (±TLR1 or TLR2)	Cell membrane	MyD88	Lipopeptides (prediction)	
TLR11	Endolysosomal	MyD88	Flagellin	Flagellated bacteria
TLR12	Endolysosomal	MyD88	Profilin	Apicomplexan parasites
TLR13	Endolysosomal	MyD88	23S RNA	Bacteria

(47)

Muchos de estos receptores han demostrado tener una función relevante en ciertas enfermedades por lo que han sido ampliamente estudiados hasta hoy. En el ámbito de la cirrosis sobresale el papel que juegan los receptores TLR 2, TLR4 y TLR9.

TLR 2

Este receptor, cuyo gen codificante está situado en la posición 4q31.3, se encarga principalmente de la detección de bacterias Gram-positivas, al reconocer el ácido lipoteicoico (LTA) de su pared. Si bien no es este el único ligando del receptor, capaz de reconocer un elevado número de moléculas gracias a su habilidad para formar heterodímeros con el TLR 1 y el TLR 6 (48, 49)

TLR4

Al igual que el TLR 2, este receptor se localiza en la superficie celular. Sin embargo, al contrario que el anterior, se encarga de la detección de bacterias Gram-negativas. Su función es la de reconocer el LPS bacteriano, considerado el inductor más potente de citocinas inflamatorias, especialmente TNF, en monocitos y macrófagos (50). El gen codificante del TLR 4 se localiza en la posición cromosómica 9q33.1.

TLR9
El TLR 9 es un receptor de tipo intracelular que se encuentra en las membranas endosomales. Reconoce las islas CpG no metiladas presentes en el ADN bacteriano, activando por tanto al sistema inmune ante la presencia de cualquier tipo de bacterias. Este gen se encuentra en la posición 3p21.2. En ausencia de ligandos se encuentra en forma monomérica, produciéndose su homodimerización tras la unión a un ligando CpG no metilado que conducirá a su activación.

IL-26
Esta citocina comparte con el TLR9 la capacidad de reconocer el ADN bacteriano; pudiendo además formar complejos con éste, proceso que ha demostrado conferir inmunogenicidad al ADN (51, 52). Estos complejos son endocitados por las células dendríticas plasmacitoides (pDC) y desencadenan la producción de IFN-α vía activación del TLR9 (53).

En el contexto de las EIIs, cada día se perfila más claramente el papel destacado que ocupa la IL-26. Se trata de una proteína anfipática que destruye bacterias extracelulares mediante la formación de poros en su membrana. La expresión de esta molécula se limita únicamente a células T CD4+ y dentro de este grupo es producida principalmente por las células Th17, las cuales contribuyen a la patogénesis de muchas enfermedades inflamatorias autoinmunes como la artritis reumatoide (54) o la EII (55). Se ha descrito una elevada presencia de IL-26 en suero de pacientes con la EC frente a controles, así como un incremento en la expresión de ARNm de IL-26 en lesiones colónicas inflamadas, correlacionados estrechamente con los de IL-8 e IL-22 (56).

1.3.2. Respuesta inflamatoria

Una vez identificada la presencia de un insulto, el sistema inmunitario responde de manera específica. Cada estímulo propicia el comienzo de una cascada de señalización que culminará con la activación de diversos factores de transcripción que promoverán la expresión de los genes implicados en la respuesta.

La señalización a través de los TLR se produce mediante el reclutamiento inicial de moléculas adaptadoras con un dominio TIR (MyD88, TIRAP, TRIF o TRAM). Según cuál de estas moléculas participe en el proceso de señalización, se producirá la activación de la vía de señalización correspondiente: una vía dependiente de MyD88 (común a todos los TLRs, excepto el TLR3) y otra independiente (exclusiva de TLR3 y TLR4). Estas vías llevaran a la producción diferencial de citocinas proinflamatorias y/o interferones de tipo 1 (46, 47).

La expresión de los TLRs es especialmente significativa en las CPAs, entre las cuales tienen un papel primordial las células dendríticas ya que son las únicas que pueden inducir respuestas inmunes primarias, lo que permite el establecimiento de la memoria inmunológica (58). Dependiendo del tipo de célula dendrítica y de la señal transmitida a las células T CD4+ «naive», estas se diferenciarán en distintos subtipos de células Th, que se caracterizan por poseer diferentes perfiles de citoquinas y funciones efectoras (59).

Las citocinas son una amplia categoría de proteínas relativamente pequeñas (<40 kDa) que se producen y liberan con el objetivo final de la señalización celular (60). Dentro de esta amplia categoría, las interleucinas son la familia más importante de citoquinas liberadas durante los procesos infecciosos y según su función se dividen en dos grandes grupos: pro-inflamatorias y anti-inflamatorias.

Estas moléculas son reconocidas por receptores específicos a través de los cuales transmiten sus

mensajes a las correspondientes células diana. Sin embargo, algunas de ellas van más allá, pudiendo ejercer una acción directa, como es el caso de la IL-26, que tiene la capacidad de actuar como ligando del ADN bacteriano, llevando a la activación de la ruta de señalización del TLR9, como ya se ha mencionado anteriormente.

Así mismo, al igual que el resto de citocinas, la IL-26 también es reconocida por su receptor especifico, un complejo heterodimérico formado por dos subunidades: IL-10R2 e IL-20R1 que se expresa especialmente en células epiteliales (56).

La unión de la IL-26 a este receptor promueve la fosforilación de las kinasas JAK1 y Tyk2 y seguidamente los factores de transcripción STAT1 y STAT3 (61, 62) que promueven la secreción de IL-10 e IL-8 y facilitan la expresión de CD54 en la superficie de las células epiteliales (63).

1.4. Alteraciones genéticas en el sistema inmune: Polimorfismos y enfermedad

La respuesta frente a una infección depende de numerosos factores, entre ellos los polimorfismos genéticos, cuya presencia ha demostrado estar asociada con los resultados clínicos (64). Los polimorfismos son variaciones en la secuencia del ADN que aparece con una frecuencia superior al 1% en la población. Los más comunes dentro de esta categoría son los polimorfismos de un solo nucleótido o SNPs.

Se han identificado centenares de estas variantes genéticas que tienen impacto en el desarrollo de todo tipo de infecciones. A nivel de reconocimiento, destacan un gran número de SNPs localizados en los TLRs cuyo efecto provoca el aumento en la susceptibilidad a un amplio número de enfermedades infecciosas e inflamatorias (65), sepsis (66, 67) e incluso cáncer (68).

La elevada incidencia de infecciones bacterianas en la cirrosis hepática se debe en gran medida al estado de 'inmunodeficiencia' que presentan los pacientes que la padecen (69), en el cual podría ser determinante la influencia de polymorfismos en los TLRs así como los receptores tipo NOD (70).

El TLR4 ha sido ampliamente estudiado y se conozcan numerosos SNPs implicados en el transcurso de todo tipo de enfermedades. Las variantes más destacadas a nivel del TLR 4 son el rs4986790 (Asp299Gly) y el rs4986791 (Thr399Ile), las cuales se asocian con una respuesta deficiente frente al LPS (71) y por tanto un mayor riesgo de infecciones causadas por bacterias gram-negativas (72).

Los estudios realizados sobre TLR2 han descrito variantes que parecen atenuar la activación del NF-κB por parte de determinadas cepas bacterianas (73) y otras que se han asociado con un deterioro en la respuesta frente a diversas lipoproteínas bacterianas (74). En pacientes cirróticos se ha observado una mayor frecuencia en la aparición de PBE en portadores de variantes de riesgo en TLR2 (75).

Entre las variantes descritas en TLR9, el polimorfismo rs187084 parece ser funcionalmente relevante regulando negativamente su expresión (76). Además, su presencia se asocia con un mayor riesgo de infecciones tales como malaria (77) o hepatitis neonatal severa (78).

Igualmente tienen un papel destacado los polimorfismos presentes en genes codificantes de citocinas, los cuales se encuentran bien representados en el amplio catálogo de SNPs asociados con enfermedades, sobre todo relacionadas con la inflamación, descritos hasta la fecha (79).

Es especialmente destacado el rol que ocupan estas moléculas en el transcurso de la EII, abarcando desde su aparición hasta el término de las mismas. Para el mantenimiento de la homeostasis y la correcta función de la barrera intestinal es esencial el mantenimiento de un delicado equilibrio entre citocinas pro y anti-inflamatorias a lo largo del tubo digestivo (80). Estudios recientes aportan cada vez mayor relevancia al papel de la IL-26 en el desarrollo de enfermedades inflamatorias (54) y parece ser especialmente determinante su papel en la EII (55).

El gen de la IL-26 se encuentra en el cromosoma 12q15, entre los genes que codifican para otras dos importantes citocinas de la clase 2, el IFN-γ y la IL-22, que demás se transcriben en la misma dirección y comparten ciertos elementos regulatorios (81, 82). Varios grupos han identificado polimorfismos tanto en el gen codificante de la IL-26 como regiones colindantes que se encuentran asociados con un mayor riesgo de desarrollar ciertas enfermedades autoinmunes (83, 84). Entre todas estas asociaciones hay datos que indican una fuerte relación entre la presencia del polimorfismo rs2870946 y el desarrollo de EII (85).

HIPÓTESIS Y OBJETIVOS

Los episodios de traslocación bacteriana han mostrado una gran relevancia en el desarrollo y mantenimiento de la inflamación crónica en el eje hígado-intestino. Las variantes alélicas en genes que interaccionan con estos productos modifican la capacidad de respuesta inmunológica de los pacientes, complicando el curso de la enfermedad.

- Evaluar la incidencia de polimorfismos relevantes en los receptores TLR-2, TLR-4 y TLR-9 y valorar su efecto sobre la respuesta inflamatoria frente a episodios de traslocación de productos bacterianos en pacientes con cirrosis.

- Determinar el papel de los polimorfismos en el gen IL-26 en el aclaramiento sistémico de ADN bacteriano en pacientes con enfermedad de Crohn.

- Reforzar los eventos de TB como un elemento central común que altera la respuesta inmunológica, especialmente en individuos genéticamente predispuestos, en enfermedades inflamatorias crónicas relacionadas con el eje hígado-intestino.

MÉTODOS

3.1. Pacientes

3.1.1 Pacientes con cirrosis

La muestra de estudio la conformaron un total de 114 pacientes con cirrosis y liquido ascítico no infectado. El diagnóstico se llevo a cabo mediante los análisis hematológicas, bioquímicas y serológicas establecidos, junto a los que se realizó un examen físico, así como pruebas ecográficas complementarias en caso de ser requeridas.

Los criterios de exclusión, cuyo cumplimiento determinó la imposibilidad de participar en el estudio fueron:

- PBE (peritonitis bacteriana espontánea)
- Infecciones
- Carcinomas multinodulares
- Trombosis portal
- Hepatitis alcohólica
- Trasplante hepático previo
- Shunt portosistémico intrahepático transyugular

La PBE se definió como la presencia de más de 250 células polimorfonucleares por microlitro en el líquido ascítico.

Se obtuvieron muestras de sangre y líquido ascítico de todos los pacientes a su ingreso para llevar a cabo análisis bioquímicos y citológicos rutinarios. En todos los casos se realizaron cultivos de sangre y líquido ascítico, además de inocularse alícuotas de sangre y líquido ascítico en tubos estériles sellados Vacutainer SST II (BD Diagnostics, Erembodegem, Belgium).

El protocolo de este estudio contó con la aprobación del Comité Ético para la Investigación Clínica (CEIC) del Hospital General Universitario de Alicante.

3.1.2. Pacientes con enfermedad de Crohn

Se incluyeron pacientes con enfermedad de Crohn en remisión, diagnosticada de acuerdo a los estándares clínicos, endoscópicos, histológicos y radiográficos (86). La remisión se certificó en aquellos pacientes que presentaron un valor CDAI (Crohn's Disease Activity Index) inferior a 150 y la ausencia total de síntomas clínicos de recaída.

Como criterios de exclusión se establecieron:

- Infecciones
- Tratamiento con antibióticos 4 semanas previas a la obtención de muestras
- Rechazo a firmar el consentimiento informado

Todos los pacientes finalmente incluidos recibieron diarios para el registro de síntomas durante la semana previa a la toma de muestras. Así mismo, se recopilaron variables clínicas y analíticas habituales en el tratamiento de los pacientes con Enfermedad de Crohn.

En la inclusión se obtuvieron muestras de sangre para estudios hematológicos y bioquímicos rutinarios y se inocularon en frascos de hemocultivo aeróbico y anaeróbico de 10 mL cada uno. Simultáneamente se obtuvieron 2 muestras de sangre en condiciones asépticas y se depositaron en tubos estériles sellados Vacutainer SST II y K_3EDTA (BD Diagnostics, Erembodegem, Belgium).

El protocolo de este estudio contó con la aprobación del Comité Ético para la Investigación Clínica (CEIC) del Hospital General Universitario de Alicante.

3.2. Técnicas empleadas

3.2.1. Estudio genotípico

Se llevó a cabo una extracción de ADN genómico empleando el kit comercial QIAmp DNA Blood Minikit (Qiagen, Hilden, Germany) a partir de PBMCs (células mononucleares de sangre periférica) aisladas mediante separación por gradiente de densidad.

Los SNPs estudiados en los receptores Toll-like fueron: TLR4 rs4986791, localizado en el cromosoma 9q33.1; TLR9 rs187084, en el cromosoma 3p21.2 y TLR2 rs4696480, en el cromosoma 4q31.3.
En el caso del gen de la IL-26, situado en el cromosoma 12q15, examinamos los siguientes polimorfismos: rs2870946, rs1558744 y rs7134599.

Se llevó a cabo una amplificación parcial de las regiones genómicas en las que se encuentran estos polimorfismos mediante el empleo de primers específicos, cuyas secuencias se recogen en la Tabla 2. Una vez realizadas las PCRs y habiendo comprobado el éxito en la amplificación, procedimos a la purificación de los amplicones empleando ExoSAP-IL PCR Product Cleanup (Affymetrix).

La incidencia de los polimorfismos se detectó por secuenciación nucleotídica de los productos de PCR empleando los mismos primers anteriormente indicados. El proceso de secuenciación se realizó en el servicio de secuenciación de Secugen S.L. Los resultaros se analizaron con el software FinchTV versión 1.5 (Geospiza).

Los datos de incidencia de las variantes en población control sana se extrajeron de The 1000 Genomes Project Consortium (87).

Tabla 2. Primers empleados en el genotipado.

SNP	Gen	Forward	Reverse	PCR product
rs4986791	TLR 4	CTACCAAGCCTTGAGTTTCTAG	AAGCTCAGATCTAAATACCT	110 pb
rs187084	TLR 9	CATTCATTCAGCCTTCACTC	ATGTGCTGTTCCCTCTGC	419 pb
rs4696480	TLR 2	GGGACAAGAATAAAGTACATAGTTG	GGCTGTACCCTCATAAATGGA	297 pb
rs2870946	IL-26	GCTTAATTGCTCCAGCCATGC	GAAATGGGAAGGCACAGGCTA	174 pb
rs1558744	IL-26	CTCCCACCCACCCCAATTTA	ACGGGTTGACCTGTTCAGAG	182 pb
rs7134599	IL-26	AGAAGTGAGCTTGCTTGTCTGT	GCAGGATCAAAATGTCAAGCAGT	153 pb

3.2.2 Análisis de expresión génica

Se extrajo ARN total a partir de $1x10^6$ PBMCs de pacientes usando el kit QIAmp RNA Blood Mini Kit (QIAGEN) y se evaluó su concentración por absorbancia a 260 nm en un espectrofotómetro NanoDropR ND- 1000 (NanoDrop Technologies, Wilmington, USA).

A partir del ARN extraído se realizaron PCRs cuantitativas para evaluar la expresión de los receptores Toll- like estudiados, así como dos de los intermediarios clave implicados en su cascada de señalización: MyD88 y NF-kB . Las reacciones se desarrollaron en un termociclador IQ5 Real- Time PCR (BioRad, Hercules, CA, USA) utilizando qScript One- Step SYBR Green- qPCR (Quanta BioScience, Gaithesburg Maryland) y la GAPDH como gen de expresión constitutiva.

Los ensayos se hicieron por duplicado y los resultados se normalizaron atendiendo a la expresión del gen de referencia.

3.2.3 Análisis de expresión proteica

Western Blot

Se realizó un lisado de PBMCs utilizando el buffer RIPA (CellSignaling, Leiden, Holanda) suplementado con Protease Inhibitor Cocktail (SIGMA, St. Louis, MO, USA) para la extracción de proteínas celulares. La concentración proteica de los lisados se cuantificó mediante el método de Bradford.

Los extractos de proteína se cargaron en un gel de SDS-poliacrilamida al 8% y posteriormente se transfirieron a una membrana de PVDF (Immobilon- FL). Para el bloqueo, la membrana se incubó a temperatura ambiente en una solución de TBS con Tween-20 al 0,05% y BSA al 3% durante 1 hora en agitación.

Los anticuerpos primarios utilizados para la técnica fueron anti-TLR 9 ab12121 (abcam) y anti- β-actina, que se utilizó como proteína de referencia.

La detección se realizó mediante el sistema de quimioluminiscencia Immobilon Western Chemiluminescent HRP solution (Millipore).

ELISA

Para la medición de proteínas, nivel de fármacos anti-TNF (infliximab y adalimumab) libres, así como la presencia de anticuerpos frente a dichos fármacos (tanto en suero como en el sobrenadante de los cultivos), se emplearon kits comerciales de ELISA (Tabla 3).

Los sueros y los sobrenadantes se conservaron a -80 ^{0}C hasta el momento del análisis, todas las muestras disponibles se midieron por triplicado y los ELISAs se llevaron a cabo siguiendo detalladamente las instrucciones de cada fabricante.

Tabla 3. Kits empleados para la cuantificación de analitos

Proteína/analito	Kit	Compañía	Muestra
TNF-α	Quantikine HS ELISA	R&D Systems	Suero/ SN cultivos
IL-6	Quantikine HS ELISA	R&D Systems	Suero/ SN cultivos
IL-10	Quantikine ELISA	R&D Systems	Suero/ SN cultivos
Nox	Parameter	R&D Systems	Suero
Infliximab	SHIKARI Q-INFLIXI	Matriks Biotek	Suero/ SN cultivos
Adalimumab	SHIKARI Q-ADA	Matriks Biotek	Suero
C3b	MicroVue C3a Plus EIA	Quidel Corporation	Suero
MAC	MicroVue SC5b-9 Plus EIA	Quidel Corporation	Suero
C5a	MicroVue C5a EIA	Quidel Corporation	Suero

Las absorbancias se determinaron empleando un lector de placas Sunrise (Tecan, Mannedorf, Switzerland). Se generaron curvas estándar para cada placa y se restó el valor de absorbancia correspondiente a 'cero' en todos los estándares, muestras, y controles previo al cálculo de las concentraciones finales en las muestras.

Cytometric Bead Array (CBA)

Para determinar los niveles de TNF-a, IFN-g e IL-12p40 en el suero de los pacientes con EC empleamos este método, basado en el uso de bolas recubiertas con anticuerpos capaces de capturar los analitos presentes en la muestra de forma altamente eficiente. Los resultados fueron medidos en un citómetro de flujo FACSCanto II (Becton Dickinson, San Jose, CA).

3.2.4. Detección de antígenos bacterianos

3.2.4.1. ADN bacteriano

La presencia de ADN bacteriano en sangre se evaluó mediante PCR empleando primers universales del reino eubacteria que amplifican cualquier 16S ARNr descrito. La secuencia de dichos primers es 5'-AGAGTTTGATCATGGCTCAG-3' y 5'-ACCGCGACTGCTGCTGGCAC-3' para Forward y Reverse repectivamente. Esta pareja de primers generó unos amplicones de 541 pb que se separaron mediante electroforesis en geles de agarosa al 2% (p/v) en TBE 0'5X (BioRad) teñidos con SYBR Safe DNA gel stain (Invitrogen, Life Technologies, Madrid, Spain). Los fragmentos migrados en los geles se visualizaron en un transiluminador ChemiDoc XRS mediante el software Image Lab (BioRad).

3.2.4.2. Lipopolisacárido

Para evaluar los niveles de endotoxina empleamos el test cromogénico de punto final Limulus Amebocyte Lysate (LAL) (Lonza, Walkersville, MD). Se trata de un ensayo que permite medir de forma cuantitativa la presencia de endotoxina de bacterias Gram- negativas en muestras biológicas.

Como resultado del test obtuvimos unos valores de absorbancia a 405 nm, medidos en un lector de placas Sunrise (Tecan, Mannedorf, Switzerland), los cuales son una representación directamente proporcional de la cantidad de endotoxina presente en una muestra. De los datos obtenidos pudo extraerse sencillamente la concentración de LPS gracias a una curva de regresión estándar.

3.2.4.3. Ácido lipoteicoico

Como uno de los principales componentes de la pared celular de las bacterias Gram-positivas y ligando específico del TLR2, determinamos la presencia de ácido lipoteicoico en suero. Para ello empleamos el Human Lipoteichoic Acid (LTA) ELISA Kit (Abbexa Ltd., Cambridge, UK), mediante un ensayo ELISA, siguiendo las instrucciones del fabricante.

3.2.5 Ensayo de la capacidad bactericida

Para evaluar la actividad bactericida, se aislaron células mononucleares de sangre periférica (PBMCs) de un subconjunto de pacientes con genotipo IL-26 salvaje e IL-26 variante. Las células aisladas se trataron con anti-IL26 o un control de isotipo anti-IgG2 (Sigma-Aldrich, Madrid, España) y se incubaron durante un período de 48 horas. Después de esta incubación se lavó y separó una porción de 2,5 x 10^6 células, las cuales se expusieron a una cantidad fija de E. coli (10^4 UFC, serotipo 0111: B4).

El ensayo de capacidad bactericida se realizó a una temperatura constante de 37 ° C en un agitador a 15 rpm durante dos tiempos diferentes; 20 min para medir la capacidad basal de las PBMCs para ingerir bacterias antes de su destrucción (Tiempo 0 o T0) y 2 h para medir la capacidad real de las PBMCs para matar bacterias (Tiempo 2 o T2).

Se llevó a cabo un lavado exhaustivo de las células para separar la E. coli extracelular y se sometió a centrifugación con sacarosa al 30% para eliminar mejor las bacterias no fagocitadas. Las células se resuspendieron en PBS con suero al 5% y luego se diluyeron en agua estéril de manera seriada. Las diluciones se sembraron en placas de agar y se incubaron 24 horas a 37 ° C, periodo tras el cual se contaron las unidades formadoras de colonias (UFC). La capacidad bactericida se extrajo de la diferencia entre el número de UFCs a TO y T2.

3.2.6. Cultivos celulares

3.2.6.1. Pacientes con cirrosis

Se aislaron células polimorfonucleares de sangre periférica (PMN) a partir de muestras de pacientes y donantes mediante el uso de la solución Polymorph Prep (Axis-Shield, Oslo, Noruega). Una vez aisladas, se realizaron dos lavados con solución salina tamponada con fosfato (PBS) a 4 °C y se evaluó su viabilidad mediante una tinción con trypan blue (Sigma, Madrid, Spain).

Las células viables obtenidas se cultivaron en medio RPMI 1640 (Gibco BRL, Life Technologies, Paisley, RU) suplementado con L-glutamina, antibióticos y un 10% de SFB a una concentración de 10^6 células / ml. Se cultivaron 4 pocillos independientes en presencia de:

- Ningún estímulo
- CpGs ODN 2395 (20 ng / ml) (InvivoGen, San Diego, CA)
- LPS (100 ng / ml) (E. coli serotipo 0111: B4; Sigma)
- Pam3CSK4 (100 ng / ml) (InvivoGen)

Los cultivos se mantuvieron durante 24 horas a 37°C y 5 % CO_2. Después de ese período, todos los sobrenadantes y las células se recogieron y almacenaron a -80ºC hasta su análisis.

3.2.6.2. Pacientes con Enfermedad de Crohn

Mediante separación por gradiente de densidad, se aislaron PBMC de pacientes que no presentaron ADN bacteriano en suero y los cuales no recibían tratamiento con biológicos o inmunosupresores.

Las células aisladas se lavaron con solución salina tamponada con fosfato (PBS) a 4ºC y se evaluó su viabilidad mediante tinción con trypan blue (Sigma, Madrid, España). Posteriormente se resuspendieron en medio RPMI 1640 (Gibco BRL, Life Technologies, Paisley, RU) complementado con suero humano AB al 10% (BioWhittaker, Walkersville, Maryland, EE. UU.).

Se incubaron 1 × 10^6 PBMCs / pocillo en un volumen final de 1 ml y en presencia de:

a. Infliximab (100 µg / ml) (Merck Sharp y Dohme of Spain, MSD, Madrid, España) más E. coli

ADN (200 ng / ml) durante 48 h.

b. IL26 humana recombinante (R & D Systems) (100 ng / 1 x 10^6 células) más DNA de E. coli (200 ng / ml) durante 24 h.

3.3. Análisis estadístico

Las variables continuas se representaron como media ± desviación estándar, como mediana y percentiles 25 a 75, o como intervalo de confianza del 95%. Las variables categóricas se expresaron como frecuencias o porcentajes.

Las diferencias entre los grupos de pacientes en las variables categóricas se analizaron usando el test Chi-cuadrado. Para determinar la normalidad de las variables continuas se empleó la prueba de Kolmogorov–Smirnov. Los datos cuantitativos que no mostraban una distribución normal se analizaron utilizando la prueba de la U de Mann-Whitney para comparaciones simples o el test de Kruskal-Wallis seguido de comparaciones por pares utilizando la prueba de la U de Mann-Whitney con la corrección post hoc de Bonferroni para comparaciones múltiples. Las correlaciones bivariadas entre las variables continuas se calcularon utilizando la prueba de Spearman. La significación estadística se consideró con valores de p inferiores a 0,05. El análisis estadístico se llevó a cabo empleando el programa IBM SPSS Statistics 19 y el software R.

RESULTADOS

ARTÍCULO 1

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Toll-like receptor polymorphisms compromise the inflammatory response against bacterial antigen translocation in cirrhosis.
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Resumen

La traslocación bacteriana se asocia con complicaciones relevantes en la cirrosis. Nuestro objetivo fue evaluar el efecto de los polimorfismos en receptores Toll-like en la respuesta soluble frente a estos episodios. Distribuimos pacientes consecutivos, con cirrosis y ascitis, según los SNPs TLR2 rs4696480, TLR4 rs4986790 y TLR9 rs187084.

Se cuantificaron los niveles de ácido lipoteicoico, lipopolisacárido, ADN bacteriano, citocinas proinflamatorias y niveles de óxido nítrico en muestras de suero. Se evaluó la respuesta in vitro de los 3 receptores frente a sus ligandos específicos. Se incluyeron ciento catorce pacientes en total. Los genotipos variantes de TLR2, TLR4 y TLR9 se asociaron con un aumento significativo de los niveles séricos de LTA, LPS y ADN bacteriano. Los niveles séricos de TNF-α, IL-6 y óxido nítrico disminuyeron significativamente en todos los pacientes con genotipo TLR variante. El incremento en los niveles de citocinas como respuesta a ligandos específicos de los receptores estudiados fue significativamente menor en los pacientes con genotipo variante frente a los de genotipo salvaje.

Toll-like receptor polymorphisms compromise the inflammatory response against bacterial antigen translocation in cirrhosis

Bacterial translocation is associated with clinically relevant complications in cirrhosis. We evaluated the effect of toll-like receptor polymorphisms in the soluble response against these episodes. Consecutive patients with cirrhosis and ascitic fluid were distributed by TLR2 rs4696480, TLR4 rs4986790, and TLR9 rs187084 single-nucleotide polymorphisms. Lipoteichoic acid, lipopolyssaccharide, bacterial-DNA, pro-inflammatory cytokines and nitric oxide levels were quantified in serum samples. In vitro response against specific ligands in variant TLR genotypes was evaluated. One hundred and fourteen patients were included. Variant TLR-2, TLR-4 and TLR-9 SNP genotypes were associated with significantly increased serum levels of LTA, LPS and bacterial-DNA. TNF-α, IL-6 and nitric oxide serum levels were significantly decreased in all variant TLR genotyped patients. Cytokine levels were significantly less upregulated in response to specific TLR-ligands in patients with all variant vs wildtype TLR genotypes. Although in vitro gene expression levels of all wildtype and variant TLRs were similar, MyD88 and NFkB were significantly downregulated in cells from TLR-variant genotyped patients in response to their ligands. Variant TLR genotypes are associated with an increased circulating antigen burden and a decreased proinflammatory response in cirrhosis. This immunodeficiency may facilitate bacteria-related complications in cirrhosis and enhance TLR targeting for its management.

The translocation of commensal bacterial antigens into blood of patients with decompensated cirrhosis is a frequent event that triggers relevant clinical complications. Several studies have related the transient passage of bacterial products into the systemic circulation of these patients with the exacerbation of the inflammatory response1–4, the prediction of infections5, an increased risk of hemodynamic disturbances4,6 or even death7. Bacterial antigenic products are specifically recognized by a host's family of receptors, both at the membrane and the intracellular levels. The Toll Like Receptor (TLR) family comprises different receptors that specifically bind unique bacterial products, launching an inflammatory signalling cascade and the mechanisms for these bacterial products clearance8,9. TLR-2 is the specific receptor for lipoteichoic acid (LTA), a product derived from gram-positive bacteria, which are also able to translocate and differentiate a specific immune response10. TLR-4 and TLR-9 are receptors for lipopolysaccharide (LPS)11 and bacterial DNA12 respectively, two of the most immunogenic bacterial products. Several studies have described the deep effects of endotoxemia or the presence of bacterial DNA on the clinical outcome of decompensated cirrhosis. Several polymorphisms in these TLR genes have been described and associated with immune alterations or clinical complications in cirrhosis and other inflammatory-related disorders. A polymorphism in the TLR-2 promoter has been linked to an increased risk for spontaneous bacterial peritonitis in cirrhosis13. The TLR-4 rs4986790 polymorphism has been found to predispose to infections in cirrhotic patients14 and to an LPS hyporresponsiveness15. These results have brought the attention into TLRs as potential targets in chronic liver disease16.

On the other hand, the TLR-9 rs187084 polymorphism has been described to be functionally relevant by downregulating TLR-9 expression17. Therefore, the genetic background may be a relevant potential modulator of the immune response in bacterial-derived complications of cirrhosis. The aim of the present study was to evaluate the incidence of relevant polymorphisms in TLR-2, TLR-4 and TLR-9, specific receptors of bacterial products which are frequently translocated from the gut in patients with cirrhosis.

Age	(years)	62 ± 10
Gender	(male/female)	76/38
Etiology	Alcohol	51 (44.7%)
	HCV	32 (28.1%)
	Alcohol + HCV	19 (16.7%)
	Alcohol + HBV	3 (2.7%)
	Other	9 (7.8%)
Previous episodes of ascites	n (%)	48 (42.1%)
Previous episodes of encephalopahty	n (%)	14 (12.3%)
Previous episodes of variceal bleeding	n (%)	20 (17.5%)
SBP (previous 6 months)	n (%)	2 (1.7%)
Infections other than SBP (previous 6 months)	n (%)	10 (8.8%)
Child-Pugh Category	A/B/C	0/66/48
Child-Pugh mean score		9.2 ± 1.8
MELD mean score		12.5 ± 3.7
Use of beta-blockers	n (%)	31 (27.2%)
Use of PPIs	n (%)	34 (29.8%)
Mean arterial pressure	(mmHg)	85.5 ± 8.5
Heart rate	(bpm)	79 ± 12
Bilirubin	(mg/dL)	3.1 ± 2.4
Albumin	(mg/dL)	2.6 ± 0.7
AST	(IU/L)	65.3 ± 45.7
ALT	(IU/L)	37.5 ± 28.8
Quick	(%)	67 ± 17
INR		1.4 ± 0.3
Serum creatinine	(mg/dL)	0.96 ± 0.4
Serum sodium	(mEq/L)	138.7 ± 4.6
Serum potassium	(mEq/L)	4.4 ± 0.6
Platelets	(/mm3)	120,474 ± 60,749
Blood WBC	(/mm3)	5645 ± 2627

Table 1. Clinical and analytical characteristics of patients. SBP: Spontaneous bacterial peritonitis; PPIs: protom-pump inhibitors; WBC: white blood cells.

Results

Characteristics of patients.

One hundred and fourteen patients with cirrhosis and non-infected AF were included in the study. All clinical and analytical characteristics of patients are detailed in Table 1. Mean age was 60±10 and 68.4% were male. Main etiology of cirrhosis was alcohol, and mean Child-Pugh score was 9.2±1.8. Thirty-six patients (31.5%) were on beta-blockers and 40 patients (35%) were taking proton pump inhibitors (PPIs).

We followed up patients for 6 months. Four patients died (all due to acute on chronic liver failure) during this time and 8 patients had an infection (1 SBP and 7 non-SBP [3 urinary tract, 2 clostridium dificile cholitis and 2 pneumonia]). We couldn't find any statistically significant relationship between any TLR variant genotype and the development of infections. However, 6 out of the 8 infectious

episodes were present in patients bearing 2 or more TLR variant genotypes.

TLR polymorphisms and bacterial antigen detection in patients with cirrhosis.

We evaluated the incidence of 3 well-known polymorphisms in TLR-2, TLR-4 and TLR-9 in patients with cirrhosis. The distribution of genotypes and allelic frequencies in patients and controls are shown in Table 2. All variants were found to be in the Hardy–Weinberg equilibrium in the controls. Of interest, no homozygous variant genotype for TLR-4 rs4986790 was found in the cirrhotic population.

Figure 1 shows the concentration of different bacterial antigens according to the polymorphisms studied in their specific receptor. As can be observed, the loss of the wild-type allele in the studied polymorphisms of TLR-2, TLR-4 and TLR-9 was associated with significantly increased serum levels of LTA, LPS and bacterial DNA,

	Genotype n (%) patients/controls			Variant allele frequency (%)
	Homozygous wild type	Heterozygous	Homozygous variant	
TLR-2 rs4696480 (T/A)	25 (21.9%)/121 (24.1%)	55 (48.2%)/243 (48.3%)	34 (29.8%)/139 (27.6%)	54.9/51.8
TLR-4 rs4986790 (A/G)	98 (85.9%)/448 (89.1%)	16 (14.1%)/52 (10.3%)	0 (0%)/3 (0.6%)	5.0/5.0
TLR-9 rs187084 (T/C)	44 (38.5%)/161 (32.0%)	53 (46.4%)/254 (50.5%)	17 (14.9%)/88 (17.5%)	37.0/42.0

Table 2. Genotype and allele frequencies for studied TLR polymorphisms.

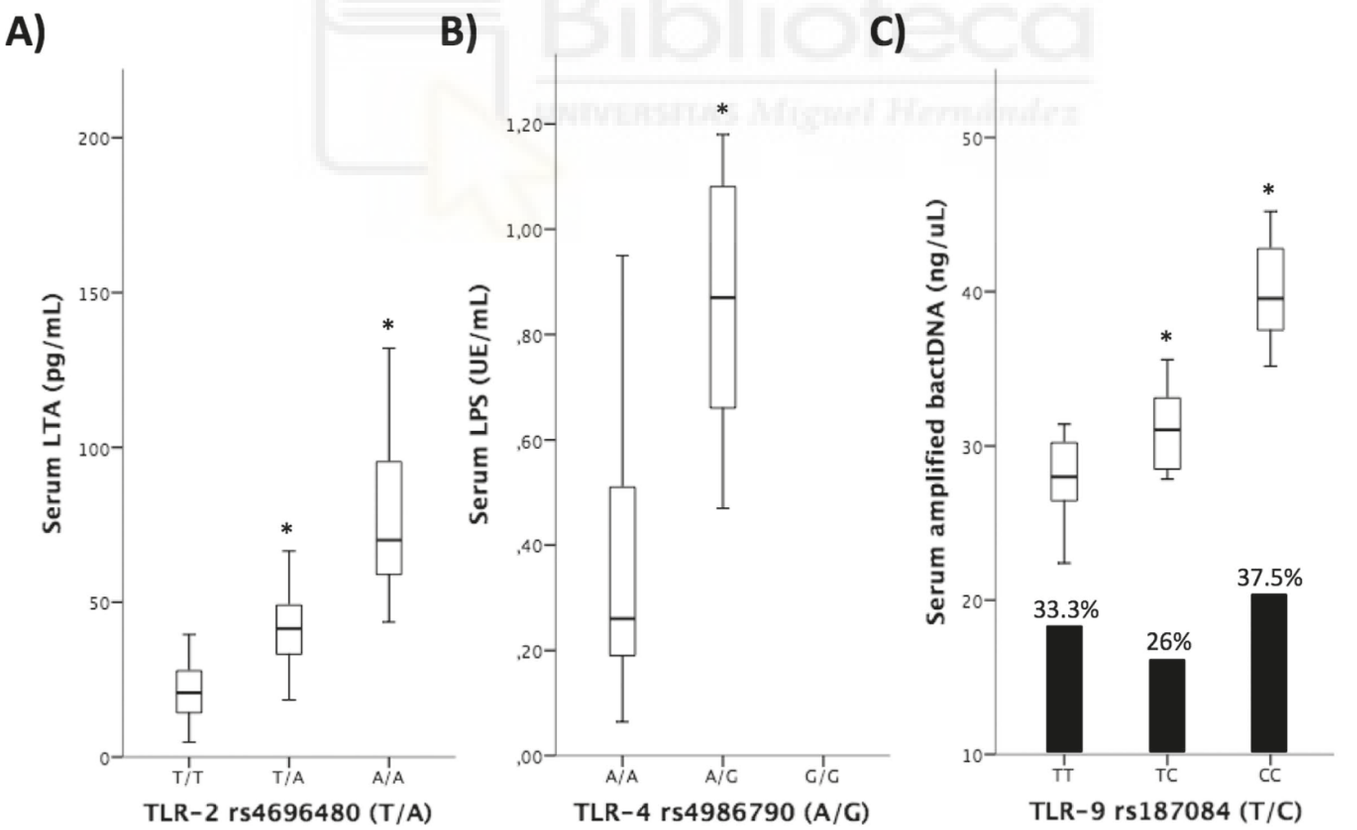

Figure 1. Serum levels of LTA (**A**), LPS (**B**), and amplified bacterial DNA (**C**) in blood of patients with cirrhosis distributed by TLR-2, TLR-4, and TLR-9 genotypes. The percentage of bacterial DNA translocation is also represented in panel *C*. *$p < 0.05$ compared to the wildtype genotypes.

(Fig. 1A,B and C, respectively). Patients with the homozigotic variant showed the highest load of circulating bacterial antigens in blood samples. In the case of TLR-9 rs187084, although the variant genotype was associated with an increased amplified bacterial DNA load in patients' samples, it was not associated with an increased rate of bacterial translocation, as represented by the bars in Fig. 1C. There were not statistical differences, either, in the sequenced species from bacterial DNA between patients distributed by TLR-9 rs187084 genotype, with a similar percentage of gram-positive microorganisms in all three groups (Supplementary Table 1). Clinical and analytical characteristics of patients distributed by each TLR polymorphism genotype showed no clinical or analytical differences (data not shown).

Soluble inflammatory response is influenced by TLR polymorphisms.

Serum pro-inflammatory mediators were evaluated in all patients and compared by the genotype of studied polymorphisms. Figure 2 shows that TNF-α and IL-6 were significantly decreased in patients bearing the variant genotype of TLR-2 rs4696480 (Fig. 2A and E). When distributing the cytokine response by the variant TLR-4 rs4986790 (Fig. 2B and F) and by TLR-9 rs187084 (Fig. 2C and G) genotypes, both cytokines revealed a similar behaviour to that shown by TLR-2 rs4696480 genotype, showing significantly decreased serum levels of both mediators in patients with the variant genotypes. Patients were further distributed by the presence of detectable bacterial DNA in blood. The differences in TNF-α and IL-6 levels in patients distributed by TLR-9 rs187084 genotype were, as expected, due to those with circulating bacterial DNA in blood (Fig. 2D and H).

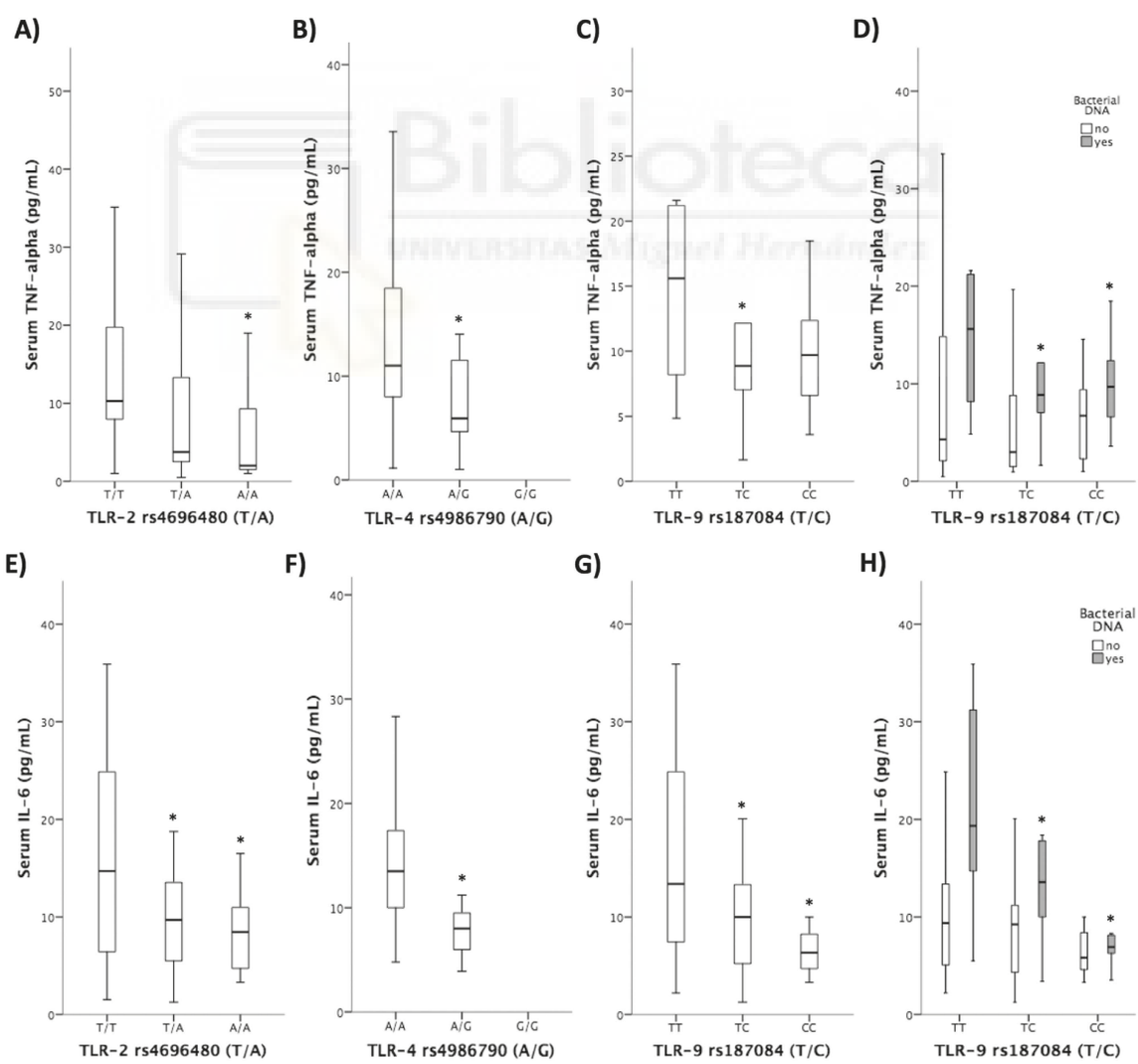

Figure 2. Serum levels of TNF-alpha (**A**–**C**) and IL-6 (**E**–**G**) in patients with cirrhosis distributed by TLR-2, TLR-4 and TLR-9 genotypes. (**D**,**H**) Serum levels of TNF-alpha and IL-6 in patients with cirrhosis distributed by TLR-9 genotype and the presence of bacterial DNA translocation. *$p < 0.05$ compared the wildtype genotypes.

A potent immune modulator such as NOx showed similar results to those observed for TNF-α and IL-6. The wild-type genotypes of all TLRs were significantly associated with increased serum NOx levels (Fig. 3A–C). As shown for the cytokine pattern, the differences in NOx levels in TLR-9 rs187084 genotyped patients were associated with circulating bacterial DNA in blood (Fig. 3D).

We further analysed the inflammatory milieu in the serum of patients grouped by the number of variant TLR genotypes. As observed in Table 3, the accumulation of variant TLR genotypes was associated with significantly decreased pro-inflammatory cytokine and NOx levels.

Immunodeficiency in decompensated cirrhosis is associated with variant TLR-2, TLR-4 and TLR-9 genotypes.

We evaluated the in vitro response to specific TLR ligands to confirm the downregulation of the inflammatory milieu in patients and donors bearing variant TLR genotypes. TNF-α, IL-6 and IL-10 levels in patients' PMNs supernatants were significantly upregulated in all cases after stimulation with Pam3-Cys, LPS and CpGs compared with resting conditions. However, TNF-α and IL-6 levels were significantly lower and IL-10 was significantly higher in response to TLR-specific ligands in patients with variant TLR-2 rs4696480, TLR-4 rs4986790 and TLR-9 rs187084 genotypes compared with those bearing the wild-type alleles (Table 4). Results on donors are shown in Table 5 and, as well as for patients, the presence of variant genotypes in all studied TLRs was associated with significantly reduced pro-inflammatory cytokine levels and increased IL-10 compared with the wild-type genotypes.

Interestingly, the gene expression levels of variant TLR-2 (Fig. 4A), TLR-4 (Fig. 4B), and TLR-9 (Fig. 4C) were not significantly different in response to their specific ligands compared with those observed in cells from patients with the wild-type alleles. However, when evaluating the gene expression levels of downstream signaling molecules, the presence of studied SNPs in TLR-2, TLR-4 and TLR-9 were associated with a significant decrease in MyD88 (Fig. 4D–F) and NFkB (Fig. 4G–I) compared with cells from patients with wildtype alleles after stimulation with their respective specific ligands. Statistical correlations between MyD88 and NFkB gene expression levels and the concentration of in vitro secreted cytokines are shown in Table 6.

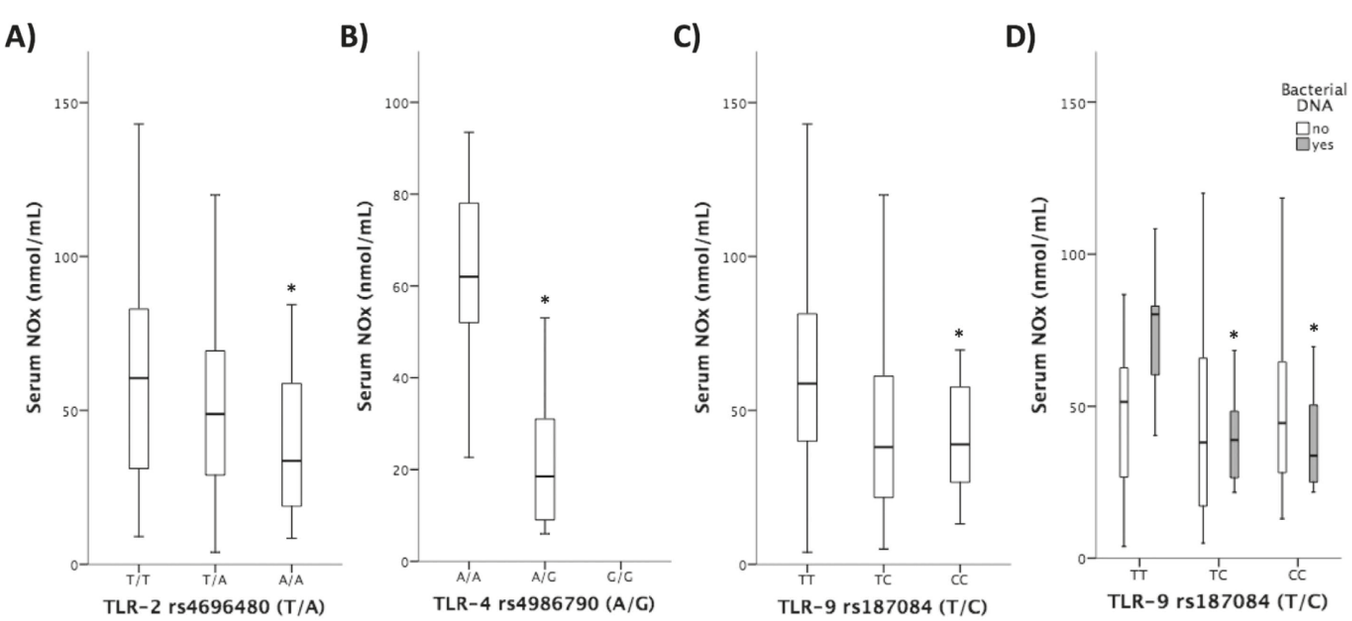

Figure 3. Serum levels of nitric oxide metabolites (NOx) in patients with cirrhosis distributed by TLR-2, TLR-4 and TLR-9 genotypes (**A**–**C**). (**D**) Serum levels of NOx in patients with cirrhosis distributed by TLR-9 genotype and the presence of bacterial DNA translocation. *$p < 0.05$ compared the wildtype genotypes.

		TNF-α (pg/mL)	IL-6 (pg/mL)	NOx (nmol/mL)
Wildtype TLRs	n = 16	16.5 ± 7.9	18.4 ± 9.1	67.4 ± 18.5
One variant TLR	n = 46	11.5 ± 6.8*	13.7 ± 7.5	56.0 ± 23.7
Two variant TLRs	n = 42	7.8 ± 3.8*	10.3 ± 3.9*	55.8 ± 19.5*
Three variant TLRs	n = 10	2.8 ± 2.5*	8.5 ± 1.4*	33.6 ± 17.1*

Table 3. Serum cytokine and nitric oxide levels in patients grouped by the number of variant TLR genotypes. TNF-α: tumor necrosis factor alpha; IL: interleukin; NOx: nitric oxide metabolites; *$p < 0.01$ compared with wildtype TLRs.

		TNF-α (pg/mL)		IL-6 (pg/mL)		IL-10 (pg/mL)	
	Total n = 114	Not stimulated	Stimulated#	Not stimulated	Stimulated#	Not stimulated	Stimulated#
TLR2 rs4696480	T/T (n = 25)	9.0 ± 2.7	270.0 ± 69.3*	26.8 ± 8.6	522.0 ± 34.6*	5.1 ± 3.2	5.6 ± 3.9
	T/A (n = 55)	8.6 ± 2.5	66.3 ± 17.8*,$	26.2 ± 4.9	446.3 ± 12.2*,$	3.9 ± 3.2	10.8 ± 4.9*,$
	A/A (n = 34)	6.9 ± 3.1	29.7 ± 6.1*,$	30.3 ± 3.8	332.6 ± 28.4*,$	4.3 ± 2.6	16.8 ± 7.2*,$
TLR4 rs4986790	A/A (n = 98)	8.9 ± 5.8	378.3 ± 41.6*	22.2 ± 7.9	360.7 ± 39.5*	4.0 ± 3.5	8.5 ± 5.3*
	A/G (16)	8.5 ± 3.3	338.6 ± 34.4*	26.8 ± 4.8	353.3 ± 43.9*,$	4.2 ± 2.9	16.3 ± 8.1*,$
TLR9 rs187084	T/T (n = 44)	8.9 ± 5.5	77.3 ± 37.5*	28.6 ± 6.5	422.0 ± 23.4*	4.3 ± 3.8	10.1 ± 6.5*
	T/C (n = 53)	10.8 ± 3.3	39.0 ± 14.1*,$	27.1 ± 4.4	338.0 ± 41.1*,$	3.8 ± 2.9	15.4 ± 6.6*,$
	C/C (n = 17)	7.3 ± 3.0	19.2 ± 2.6*,$	28.0 ± 2.8	213.0 ± 62.9*,$	3.7 ± 3.6	19.6 ± 7.5*,$

Table 4. Secreted levels of cytokines in the supernatants of cultured PMNs from patients distributed by the genotype of TLR polymorphisms. #Stimuli: Pam3CSK4 (100 ng/ml) for TLR-2; LPS (100 ng/ml) for TLR-4; and CpGs ODN 2395 (20 ng/ml) for TLR-9. *$p < 0.01$ compared with the unstimulated condition; $$p < 0.01$ compared with the stimulated wildtype TLR genotype.

Discussion

In this study, we demonstrate that variant genotypes in TLR-2, TLR-4 and TLR-9 genes are associated with an increased bacterial antigen burden and a decreased pro-inflammatory cytokine profile in blood of patients with cirrhosis and non-infected AF, suggesting that these genetic variants may compromise bacterial antigen interaction with their specific receptors and limit the innate soluble inflammatory response in these patients. This genetically derived immunodeficiency might have consequences in bacterial antigen clearance and contribute to the clinically relevant complications that are frequently developed in patients with cirrhosis.

A compromised immunological status is one of the three main aspects classically proposed for the development of bacterial antigen translocation episodes in cirrhosis, which in turn, is proposed to be either responsible or a contributor to life-threatening complications in these patients such as SBP, hepatic encephalopathy or haemodynamic disturbances. TLRs take part in the specific recognition of antigenic molecules and trigger early events in the immunological response. We have evaluated the effect of TLR2 rs4696480, TLR4 rs4986790 and TLR9 rs187084 polymorphisms in the bacterial antigen load and the pro-inflammatory mediator levels in the blood of a consecutive series of patients with cirrhosis and AF

	Total n = 20	TNF-α (pg/mL)		IL-6 (pg/mL)		IL-10 (pg/mL)	
		Not stimulated	Stimulated[#]	Not stimulated	Stimulated[#]	Not stimulated	Stimulated[#]
TLR2 rs4696480	T/T (n = 8)	6.2 ± 3.3	186.4 ± 74.6*	8.5 ± 5.3	410.0 ± 224.2*	3.5 ± 3.4	12.6 ± 6.8*
	T/A (n = 6)	5.2 ± 2.8	88.2 ± 32.8*,$	7.2 ± 4.8	250.4 ± 119.6*,$	5.3 ± 4.1	19.4 ± 9.2*,$
	A/A (n = 6)	6.1 ± 3.1	35.1 ± 20.6*,$	9.4 ± 6.2	100.8 ± 80.4*,$	3.4 ± 2.7	25.6 ± 12.4*,$
TLR4 rs4986790	A/A (n = 13)	7.3 ± 3.6	364.6 ± 104.6*	7.7 ± 5.4	450.4 ± 168.3*	3.5 ± 2.6	9.4 ± 3.8*
	A/G (7)	5.8 ± 4.1	188.6 ± 75.7*,$	10.1 ± 7.3	286.5 ± 104.7*,$	4.1 ± 3.2	15.8 ± 6.2*,$
TLR9 rs187084	T/T (n = 7)	6.6 ± 3.2	72.2 ± 26.5*	6.9 ± 3.5	402.6 ± 175.4*	4.9 ± 3.3	13.5 ± 8.5*
	T/C (n = 7)	5.5 ± 3.5	45.3 ± 22.1*,$	7.3 ± 4.5	283.3 ± 98.8*,$	3.9 ± 2.4	18.6 ± 10.1*
	C/C (n = 6)	5.2 ± 3.0	19.2 ± 8.8*,$	6.6 ± 3.8	137.5 ± 77.3*,$	3.6 ± 3.0	26.6 ± 11.2*,$

Table 5. Secreted levels of cytokines in the supernatants of cultured PMNs from donors distributed by the genotype of TLR polymorphisms. [#]Stimuli: Pam3CSK4 (100 ng/ml) for TLR-2; LPS (100 ng/ml) for TLR-4; and CpGs ODN 2395 (20 ng/ml) for TLR-9. *$p < 0.01$ compared with the unstimulated condition; [$]$p < 0.01$ compared with the stimulated wildtype TLR genotype.

We first show that TLR variant genotypes are associated with significantly increased serum levels of their specific antigenic ligands (LTA, LPS and bacterial DNA, respectively). This would be in line with previous findings showing an increased risk of SBP in patients with cirrhosis bearing TLR-2 polymorphisms13 or the predisposition to infections in cirrhotic patients with a variant TLR-4 genotype14. Interestingly, although the rate of bacterial DNA translocation is not increased in patients with a variant TLR9 genotype, there is a significant increment in the amount of amplified bacterial DNA in these patients compared with those bearing the wild-type TLR-9 genotype. This is relevant, as it has been proven that the grade of soluble inflammatory response is significantly affected by bacterial DNA concentrations in patients with cirrhosis18.

However, an important result of the study is that evaluated TLR variant genotypes show a reduced secretion of pro-inflammatory mediators in the serum of cirrhotic patients, despite their significantly increased antigenic burden (Fig. 2). Moreover, the presence of several variant TLR polymorphisms is associated with a progressive, significantly reduced inflammatory milieu. To confirm our results, we evaluated the soluble response to TLR-specific ligands in in vitro cultured neutrophils from cirrhotic patients and donors with different TLR genotypes. As shown in vivo, although TLR stimulation resulted in significantly increased supernatant levels of pro-inflammatory cytokines compared with resting cells, the variant genotypes showed a significantly decreased ability to induce TNF- and IL-6 in response to Pam3-cys, LPS and CpGs compared with wild-type TLR cells, both in patients (Fig. 4) and donors (Table 3), suggesting that the impact of studied polymorphisms on the immune system is independent of the pathological context.

These results would restrict findings in previous studies to wild-type TLR-genotyped patients and they would suggest either a lack of the antigenic ligand recognition by TLR or a TLR variant genotype-induced breakdown in the pro-inflammatory signalling pathway. Our results in vitro show no differences in TLR gene expression levels, suggesting a competent stimulation of these receptors despite their polymorphic regions (Fig. 4A–C). On the contrary, downstream signalling molecules were reduced in cells from TLR variant vs wildtype genotyped patients (Fig. 4D–I), supporting the second hypothesis. Either way, as a result, bacterial antigenic internalization and/or cellular signalling events would be compromised, leading to serum free antigen accumulation and low pro-inflammatory cytokine levels. In fact, hyporresponsiveness to LPS has been described in the presence of TLR-4 variant genotypes15 and TLR-4 expression downregulation19. This resultant "tolerant state" might account for the increased risk of infections commented before.

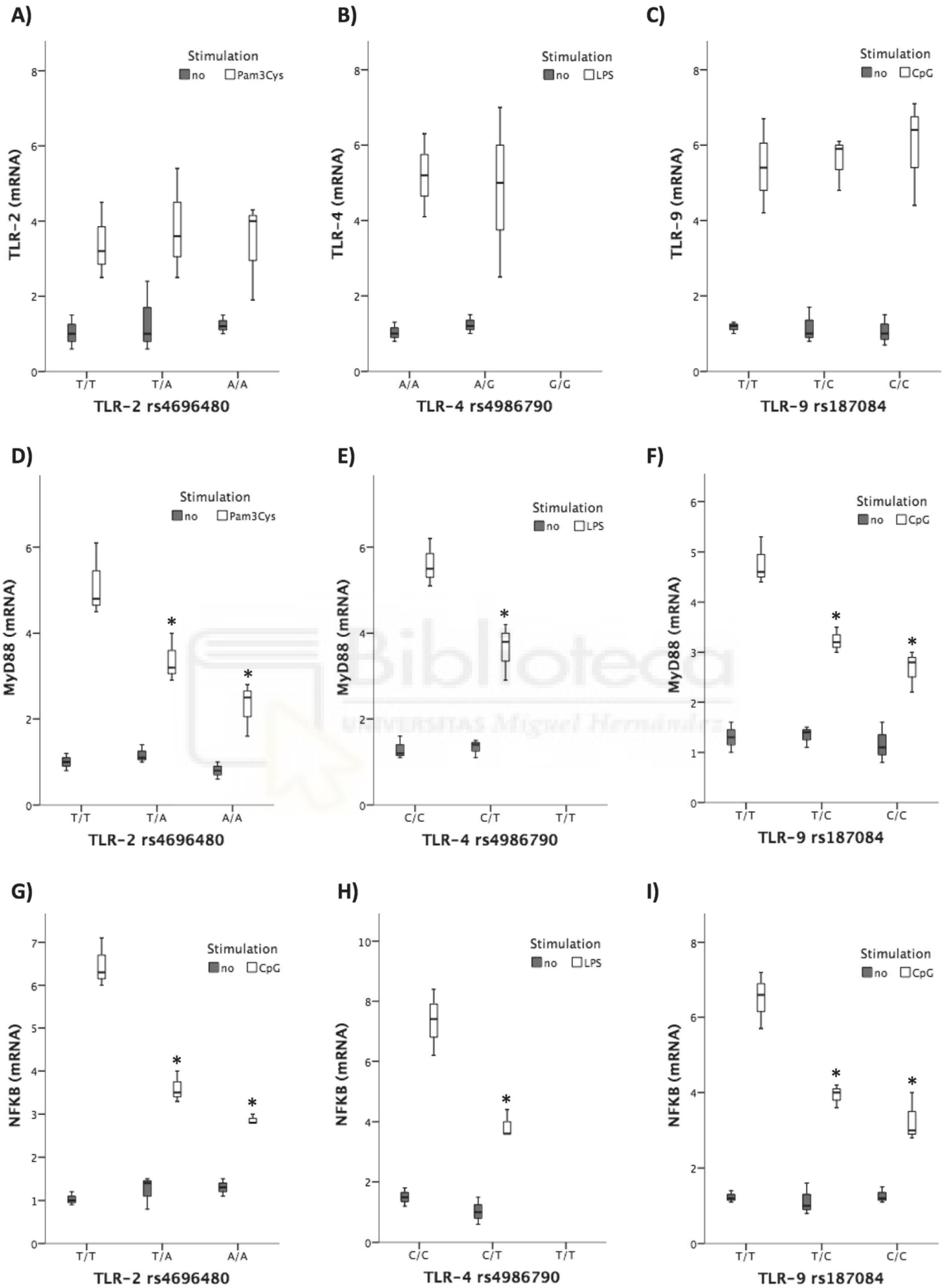

Figure 4. Gene expression levels of TLR-2, TLR-4, TLR-9, MyD88 and NFkB in cultured PMNs from patients with cirrhosis distributed by TLR-2 rs4696480 (**A**,**D**,**G**), TLR-4 rs4986790 (**B**,**E**,**H**) and TLR-9 rs187084 (**C**,**F**,**I**) genotypes. *$p < 0.05$ compared with non-stimulated conditions.

The mechanisms by which the TLR polymorphisms may condition receptor are not fully elucidated. Several controversial examples were nicely reviewed in the recent past by Medveded A20. For example, the R753Q TLR-2 polymorphism does not significantly affect TLR2 expression, but induces the suppression of NF-kB activation and cytokine expression by affecting the electrostatic potential of the TIR domain21. Other TLR2 variants, such as P681H, do not change wild-type TLR-2 expression levels22, but it is associated with slower rates of internalization from the cell surface to endosomes23. The D299G TLR-4 variant is associated with decreased TLR-4 expression levels in response to LPS15. In contrast, others have reported no changes in expression levels of WT, D299G, or T399I TLR4 variants in HEK293T/CD14/MD2 transfectants, but a compromised ability to elicit NFkB activation in response to LPS in the TLR-4 variants24,25. In fact, despite similar TLR-4 expression levels, PMNs from individuals expressing the D299G TLR4 variant showed reduced phosphorylation of IkB-a and secretion of IL-12 p17 upon stimulation with LPS compared with cells expressing WT TLR426. D299G polymorphism has also been described to inhibit LPS-induced association of TLR-4 with adapters MyD88 and TRIF, resulting in suppressed activation of the transcription factors NF-kB and IRF-3, p38 MAPK phosphorylation, and induction of MyD88- and TRIF-dependent cytokines27.

From a clinical standpoint, TLR polymorphisms have widely been associated with human disease28. Particularly, bacteria-related complications and the associated inflammatory response are relevant to the TLR polymorphism evaluation, as several members of this family are specific receptors of their products. In fact, previously TLR-2 and TLR-4 polymorphism have been associated with spontaneous bacterial peritonitis or bacteremia in cirrhotic patients13,14 and the immunological hyporesponsiveness to LPS in TLR-4 variants has been previously reported15. As an explanation for those associations, we now demonstrate that the circulating antigen burden is significantly increased in variant-TLR cirrhotic patients. As the translocation of bacterial products is frequent and recurrent in cirrhosis, it is likely that genetically deficient TLR signalling might be involved and, therefore, provide new targets for treating bacteria-derived complications in cirrhosis.

	Secreted TNF-α ($pg/10^6$ cells)	Secreted IL-6 ($pg/10^6$ cells)	Secreted IL-10 ($pg/10^6$ cells)
MyD88 mRNA	$r = 0.817$; $p < 0.001$	$r = 0.834$; $p < 0.001$	$r = -0.616$; $p < 0.001$
NFkB mRNA	$r = 0.789$; $p < 0.001$	$r = 0.815$; $p < 0.001$	$r = -0.685$; $p < 0.001$

Table 6. Correlations between *in vitro* secreted cytokine levels and TLR signalling molecules. TNF-α: tumor necrosis factor alpha; IL: interleukin; MyD88: Myeloid differentiation primary response gene 88; NFkB: nuclear factor kappa B.

In summary, polymorphisms in TLR-2, TLR-4 and TLR-9 genes are associated with an increased bacterial antigen burden and a deficient pro-inflammatory cytokine response in patients with cirrhosis. Both aspects may delay bacterial antigen clearance in blood and contribute to clinically relevant bacteria-derived complications in patients with cirrhosis.

Patients and Methods

Patients and study design.

We conducted a prospective observational study in patients with cirrhosis and non-infected ascitic fluid (AF) consecutively admitted or followed at our Hospital from January 2014 to January 2016.

Exclusion criteria were the presence of spontaneous bacterial peritonitis (SBP) or infections, multinodular

hepatocellular carcinoma, portal thrombosis, alcoholic hepatitis, previous liver transplantation or previous transjugular intrahepatic portosystemic shunt. SBP was defined as the presence of >250 polymorphonuclear cells/μL in AF. The Ethics committee of Hospital General Universitario de Alicante approved the study protocol, and all patients gave informed consent to participate in the study. All methods described herein were performed in accordance with the relevant guidelines and regulations.

Blood and AF were obtained from all patients at admission and analyzed for routine biochemical and cytological studies. Blood and AF cultures were performed in all cases. Aliquots of blood and AF were inoculated under aseptic conditions in sterile, rubber-sealed Vacutainer SST II tubes (BD Diagnostics, Belgium) that were never exposed to free air.

Patients were followed-up for 6 months. Incidence of infections, successive hospitalizations and mortality were registered.

TLR polymorphisms genotyping.

The studied SNPs were TLR4 rs4986790 on chromosome 9q33.1, TLR9 rs187084 on chromosome 3p21.2 and TLR2 rs4696480 on chromosome 4q31.3. Genotyping was performed in genomic DNA extracted from peripheral blood samples by using the QIAamp DNA Blood Mini Kit (Qiagen) according to manufacturer's recommendations. Partial amplification of the genes containing the different polymorphisms was performed using specific primers as follows: TLR4 rs4986790 forward 5′-CTACCAAGCCTTGAGTTTCTAG-3′, reverse 5′-AAGCTCAGATCTAAATACCT-3′; TLR9 rs187084 forward 5′-CATTCATTCAGCCTTCACTC-3′, reverse: 5′-ATGTGCTGTTCCCTCTGC-3′; TLR2 rs4696480 forward 5′-GGGACAAGAATAAAGTACATAGTTG-3′, reverse 5′-GGCTGTACCCTCATAAATGGA-3′. PCR product sizes (110 bp, 419 bp and 297 bp, respectively) were purified using ExoSAP-IT PCR Product Cleanup (Affymetrix). The incidence of polymorphisms was detected by nucleotide sequencing of PCR products using the same primers as for the amplification. The sequencing process was performed by Secugen SL. The results were analysed with FinchTV software version 1.5 (Geospiza). The incidence of all three polymorphisms in control population was obtained from The 1000 Genomes Project Consortium29 (www.1000genomes.org).

Bacterial antigen measurement in patients' samples.

Samples and reagents were handled in an airflow chamber and processed with pyrogen-free material tested by manufacturers. To determine lipoteichoic acid (LTA), the specific ligand of TLR-2, a Human lipoteichoic acid elisa kit (Abbexa Ltd., Cambridge, UK) was used according to manufacturer's instructions. A quantitative chromogenic limulus amebocyte lisate (LAL) test (BioWhittaker, Nottingham, UK) was followed to evaluate endotoxin levels in blood and AF samples as previously described30. To detect the presence of bacterial DNA fragments in blood and AF, a broad-range polymerase chain reaction (PCR) was performed according to the methodology described elsewhere31. PCR amplicons were loaded onto DNA Laboratory-on-chips (Agilent Technologies, Palo Alto, CA) and analyzed with an Agilent 2100 BioAnalyzer.

Inflammatory mediators quantification.

Enzyme-linked immunosorbent assays (ELISAs) for the quantitative measurement of TNF-α and IL-6 levels were carried out in serum samples of patients by handling Human Quantikine kits (R&D Systems, Minneapolis, MN), according to manufacturer's instructions. All samples were tested in triplicate and read in a microplate reader. Lower limits of detection of all cytokine assays were 5–8pg/mL.

Standard curves were generated for each plate, and the average zero standard optical densities were subtracted from the rest of standards, controls, and samples to obtain a corrected concentration for both cytokines. The sum of the NO metabolites nitrite (NO_2^-) and nitrate (NO_3^-) is widely used as an index of NO generation32

and expressed as NOx levels (nmol/ml). NOx levels were calculated by measuring conversion of NO3 – to NO2– by the enzyme nitrate reductase using an ELISA assay (R&D Systems, Minneapolis, USA). All samples were tested in duplicate and values were corrected by running samples with culture media without cells to assess background NOx levels.

Cell cultures.

Human peripheral blood polymorhonuclear cells (PMNs) were isolated from patients and donors by Polymorph Prep solution (Axis-Shield, Oslo, Norway). Cells were washed twice with freshly made phosphate-buffered saline (PBS) at 4 °C and viability was evaluated by trypan blue (Sigma, Madrid, Spain). Cells were cultured in phenol red free RPMI 1640 medium (Gibco BRL, Life Technologies, Paisley, UK) supplemented with L-glutamine, antibiotics and 10% FBS at a concentration of 106 cells/ml in presence of no stimulus, CpGs ODN 2395 (20ng/ml) (InvivoGen, San Diego, CA), LPS (100ng/ml) (E. coli serotype 0111:B4; Sigma) or Pam3CSK4 (100 ng/ml) (InvivoGen) during 24 hours. After that period, all supernatants and pellets were collected and stored at −20 °C.

Quantitative PCR analysis.

Total RNA was extracted using QIAamp RNA Blood Mini Kit (QIAGEN) and quantitative PCRs were performed in order to evaluate the expression of Toll-like receptors studied, as well as the key genes in their signaling cascade. The reactions were performed in a 12.5 uL PCR mixture using qScript One-Step SYBR Green RT-qPCR (Quanta BioScience, Gaithesburg, Maryland). The specific primers used were: 5′ TGTGACCGCAATGGTATCTG 3′ (forward) and 5′TGTTGTTGGACAGGTCAAGG 3′ (reverse) for TLR2, 5′ TCCATAAAAGCCGAAAGGTG 3′ (forward) and 5′ GATACCAGCACGACTGCTCA 3′ (reverse) for TLR4, 5′ GGGAGCTACTAGGCTGGTATAAAAATC 3′ (forward) and 5′GCTACAGGGAAGGATGCTTCAC 3′ (reverse) for TLR9, 5′ GGACCCAGCATTGAGGAG 3′ (forward) and 5′ ACAGCGGCCACCTGTAAA 3′ (reverse) for MyD88, 5′ TCATGAAGAAGAGTCCTTTCAGC 3′ (forward) and 5′ CTGGCTTGGGGACAGAAG 3′ (reverse) for nF-KB, 5′ GACTCCATCTTGGCTGTGA 3′ (forward) and 5′ TGATTTCTGCTCTGACAACCT 3′ (reverse) for IFN-α, 5′ AGGACAGGATGAACTTTGAC 3′ (forward) and 5′ TGATAGACATTAGCCAGGAG 3′(reverse) for IFN-β. Relative mRNA levels were calculated by normalizing to an endogenous reference gen (GAPDH).

Statistical analysis.

Continuous variables are reported as mean ± standard deviation (or as median [25th–75th percentiles] in Figures) and categorical variables as frequency or percentages. Quantitative data were analysed using the Mann-Whitney U test for simple comparisons or the Kruskal-Wallis test followed by pairwise comparisons using the Mann-Whitney U test with the post-hoc Bonferroni correction for multiple comparisons. Differences in qualitative variables were analysed using the $\chi 2$ test. Bivariate correlations between continuous variables were calculated using the Spearman test. All reported p values are 2-sided, and p values lower than 0.05 were considered to indicate significance. All calculations were performed using the IBM SPSS Statistics 19.

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Author Contributions

P.P., O.J.: experimental work, data collection. P.G.: sample processing and storage. P.Z.: statistical analysis of data. E.C., J.M.G.N., J.S.: basic and clinical validity of results. R.F.: study design, data interpretation and manuscript writing.

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IL26 modulates cytokine response and anti-TNF consumption in Crohn's disease patients with bacterial DNA.

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Resumen

La interleucina (IL26) interviene en la destrucción de microbios y la detección innata de ADN de bacterias (ADNbact). Se evaluó la relación entre los niveles séricos de IL26 y la traslocaciónde ADNbact en la enfermedad de Crohn (EC). Realizamos un estudio prospectivo en pacientes con enfermedad de Crohn en remisión. Se evaluaron polimorfismos comunes en el gen de la IL26, niveles de citocinas séricas, proteínas del complemento, ADNbact amplificado y anti-TNF-α. Se realizó además un análisis in vitro con PBMCs de pacientes.

Se incluyeron un total de trescientos trece pacientes (CDAI media: 83.6 ± 32.8; calprotectina fecal media: 55.4 ± 35.3 μg/g). Detectamos la presencia de ADNbact en 106 de los pacientes estudiados (33,8%) y 223 (71%) presentaban un genotipo variante para la IL26. La presencia de ADN bacteriano en suero se correlacionó significativamente con un aumento en los niveles de IL26 en comparación con los pacientes sin ADNbact. Las PBMCs de los pacientes con genotipos variantes mostraron una capacidad significativamente reducida para eliminar E. coli en comparación con los pacientes con genotipo salvaje. La estimulación con una proteína IL26 recombinante redujo la presencia de citocinas proinflamatorias en respuesta a E. coli en los sobrenadantes de las células procedentes de pacientes con genotipos variantes. Los niveles de anti-TNF-α, medidos en el suero de pacientes en tratamiento con biológicos con genotipo varIL26 frente a wtIL26, fueron significativamente más bajos en presencia de ADNbact.

Las células de los pacientes con genotipos polimórficos frente a los de genotipo salvaje, cultivadas con ADN de E. coli e infliximab, mostraron una disminución significativa en la concentración de anti-TNF-α libre. El hecho de poseer un genotipo variante para la IL26 se asoció con el comienzo de tratamientos anti-TNF-α durante el seguimiento a 6 meses de los pacientes. Los polimorfismos en este gen pueden prevenir el aclaramiento de ADNbact e identificar a pacientes de Crohn con una evolución inflamatoria y respuesta al tratamiento empeoradas.

IL26 modulates cytokine response and anti-TNF consumption in Crohn's disease patients with bacterial DNA

Abstract

Interleukin IL26 supports killing of microbes and the innate sensing of bacterial-derived DNA (bactDNA). We evaluated the relationship between IL26 serum levels and bactDNA translocation in Crohn's disease (CD). We ran a prospective study on CD patients in remission. IL26 common polymorphisms, serum cytokines and complement protein, amplified-bactDNA, and anti-TNF-α were evaluated. In vitro PBMC analysis was performed. Three hundred and thirteen patients were included (mean CDAI: 83.6 ± 32.8; mean fecal calprotectin: 55.4 ± 35.3 μg/g). A total of 106 patients (33.8%) showed bactDNA and 223 patients (71%) had a varIL26 genotype. BactDNA significantly correlated with increased IL26 levels compared with bactDNA-negative patients. PBMCs from varIL26 patients significantly reduced E. coli killing capacity compared with wtIL26-genotyped patients. The stimulation with a recombinant IL26 protein reduced proinflammatory cytokines in response to E. coli in the varIL26 cell supernatants. Serum anti-TNF-α levels in varIL26 vs wtIL26-genotyped patients on biologics were significantly lower in the presence of bactDNA. Cells from varIL26 vs wtIL26-genotyped patients cultured with E. coli DNA and infliximab showed a significant decrease in free anti-TNF-α concentration. A varIL26 genotype was associated with the initiation of anti- TNF-α in CD patients during the 6-month follow-up. IL26 polymorphisms may prevent bactDNA clearance and identify CD patients with a worse inflammatory evolution and response to therapy.

Key messages

- BactDNA translocation in CD is associated with an increased risk of relapse.
- IL26 is sensitive to bactDNA and modulates the inflammatory response in CD patients.
- The varIL26 genotype is associated with reduced PMN capacity to kill bacteria.
- AvarIL26 genotype is associated with decreased levels of anti-TNF-α in CD patients.
- IL26 may help explain the role of bactDNA as a risk factor of flare in CD patients.

Keywords

Crohn's disease, Bacterial translocation, Interleukin 26.

Introduction

The translocation of bacterial DNA (bactDNA) into the blood is a frequent and clinically relevant event in up to 40% of patients with Crohn's disease (CD) [1, 2], which is driven by an unbalanced interaction between the intestinal microbiota and the mucosal immune system in a genetically predisposed individual [3–6], leading to a sustained inflammatory milieu in these patients [7, 8]. The translocation of bactDNA has been associated with increased disease activity, and it has been identified as an independent risk factor of flare in the short term[9].

Interleukin 17-producing helper T (TH) cells are involved in defense against fungi and extracellular bacterial infections, are increased in numbers in active CD patients [10], and produce cytokines associated with disease activity [11]. Interleukin IL26 is produced by TH17 cells in colonic lessions of patients with active inflammatory bowel disease (IBD) [12]. IL26 is a 19 kDa member of the IL-20 cytokine family that signals through the IL-10R2-IL-20R1 heterodimeric receptor [13]. It facilitates killing of microbes, senses extracellular bactDNA, and promotes a potent proinflammatory response

by inducing plasmocytoid dendritic cells (pDCs) to secrete interferon (IFN)-α [14]. The clearance of bactDNA might be compromised in the absence of IL26 availability and account for disease flares. Actually, the blockade of IL-17A with the human anti-IL-17A monoclonal antibody secukinumab leads to increased disease activity in CD patients [15].

Genome-wide association studies have identified singlenucleotide polymorphisms (SNPs) in the IL26 gene that were associated with IBD [16, 17] and other inflammatory disorders, such as rheumatoid arthritis [18]. As IL26 promotes bacterial killing [14], we hypothesize that CD patients with a variant (var) IL26 genotype may not adequately clear bactDNA fragments, facilitating upheld levels of proinflammatory mediators such as TNF-α. This might contribute to the significantly reduced levels of anti-TNF-α observed in patients with bactDNA compared to patients without bactDNA, considered as an indirect evidence of increased drug consumption in these patients [8], and would support the role of bactDNA translocation as a risk factor for relapse in CD patients [9].

In the present study, we have investigated the role of IL26 on the inflammatory and anti-TNF-α levels in response to bactDNA translocation in a large series of CD patients in remission.

Material and methods

Patients and study design

In this prospective, observational study, CD patients in remission, diagnosed and followed at Hospital General Universitario de Alicante, Spain, were consecutively included. Remission was defined as a CDAI < 150 and absence of clinical symptoms of relapse. Only patients, who did not change their established therapy in the previous month, were included. The diagnosis of CD was established according to standard clinical, endoscopic, histological, and radiographical criteria [19]. Patients treated with antibiotics in the previous 4 weeks, patients with signs of active infection, and those who refused to sign informed consent to participate in the study were excluded. The incidence of IL26 gene polymorphisms in the control population was obtained from The 1000 Genomes Project Consortium [20] (www.1000genomes.org). The Ethics Committee of Hospital General Universitario de Alicante approved the study protocol.

Usual clinical and analytical variables in the management of CD patients, including fecal calprotectin, were recorded at baseline in all patients. All patients were Caucasian of Mediterranean ethnicity and were classified according to the Montreal classification [21]. All included patients received diaries to record symptoms 1 week prior to inclusion and sample collection.

Blood samples were obtained for routine hematological and biochemical studies at inclusion and inoculated in aerobic and anaerobic blood culture bottles, 10 ml each. Simultaneously, two separate blood samples were inoculated under aseptic conditions in rubber-sealed sterile Vacutainer SST II and K3E tubes, respectively (BD Diagnostics, Erembodegem, Belgium), which were never exposed to free air. Peripheral blood mononuclear cells (PBMCs) were isolated using Biocoll Separating Solution (Biochrom GmbH, Berlin, Germany) according to manufacturer's instructions.

Identification of bactDNAfragments and IL26 genotyping

Genomic DNA was isolated from 5 × 106 cells with the QIAmp DNA Blood Minikit (Qiagen, Hilden, Germany). BactDNAwas identified by running a broad-range PCR with universal eubacterial primers of a conserved region of 16SrRNA gene followed by partial nucleotide sequencing, as previously described [22].

We studied rs2870946, rs1558744, and rs7134599 SNPs in the IL26 gene, located on chromosome 12q15. After extraction of genomic DNA from the peripheral blood with the QIAamp DNA Blood Mini Kit (Qiagen), partial amplification of the regions containing the different polymorphisms was performed using the following specific primers: rs2870946 forward 5′-GCTTAATTGCTCCAGCCATGC-3, reverse 5′-GAAATGGGAAGGCACAGGCTA-3′; rs1558744 forward 5′-CTCCCACCCACCCCAATTTA-3′, reverse 5′-ACGGGTTGACCTGTTCAGAG-3′; and rs7134599 forward 5′-AGAAGTGAGCTTGCTTGTCTGT-3′, reverse 5′-GCAGGATCAAAATGTCAAGCAGT-3′. The PCR products, of 174, 182, and 153 bp length, respectively, were purified using ExoSAP-IT PCR Product Cleanup (Affymetrix). The incidence of polymorphisms was detected by nucleotide sequencing of PCR products using the same primers as for the amplification. Subsequently, the sequencing process was performed in the sequencing service of Secugen S.L. The results were analyzed with FinchTV software version 1.5 (Geospiza). Patients bearing either one or more of the studied SNPs were grouped as variant (var) IL26 patients and compared with wild-type (wt) IL26 patients.

Serum cytokines, complement proteins and free anti-TNF-α levels. Presence of anti-drug antibodies

IL26 levels in serum samples were determined by handling an enzyme-linked immunosorbent assay (ELISA) for human IL26 measurement (Cloud-Clone Corp. Houston, TX) according to the manufacturer's instructions. Sensitivity assays were run to evaluate possible differences between wt and varIL26-genotyped patients and between IL26 either alone or in the presence of bactDNA (Supplementary Fig. 1). Serum TNF-α, IFN-γ, and IL-12p40 levels were determined by cytometric bead arrays (CBA) and measured with a FACSCanto II flow cytometer (Becton Dickinson, San Jose, CA).

ELISA kits were also carried out to measure free infliximab and adalimumab levels and to detect anti-drug antibodies (Matriks Biotek, Ankara, Turkey) according to the manufacturer's instructions. Proteins of the complement system C3b, the membrane attack complex (MAC), and C5a were evaluated by MicroVue EIA kits (Quidel Corporation, San Diego, CA) in serum samples of CD patients according to manufacturer's instructions, as previously described [23]. All samples were tested in triplicate and read in a Sunrise Microplate Reader (Tecan, Männedorf, Switzerland). The detection limits for each cytokine assay varied between 2 and 5 pg/mL, between 10 and 30 ng/mL in the case of free nti- TNF-α kits, and between 5 and 8 pg/mL for the complement protein assays. Standard curves were generated for every plate, and the average zero standard optical densities were subtracted from the rest of the standards and samples to obtain a corrected concentration for all parameters. The presence of anti-drug antibodies was evaluated by a cut-off value estimated by multiplying the optical density (OD) of the zero standard by 3, as indicated by the manufacturer's. Samples were considered positive when the ratio sample OD/zero standard OD was higher than 3.

Peripheral blood mononuclear cell killing assays

To evaluate blood PBMC bactericidal activity, cells from a subset of wtIL26 and varIL26 genotyped patients were isolated and seeded left untreated or treated with anti-IL26 or an anti-IgG2 isotype control (Sigma-Aldrich, Madrid, Spain) for a 48h period. Following this incubation, 2.5 × 106 cells were washed and exposed to the same amount of E. coli (104 CFU; serotype 0111:B4). The killing assay was performed at 37 °C in a shaker at 15 rpm for 20 min to measure the basal capacity of PBMCs to ingest bacteria before killing occurred (T0), or 2 h to measure the real PBMC killing ability (T2). Extracellular E. coli were thoroughly washed and subjected to a 30% sucrose centrifugation to better eliminate bacteria. Cells were resuspended in PBS 5% serum and then diluted in sterile water, to be

seeded in agar plates at the estimated dilution ratio. Plates were left 24 h at 37 °C and colony-forming units counted afterwards.

Cell cultures

PBMCs from patients without bactDNA not receiving biologics or immunosuppressors were washed with phosphatebuffered saline (PBS) at 4 °C. Viability of isolated cells was evaluated by trypan blue staining (Sigma, Madrid, Spain). Cells were resuspended in phenol red-free RPMI 1640 medium (Gibco BRL, Life Technologies, Paisley, UK) supplemented with 10% human serum AB (BioWhittaker, Walkersville, Maryland, USA). PBMCs (1 × 106 cells/well) were incubated with the following: (a) infliximab (100 μg/ mL/106 cells) (infliximab was kindly provided by Merck Sharp and Dohme of Spain MSD, Madrid, Spain) plus E. coli DNA (200 ng/mL/106 cells) for 48h; (b) recombinant human IL26 (R&D Systems) (100 ng/1 × 106 cells) plus E. coli DNA (200 ng/mL/106 cells) for 24 h.

Statistical analysis

Continuous variables were reported as mean ± standard deviation or 95% confidence interval, and categorical variables were expressed as frequencies and percentages. Differences between patient groups were analyzed using the U-Mann Whitney test for quantitative data and Chi-square test for qualitative data. The Kolmogorov–Smirnov test was used to test normality of continuous variables. Statistical differences were analyzed using the $\chi 2$ test for categorical data, and analysis of variance (ANOVA) test for quantitative data followed by the post hoc Bonferroni correction for multiple comparisons. Quantitative data showing non-normal distribution were analyzed using the Mann–Whitney U test or Kruskal–Wallis test followed by pairwise comparisons using the Mann–Whitney U test with the post hoc Bonferroni correction for multiple comparisons. Bivariate correlations were analyzed using the Spearman test. Statistical significance was considered at pvalues less than 0.05. Statistical analysis was performed using SPSS v15 and R software.

Results

Characteristics of patients

Three hundred and thirteen CD patients in remission were included in the study. Clinical and analytical characteristics of patients are shown in Table 1. Briefly, mean age was 42 ± 15 years and 54% were male. Mean CDAI was 83.6 ± 32.8, and mean feca l calprote ctin was 52.4 ± 34.8 μg/g. These parameters, along with ESR and CRP levels shown in Table 1, confirm clinical remission in our series of patients. Forty percent of patients were active smokers and 25% had a history of previous surgery. Ileal disease was present in 45% of patients, whereas colonic or ileo-colonic disease was present in 50% of patients. Twenty-one percent of patients presented with perianal disease. Twenty-eight percent of patients were on anti-TNF, either alone or combined, and 55% were receiving immunosuppressors.

BactDNA translocation and IL26 SNP distribution in CD patients

One hundred and six patients had bactDNA fragments in the blood (33.8%). Supplementary Table 1 presents sequencing analysis showing the identified bacterial species and the amount of amplified bactDNA for each species. BactDNA belonged to the Enterobacteriaceae family in 78% of patients. The amount of amplified bactDNA was not significantly different between species, family, or gram distribution.

IL26 SNP distribution of genotypes and allelic frequencies among included patients can be followed in Table 2. All variants were found to be in the Hardy–Weinberg equilibrium in the controls. Variant IL26 (varIL26)-genotyped patients (n = 223) were defined as carrying any of the three variants studied either in homozygosis or heterozygosis compared with patients with a wild-type (wt) IL26 genotype (n = 90). The rate of bactDNA translocation in varIL26-genotyped patients was similar to that present in wtIL26-genotyped patients (33.6 vs 34.4%, p = ns). No significant clinical or analytical differences were found among included CD patients distributed either by bactDNA translocation or IL26 genotype (data not shown).

IL26 serum levels respond to systemic bactDNA translocation

The presence of circulating bactDNAwas associated with significantly increased levels of IL26 regardless of IL26 genotype status compared with bactDNA-negative patients (101.6 ± 25.4 vs 18.9 ± 11.2, p = 0.001). This elevation was blunted in patients with varIL26 genotype (Fig. 1a).

The amount of bactDNA was significantly increased in varIL26 vs wtIL26 patients (26.9 ± 6.0 vs 30.8 ± 7.2, p = 0.01). The overall correlation between amplified bactDNA concentration in patients with bactDNA and IL26 serum levels was statistically significant although weak (r = 0.37; p = 0.01). This correlation significantly improved when only wtIL26 patients were considered (r = 0.77; p = 0.001). The correlation between both variables was lost when patients with a varIL26 genotype (r = 0.08; p = 0.476) were considered (Fig. 1b). These results were confirmed in vitro in PBMCs from wt and varIL26 patients exposed to different concentrations of E. coli bactDNA (Fig. 1c).

Functional capacity of PBMCs to kill bacteria is decreased in varIL26-genotyped patients

These results suggest that PBMC interaction with bacteria is compromised in varIL26-genotyped patients. To confirm this, killing capacity of PBMCs from a subset of bactDNA-negative patients was evaluated. Figure 2 shows that wtIL26-genotyped patients' PBMCs are able to significantly reduce the total CFU counts 2 h after exposure to E. coli. However, the presence of a varIL26 genotype significantly worsens PBMC killing capacity, as total CFU count significantly increases at 2 h in comparison to wtIL26-genotyped PBMCs. In addition, preincubation of PBMCs from wtIL26 and varIL26-genotyped patients with anti-IL26 further reduces PBMC killing capacity.

The soluble inflammatory response to bactDNA is sensitive to IL26 genotype

We then evaluated the soluble inflammatory response to bactDNA in patients distributed by IL26 genotype. Serum levels of proinflammatory mediators in patients without bactDNA were as follows: TNF-α [44.4 ± 29.8 pg/ mL], IFN-γ [102.2 ± 68.5 pg/mL], and IL-12 [194.3 ± 70.4 pg/mL]. These levels significantly increased both in wtIL26 and varIL26 patients with bacterial DNA (Fig. 3a). However, the variant genotype was associated with significantly further increased levels of all three cytokines compared to wtIL26-genotyped patients with bactDNA. To support this result, serum levels of complement proteins were measured and showed a significant increase in the overall series of patients with vs without bactDNA (Supplementary Table 2). The presence of a varIL26 further increased complement protein levels in patients with bactDNA compared to wtIL26-genotyped patients.

We then cultured PBMCs from bactDNA-negative patients distributed by IL26 genotype with human recombinant IL26 and E. coli DNA. Cytokine levels were significantly higher in the supernatants of PBMCs cultured with bacterial DNA from varIL26 patients than in wtIL26 patients (white vs black bars), supporting the in vivo results. The addition of the recombinant IL26 protein was associated with a reduction in pro-inflammatory cytokine levels in the supernatants of cultured varIL26 cells to levels present in wtIL26 cells in response to E. coli DNA (Fig. 3b). Supplementary Table 3 shows cytokine levels in the supernatants of cultured PBMCs unstimulated and with human recombinant IL26 alone.

Table 1 Clinical and analytical characteristics of patients

	Patients (n = 313)
Age (years)	42 ± 15
Weight (kg)	70.24 ± 14.86
Gender (male/female), *n* (%)	167 (53.4%)/146 (46.6%)
Smoking habit (yes/no/ex), *n* (%)	126 (40.2%)/119 (38.0%)/68 (21.8%)
Disease duration (months)	115.15 ± 114.55
Resection, *n* (%)	71 (22.6%)
CDAI	83.6 ± 32.8
Montreal A (age of onset), *n* (%)	
A1 (≤ 16)	18 (5.8%)
A2 (17–40)	219 (70.0%)
A3 (> 40)	76 (24.2%)
Montreal L (location), *n* (%)	
L1	143 (45.6%)
L2	70 (22.3%)
L3	86 (27.4%)
L4	14 (4.5%)
Montreal B (behavior), *n* (%)	
B1 (non-stricturing, nonpenetrating)	151 (48.2%)
B1p (non-stricturing, nonpenetrating, penetrating perianal disease)	40 (12.8%)
B2 (stricturing)	49 (15.6%)
B2p (stricturing, perianal disease associated)	14 (4.5%)
B3 (penetrating)	45 (14.4%)
B3p (penetrating, penetrating perianal disease)	14 (4.5%)
Therapy, *n* (%)	
Mesalazine	70 (22.4%)
Azathioprine	104 (33.2%)
Metotrexate	9 (2.9%)
Mesalazine and azathioprine	17 (5.4%)
Mesalazine and steroids	4 (1.3%)
Azathioprine + steroids	4 (1.3%)
Metotrexate + steroids	1 (0.3%)
Infliximab	27 (8.6%)
Adalimumab	29 (9.3%)
Infliximab + azathioprine	16 (5.1%)
Adalimumab + azathioprine	11 (3.5%)
Infliximab + steroids	2 (0.6%)
Adalimumab + steroids	4 (1.4%)
Infliximab + metotrexate	2 (0.6%)
Infliximab + azathioprine + steroids	1 (0.3%)
No therapy	12 (3.8%)
CRP (mg/dL)	0.58 ± 1.10
Fecal calprotectin (μg/g)	52.40 ± 34.80
ASCAs, *n* (%)	118 (39.6%)
Hemoglobin (g/dL)	13.95 ± 2.35
ESR (mm)	19.3 ± 15.4
Albumin (g/dL)	4.12 ± 4.50
Total WBCs (mm^3)	6892.5 ± 2636.8
Temperature (°C)	36.07 ± 0.22
Pulse rate (bpm)	70.68 ± 6.11

Table 2 Allelic frequencies of *IL26* SNPs in included CD patients

Genotype *n* (%) patients/controls				
	Homozygous wild-type	Heterozygous	Homozygous variant	Variant allele frequency (%)
IL26 rs1558744 (A > G)	55 (17.5%)/88 (17.5%)	178 (56.8%)/229 (45.5%)	80 (25.5%)/186 (37.0%)	53.9/59.7
IL26 rs2870946 (T > C)	243 (77.6%)/431 (85.7%)	67 (21.4%)/68 (13.5%)	3 (0.9%)/4 (0.8%)	11.6/7.6
IL26 rs7134599 (G > A)	88 (28.1%)/208 (41.4%)	170 (54.3%)/76 (15.1%)	55 (17.5%)/219 (43.5%)	44.7/36.9

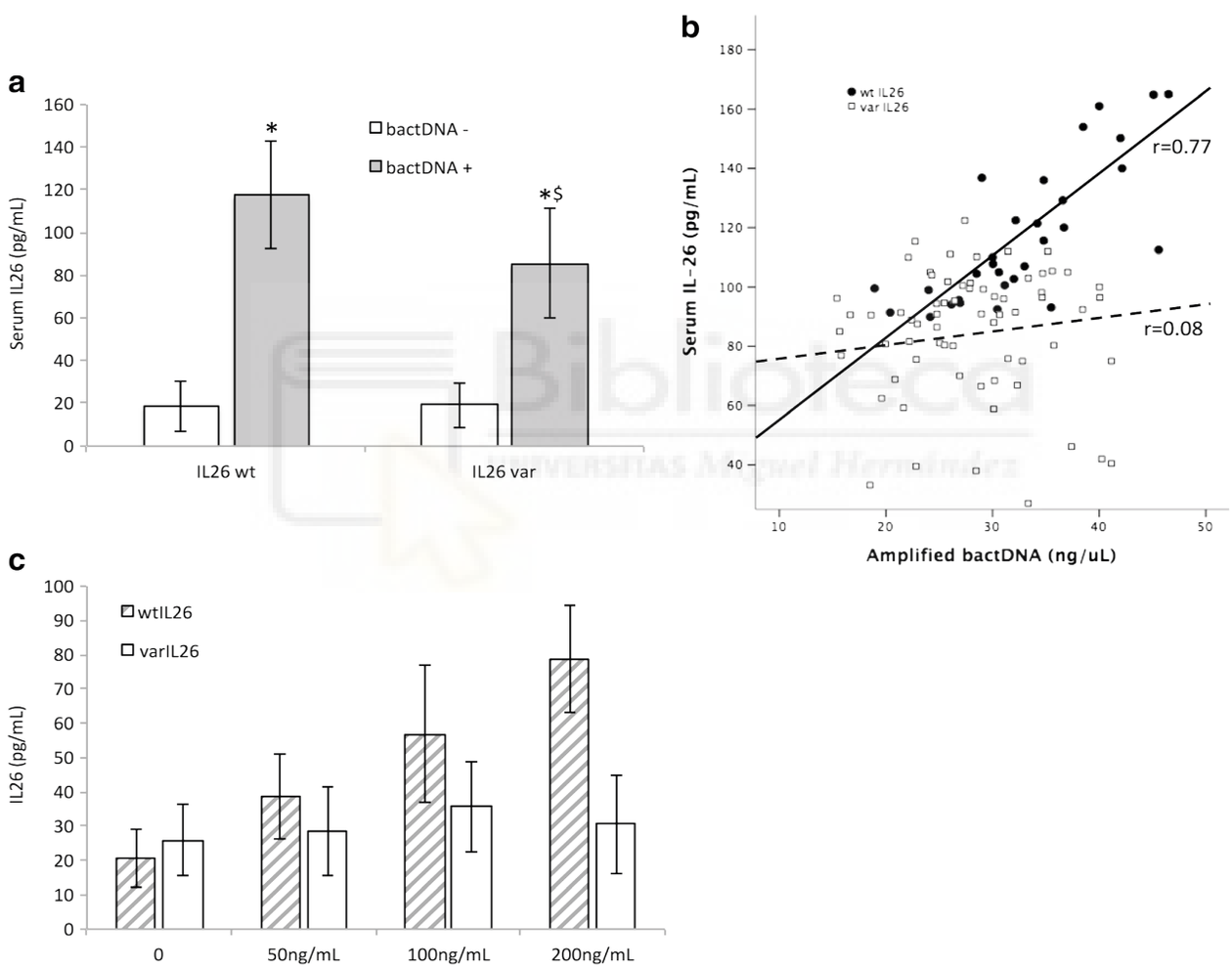

Fig. 1 Serum IL26 levels in CD patients. **a** Serum IL26 levels in CD patients distributed by *IL26* genotype and the presence of bacterial DNA in blood. **b** Correlation between IL26 serum levels and amplified bactDNA in patients with bactDNA in blood distributed by *IL26* genotype. *$p < 0.01$ compared with patients without bactDNA; $^{S}p = 0.01$ compared with wt*IL26* with bacterial DNA. **c** IL26 levels in the supernatants of PBMCs from wild-type and variant *IL26*-genotyped patients cultured with increasing amounts of *E. coli* DNA. Figure shows mean ± SD from 10 patients from each group. *$p < 0.01$ compared with wt*IL26*. *wt* wild-type, *var* variant

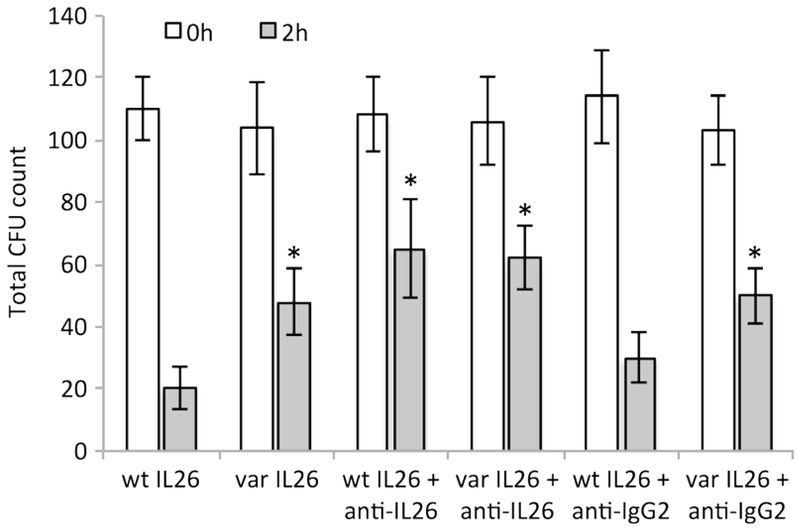

Fig. 2 *E. coli* killing assays on blood PBMCs. Total CFUs before ($T = 0$) and 2 h after *E. coli* exposure ($T = 2$) are represented. Results were obtained in experiments using 2.5×10^6 cells from 10 wt*IL26* and 10 var*IL26* patients. $^*p < 0.01$ compared with wt*IL26* patients and wt*IL26* patients incubated with an IL26 isotype control

Anti-TNF treatment efficacy is affected by IL26 genotype in patients with bacterial DNA

As varIL26 was associated with elevated TNF-α concentrations in patients with circulating bactDNA, we studied varIL26 effects on the treatment with anti-TNF-α drugs. Six out of 92 patients on biologics (4 wtIL26 and 2 varIL26) had anti-drug antibodies in the blood and were excluded from this analysis. Figure 4 shows that serum free anti-TNF-α levels in varIL26- genotyped patients were significantly lower than levels in wtIL26-genotyped patients in the presence of bactDNA.

To confirm the further reduction of serum free anti-TNF-α levels associated with a varIL26 genotype and the presence of bactDNA, we cultured PBMCs from bactDNA-negative patients distributed by IL26 genotype with E. coli DNA and infliximab (Fig. 5). While TNF-α levels significantly increased, the concentration of infliximab significantly decreased in the supernatant of cultured cells from patients with a varIL26 genotype stimulated with E. coli DNA compared with wtIL26- genotyped patients. IL26 blockade in wtIL26-genotyped patients further increased TNF-α levels in the supernatants and signficantly reduced free anti-TNF-α levels. On the contrary, IL26 supplementation to PBMCs from varIL26 patients resulted in the partial restoration of the TNF-α response and available anti-TNF-α levels shown by wtIL26-genotyped patients.

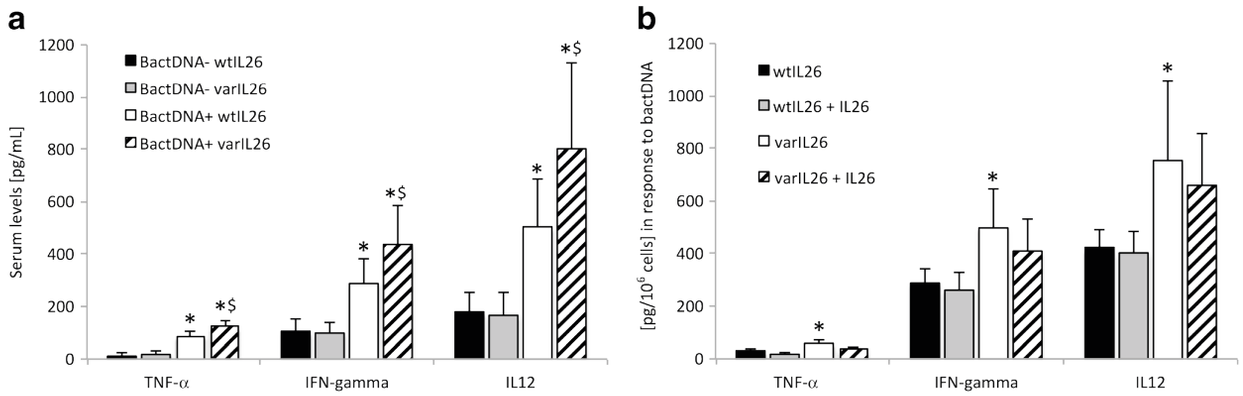

Fig. 3 **a** Serum levels of TNF-α, IFN-γ, and IL-12p40 in CD patients with bactDNA distributed by *IL26* genotype. $^{*}p < 0.01$ compared with serum levels in patients without bactDNA; $^{\$}p < 0.01$ compared with serum levels in bactDNA + wt*IL26* patients. **b** Levels of TNF-α, IFN-γ, and IL-12p40 in the supernatants of PBMCs from bactDNA-negative patients either with recombinant human IL26 or not and cultured with *E. coli* DNA (see methods). Results were obtained in experiments using 1×10^6 cells from 10 wt*IL26* and 10 var*IL26* patients. $^{*}p < 0.01$ compared with the rest of conditions

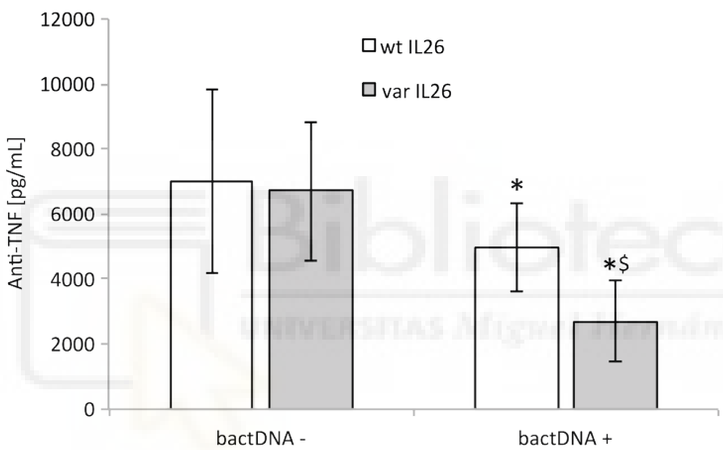

Fig. 4 Free anti-TNF-α serum levels in CD patients on biologics distributed by the *IL26* genotype and the presence of bactDNA. $^{*}p < 0.01$ compared with levels in patients without bacDNA. $^{\$}p < 0.01$ compared with levels in wt*IL26*-genotyped patients

A varIL26 genotype is associated with the initiation of anti-TNF-α therapy in CD patients in remission

Patients were followed up for 6 months. None of the patients were lost during the follow-up period. Forty patients relapsed in this time frame, with no differences according to IL26 genotype (11 of 90 wtIL26 [12.2%] patients vs 35 of 223 [15.7%] varIL26 patients, p = 0.104). Figure 6 shows the percentage of patients who required either initiation, intensification, or switch of biologics. The initiation of biologics, the need for intensification, and switch of biologic drug were more frequent in varIL26-genotyped patients. However, the initiation of biologics was the only statistically significant variable between groups.

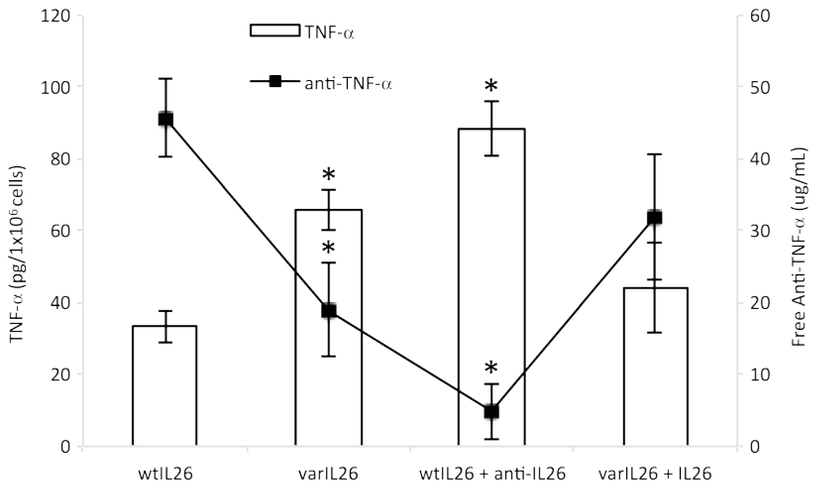

Fig. 5 In vitro analysis of TNF-α and free anti-TNF-α levels in the supernatants of PBMCs from wt*IL26* and var*IL26* patients cultured with *E. coli* DNA (200 ng/mL/10^6 cells) plus infliximab (100 µg/mL/10^6 cells) in all cases. Results were obtained in experiments using 1×10^6 cells from 10 wt*IL26* and 10 var*IL26* patients. *$p < 0.01$ compared with wt*IL26*

Discussion

The present study shows that IL26 is sensitive to bactDNA translocation into blood of CD patients. In addition, a varIL26 genotype is associated with reduced IL26 serum levels in patients with this circulating antigen and the reduction of PBMC functional capacity to kill bacteria. This might compromise bactDNA clearance, inducing a sustained pro-inflammatory environment, and an increased anti-TNF-α consumption.

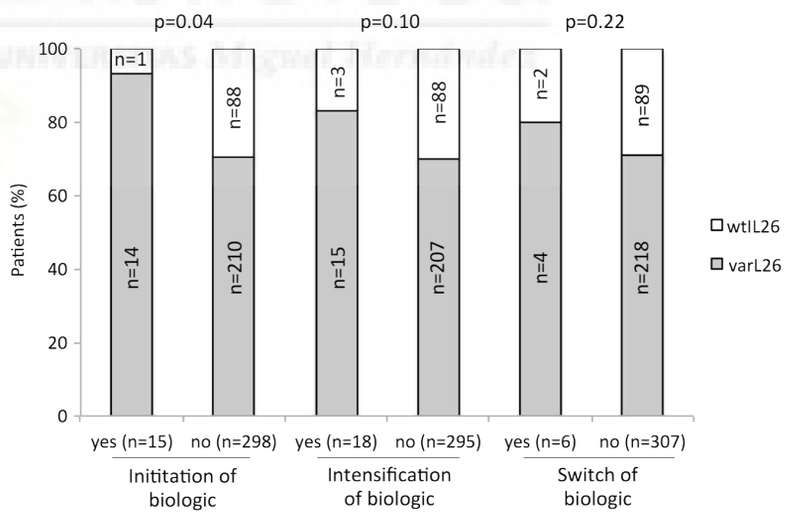

Fig. 6 Clinical evolution of patients. Initiation, intensification, and switch of biologics among included CD patients during follow-up are represented

Bacterial DNA translocation has been identified as a risk factor of relapse in CD patients in remission. In addition to this, an increased risk of other significant clinical events such as the inititation of steroids or the switch to stepped up treatments has also been associated with the detection of circulating bacterial genomic fragments in the blood of CD patients [9]. Likely, the presence of circulating bactDNA induces the secretion of pro-inflammatory cytokines leading to an increased [1] and dose-dependent production of antimicrobial peptides [24]. Its interaction with the immune system is especially relevant in patients with NOD2 gene variants, who seem to be predisposed to CD development [25, 26] and may show an impaired response against the translocation of commensal bacterial antigens [8]. These data support that the translocation of bactDNA may be a risk factor for a more severe disease course.

IL26, a member of the IL-20 cytokine family and part of the Th17 cell cytokines, has been shown to assemble with bactDNA fragments and promote TLR-9 activation in antigen presenting cells to deliver a pro-inflammatory immune response [14]. This facilitates the clearance of circulating bactDNA and may prevent a sustained inflammatory environment. Several IL26 SNPs have been described, and we hypothesized that a varIL26 genotype may affect IL26 interaction with bactDNA and impair the required immune response to resolve this bacterial antigen challenge.

To our knowledge, this is the first study evaluating the effect of IL26 SNPs in CD patients. We have observed that IL26 levels are significantly increased in patients with bactDNA. However, the presence of a varIL26 genotype in patients with bactDNA is associated with significantly reduced IL26 levels compared with wtIL26 patients (Fig. 1). Although a limitation due to untested effect of IL26-DNA complexes on measured IL26 serum levels may be considered, these results suggest first that IL26 production depends in part on the presence of circulating bactDNA in CD patients. In fact, a positive correlation between IL26 and the amount of amplified bactDNA is present in wtIL26-genotyped patients, indicating that this cytokine participates in defense programs against bacterial challenges mediated by Th17 responses [27, 28]. Secondly, that the clearance of bactDNA in these patients may be affected by the presence of a varIL26 genotype, which may partially explain the observed genetic susceptibility to CD in patients with SNPs in the IL26 region [16]. In addition, the concentrations of the pro-inflammatory cytokines TNF-α, IFN-γ, and IL-12 are significantly increased in bactDNA-positive CD patients with a varIL26 genotype (Figure 3a). Potentially, the increased exposure of bactDNA could stimulate the innate immune machinery, in which activated macrophages are recruited, the complement cascade is initiated, and endothelial cells are activated to induce TNF-α and other inflammatory mediators that further contribute to sustain an upheld pro-inflammatory environment [29]. In agreement with this, complement proteins are significantly increased in response to bactDNA in patients with a varIL26 genotype compared to patients with a wtIL26 genotype.

The relevance of an increased systemic immune response in CD patients may be reflected in their need for intensified anti-TNF-α therapy. In the present investigation, the significant reduction in serum TNF-α levels in patients on biologics was only achieved in wtIL26-genotyped patients. In consequence, we found that free anti-TNF-α trough levels are significantly reduced in varIL26 patients (Fig. 4). To model the in vivo situation in vitro, PBMCs from patients without bactDNA were stimulated with E. coli in the presence of infliximab. Cultures of PBMCs from varIL26 patients showed significantly lower amounts of free anti-TNF-α of PBMCs from wtIL26 patients (Fig. 5).

From a clinical point of view, the classification of CD patients by their IL26 genotype could be of interest, not only in terms of their predisposition to an exacerbated inflammatory response, as theymay be exposed to longer antigen circulation in the case of bacterial translocation, but also for the identification of patients who might respond worse to established drug schedules. In fact, the percentage of patients who required initiation of anti-TNF-α was significantly higher in varIL26 vs wtIL26-genotyped patients (Fig. 6). Also, an increased percentage of varIL26 patients included in our series, compared to wtIL26 patients, required intensification or switch of biologic drug, although probably larger series of patients would be necessary to find a statistically significant association. Although results in this regard are merely exploratory and present several limitations that would require a specifically designed study, these patients might benefit from early therapies in the so-called top-down approach [30, 31], especially those subgroups with translocation of bacterial DNA, NOD2, or other CD-related gene variants.

In summary, IL26 participates in the modulation of the proinflammatory soluble response in CD patients in remission with bactDNA translocation. However, the presence of a varIL26 genotype is associated with a reduced PBMC functional capacity to kill bacteria, the stimulation of an exacerbated

inflammatory response, and the increment of anti- TNF-α consumption in these patients. These results may help in explaining the identification of bactDNA in blood as an independent risk factor of flare at short term in CD patients. Further studies on IL26 signaling restoration may provide new therapeutic approaches to control bacterial translocation and inflammation in this subgroup of CD patients.

Acknowledgements

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Compliance with ethical standards

The Ethics Committee of Hospital General Universitario de Alicante approved the study protocol.

Conflict of interest

The authors declare that they have no conflicts of interest.

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DISCUSIÓN

Los episodios de traslocación bacteriana son un fenómeno frecuente en pacientes con trastornos inflamatorios crónicos entorno al eje intestino-hígado. Se conocen los factores que influencian este paso de antígenos desde la luz intestinal, como sería el aumento de la permeabilidad de su pared. Sin embargo, estos eventos no se consideran patológicos hasta el momento en que la respuesta inflamatoria que habría de combatirlos se produce de forma inadecuada y favorece la aparición de otras complicaciones. Dicha respuesta se genera a través de receptores que activan diferentes cascadas de señalización para la producción de citocinas. Considerando estos dos elementos, receptores y citocinas, puntos clave en una respuesta inmunológica correcta y eficiente, estudiamos el efecto de variantes comunes en los mismos en relación con la incidencia de traslocación bacteriana en 2 enfermedades importantes del eje hígado-intestino.

En el primer estudio hemos evaluado el efecto de los SNPs TLR2 rs4696480, TLR4 rs4986790 y TLR9 rs187084 en una serie consecutiva de pacientes con cirrosis, demostrando una asociación entre los genotipos variantes de estos genes y el aumento de la carga de antígenos bacterianos en sangre, así como la disminución del perfil de citoquinas proinflamatorias.

El primer resultado relevante del estudio es que los genotipos TLR variantes se asocian con niveles séricos significativamente aumentados de sus ligandos antigénicos específicos (LTA, LPS y ADN bacteriano, respectivamente), sugiriendo que estas inmunodeficiencias genéticas podrían tener consecuencias en la eliminación de antígenos bacterianos y contribuir a las complicaciones que se desarrollan con frecuencia en la cirrosis. Este resultado se encontraría alineado con hallazgos anteriores que pronostican un mayor riesgo de PBE en pacientes con cirrosis portadores de polimorfismos en

TLR2 (75) o la predisposición a infecciones asociada a variantes en TLR4 (88).

Aunque la tasa de traslocación de ADN bacteriano no aumentó en pacientes con genotipo variante para el TLR9, se observó un incremento significativo en la cantidad de ADN bacteriano amplificado en estos pacientes en comparación con aquellos que presentaban el genotipo salvaje. Esto es relevante ya que se ha demostrado que, en pacientes con cirrosis, el grado de respuesta inflamatoria soluble se ve significativamente afectado por la concentración de ADN bacteriano (89). Sin embargo, a pesar del significativo aumento de la carga antigénica en el suero de los pacientes (Fig. 2), los portadores de los SNPs evaluados mostraron una secreción reducida de mediadores proinflamatorios. Dichos resultados sugieren que estas variantes genéticas comprometen la interacción de los antígenos bacterianos con sus receptores específicos, limitando así la respuesta inflamatoria soluble innata en estos pacientes.

Para confirmar nuestros resultados, evaluamos la respuesta soluble a ligandos específicos de los TLR en pacientes cirróticos y donantes (con diferentes genotipos para las variantes) mediante un ensayo in vitro. La estimulación dio como resultado niveles significativamente elevados de citocinas proinflamatorias en comparación con las células en reposo pero, como ocurría in vivo, los genotipos variantes mostraron una capacidad significativamente menor para inducir TNF-α e IL-6 en comparación con las células de pacientes WT, lo que indica que el impacto de los polimorfismos estudiados en el sistema inmune es independiente del contexto patológico.

Nuestros resultados in vitro no mostraron diferencias en los niveles de expresión de los TLR, lo que se traduce en una estimulación competente de estos receptores a pesar de sus regiones polimórficas (Fig. 4A-C). Por el contrario, las moléculas de señalización aguas abajo se encontraban reducidas en las células de genotipos variantes frente a las WT (Fig. 4D-I), lo que sugiere una ruptura en la vía de señalización proinflamatoria.

Estos resultados perfilan la adopción de un "estado tolerante" que podría explicar el aumento del riesgo de infecciones en los pacientes portadores de polimorfismos en los TLRs. Sin embargo, los mecanismos por los cuales las variantes estudiadas pueden condicionar estos receptores no están completamente dilucidados. Existen datos sobre SNPs que suprimen la activación de NF-kB y la expresión de citoquinas al afectar el potencial electrostático del dominio TIR(90), provocadas por el polimorfismo R753Q en TLR2. Otras variantes en este mismo receptor, como la P681H, se asocian con tasas de internalización más lentas desde la superficie de la célula a los endosomas (91). Por otro lado, el polimorfismo D299G en TLR4 parece inhibir la asociación del receptor, inducida por LPS, con los adaptadores MyD88 y TRIF, lo que suprime la activación de los factores de transcripción NF-kB e IRF-3, la fosforilación de p38 MAPK y la inducción de citoquinas dependientes de MyD88 y TRIF(92). En el TLR9 también se conocen polimorfismos que afectan a la actividad de NF-kB; parece probable que el rs5743836, localizado en la región promotora del gen, interfiera mediante la modificación de un potencial sitio de unión para dicho factor de transcripción (93, 94).

Desde un punto de vista clínico, los polimorfismos en TLRs se han asociado ampliamente con enfermedades (95). En particular, con complicaciones relacionadas con bacterias y la respuesta inflamatoria asociada. Como explicación para esas asociaciones, ahora demostramos que la carga de antígenos circulantes aumenta significativamente en pacientes con cirrosis portadores de variantes en los TLR. Dado que la traslocación de productos bacterianos es frecuente y recurrente en la cirrosis, es probable que una señalización deficiente por parte de los TLR este involucrada, proporcionando a su vez nuevas dianas para tratar las complicaciones derivadas de bacterias en la cirrosis.

El segundo estudio está centrad en la relación existente entre la traslocación del ADN bacteriano y la enfermedad de Crohn. La presencia de DNA bacteriano en sangre de pacientes con EC en remisión

se ha identificado como un factor de riesgo de recaída a corto plazo, además de incrementar el riesgo de aparición de otros eventos clínicos, como la necesidad de iniciar el tratamiento con esteroides o el cambio a tratamientos intensivos (21). La presencia de ADNbact circulante induce la secreción de citoquinas proinflamatorias que conducirían a una incremento dosis dependiente en la producción de péptidos antimicrobianos (20, 96).

Los datos apuntan a la traslocación de ADN bacteriano como un importante factor agravante en el curso de la enfermedad y este estudio confirma que la IL26 está involucrada en la eliminación de dicho ADN inmunogénico (cuya presencia se asocia con un aumento en los niveles de IL-26 en enfermos de Crohn).

La acumulación de SNPs en el gen codificante de la IL-26 se relaciona con concentraciones reducidas de dicha citocina en pacientes con EC positivos para la presencia de ADN bacteriano en sangre, lo que puede comprometer el aclaramiento del mismo, induciendo un entorno proinflamatorio sostenido y un mayor consumo de fármacos anti-TNF-α.

Se ha demostrado que la IL-26 forma complejos con fragmentos de ADNbact y promueve la activación del TLR9 en células presentadoras de antígeno para producir una respuesta inmune proinflamatoria (53). Esto facilita el aclaramiento de ADNbact circulante y puede prevenir el desarrollo de un entorno inflamatorio sostenido. Nuestra hipótesis plantea la posibilidad de que estas variantes puedan obstaculizar la interacción de la IL-26 con el ADNbact y deteriorar la respuesta inmune requerida para responder a este insulto antigénico.

Los resultados obtenidos en este trabajo asocian la acumulación de dos o más SNPs, en pacientes positivos para ADNbact, con niveles de IL26 significativamente reducidos, en comparación con los pacientes sin SNPs (Figura 1). Estos datos sugieren, en primer lugar, que la producción de IL26 depende en parte de la presencia de ADN bacteriano circulante en pacientes con EC. De hecho, existe una correlación positiva entre IL26 y la cantidad de ADNbact amplificado, lo que indica que esta citocina participa en programas de defensa frente a bacterias mediados por respuestas Th17 (97, 98).

En segundo lugar, la acumulación de SNPs en el gen IL26 reduce la producción de esta citocina en presencia de ADN bacteriano y como consecuencia, el aclaramiento de ADNbact puede verse afectado, lo que podría explicar parcialmente la susceptibilidad genética a la EC observada en pacientes con polimorfismos en la región IL-26 (85). En línea con estos hallazgos, en los pacientes con dos o más SNP la expresión génica de TLR9 en presencia de ADNbact se encontraba reducida en comparación con pacientes con genotipo WT (Figura 2), lo que hace pensar que el pronunciado ambiente inflamatorio en estos pacientes modula la expresión de TLR9. Por otro lado, las concentraciones de las citoquinas proinflamatorias TNF-α, IFN-γ e IL-12 eran significativamente mayores en pacientes positivos para ADN bacteriano con genotipo variante (Tabla 4), lo que parece indicar que a pesar de la regulación negativa del gen TLR9, la exposición permanente de a ADN bacteriano podría estimular la maquinaria inmune innata, en la que se reclutan macrófagos activados, se inicia la cascada del complemento y se activan las células endoteliales para producir TNF-α y otros mediadores inflamatorios que contribuyen al mantenimiento de un entorno proinflamatorio (99). De acuerdo con esto, las proteínas del complemento se hallaban significativamente aumentadas en respuesta a ADNbact en pacientes con SNPs en comparación con aquellos IL-26 WT.

La manifestación de una mayor respuesta inmune sistémica en pacientes con EC puede verse reflejada en su necesidad de intensificar la terapia anti-TNF-α. En la presente investigación, la reducción significativa en los niveles séricos de TNF-α durante la terapia con productos biológicos solo se logró en ausencia de SNPs en la IL-26. En consecuencia, los niveles mínimos libres de anti-TNF-α han

de verse reducidos significativamente en pacientes con SNPs en la IL26. Para modelar la situación que ocurriría in vivo, realizamos un ensayo in vitro en el que se estimularon PBMCs de pacientes portadores de variantes en la IL-26 con ADN de E. coli y demostramos que se consumía más anti-TNF-α en los cultivos con PBMC de pacientes con SNP en comparación con los de genotipo salvaje (Figura 4).

Desde un punto de vista clínico, la identificación de pacientes con SNPs en la IL-26 podría ser de interés, no solo en términos de su predisposición a una respuesta inflamatoria exacerbada, ya que parecen estar expuestos a la traslocación de ADNbact sostenida, sino también en la selección de pacientes que podrían requerir tratamientos anti-TNF-α intensificados. De hecho, el porcentaje de pacientes en programas anti-TNF-α intensificados fue mayor en los grupos con dos o más SNPs en la IL-26 comparados con los de genotipo salvaje. Estos pacientes, especialmente aquellos subgrupos con traslocación de ADN bacteriano u otras variantes genéticas relacionadas con la EC, podrían beneficiarse de terapias tempranas en el llamado enfoque descendente (100, 101). Serian necesarios futuros estudios prospectivos aleatorizados, que estratificaran a los pacientes según concentraciones séricas de IL-26 para demostrar que los pacientes con SNP en la IL-26 pueden beneficiarse de tratamientos anti-TNF-α intensificados.

En resumen, estos trabajos muestran la importancia de los polimorfismos en los genes que codifican proteínas que interaccionan con productos antigénicos bacterianos y regulan la respuesta inflamatoria frente a lo procesos de traslocación bacteriana, frecuentes tanto en cirrosis como en EC, y con complicaciones asociadas a su desarrollo en ambos casos. Las evidencias mostradas en la alteración de la respuesta inflamatoria soluble en los pacientes cirróticos con SNPs en diferentes TLR y en los pacientes con EC con variantes alélicas en IL-26 ofrecen la oportunidad de diseñar estudios que evalúen de forma especifica su utilidad como dianas anti-inflamatorias que contribuyan a reducir las tasas de traslocación bacteriana en estos pacientes.

CONCLUSIONES

Los polimorfismos rs4696480, rs4986790 y rs187084 (en los genes TLR-2, TLR-4 y TLR-9 respectivamente) se asocian con un aumento de la carga de antígenos bacterianos circulantes en la sangre de pacientes con cirrosis.

Este fenómeno viene acompañado de una respuesta deficiente en la secreción de citoquinas proinflamatorias por parte de las células inmunes en los pacientes con genotipos polimórficos, lo que podría condicionar el aclaramiento antigénico y contribuir a complicaciones clínicamente relevantes en pacientes con cirrosis.

La traslocación de ADN bacteriano en pacientes con enfermedad de Crohn en remisión se asocia con un aumento de los niveles séricos de IL26.

La acumulación de SNP en el gen de la IL26 parece dificultar la depuración de ADN bacteriano, favoreciendo una producción exacerbada de citocinas proinflamatorias y aumentando el consumo de anti-TNF-α.

Futuros estudios enfocados a la restauración de la señalización orquestada por la IL26 podrían proporcionar nuevos enfoques terapéuticos para controlar la traslocación bacteriana y la inflamación en este subgrupo de pacientes con EC.

CONCLUSIONS

The polymorphisms rs4696480, rs4986790 and rs187084 (in the TLR-2, TLR-4 and TLR-9 genes respectively) are associated with an increased load of circulating bacterial antigens in the blood of patients with cirrhosis.

This phenomenon is accompanied by a deficient response in the secretion of proinflammatory cytokines by immune cells in patients with polymorphic genotypes, which could compromise the antigenic clearance and might contribute to clinically relevant complications in patients with cirrhosis.

The translocation of bacterial DNA in patients with Crohn's disease in remission is associated with an increase in serum IL26 levels.

The accumulation of SNPs in the IL26 gene seems to hinder the clearance of bacterial DNA, supporting an exacerbated production of proinflammatory cytokines and increasing the consumption of anti-TNF-α.

Future studies focused on the restoration of the signaling orchestrated by IL26 could provide new therapeutic approaches to control bacterial translocation and inflammation in this subgroup Crohn's disease patients.

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